The control of microbiological spoilage of food requires an understanding of a number of factors including the knowledge of possible hazards, their likely occurrence in different products, their physiological properties and the availability and effectiveness of different preventative measures. Food spoilage microorganisms focuses on all these issues in the prevention and control of microbial spoilage. The first part of the book looks at tools, techniques and methods for the detection and analysis of microbial food spoilage, with chapters focusing on analytical methods, predictive modelling and stability and shelf life assessment. The second part tackles the management of microbial food spoilage with particular reference to some of the major food groups where the types of spoilage, the causative microorganisms and methods for control are considered by product type. The following three parts are then dedicated to yeasts, moulds and bacteria in turn, and look in more detail at the major organisms of significance for food spoilage. In each chapter the taxonomy, spoilage characteristics, growth, survival and death characteristics, methods for detection and control options are discussed. Food spoilage microorganisms takes an applied approach to the subject and will be an indispensable guide both for the microbiologist and the non-specialist, particularly those whose role involves microbial quality in food processing operations.

Dr Clive Blackburn works at Unilever Colworth, and has published widely on the control of foodborne pathogens.
Food spoilage microorganisms
Related titles:

Emerging foodborne pathogens
Developments such as the increasing globalisation of the food industry, constant innovations in technologies and products, and changes in the susceptibility of populations to disease have all highlighted the problem of emerging pathogens. Pathogens may be defined as emerging in a number of ways. They can be newly discovered (e.g. through more sensitive analytical methods), linked for the first time to disease in humans, or first associated with a particular food. A pathogen may also be defined as ‘emerging’ when significant new strains emerge from an existing pathogen, or if the incidence of a pathogen increases. Designed for microbiologists and QA staff in the food industry, and food safety scientists working in governments and academia, this collection discusses ways of identifying emerging pathogens and includes chapters on individual pathogens, their epidemiology, methods of detection and means of control.

Understanding pathogen behaviour: Virulence, stress response and resistance
Pathogens respond dynamically to their environment. Understanding pathogen behaviour is critical both because of evidence of increased pathogen resistance to established sanitation and preservation techniques, and because of the increased use of minimal processing technologies which are potentially more vulnerable to the development of resistance. This collection summarises the wealth of recent research in this area and its implications for microbiologists and QA staff in the food industry.

Handbook of hygiene control in the food industry
The foundation of food safety lies in good hygiene practice. This important and wide-ranging book complements the highly successful Hygiene in food processing by reviewing some of the key recent research on improving hygiene in food processing plants. Part I considers the latest research on contamination risks such as biofilms and how they can be assessed. Part II reviews ways of improving hygienic design of both buildings and equipment, including such key topics as clean room technology. The final part of the book discusses ways of improving hygiene practice and management, with chapters on areas such as cleaning and monitoring techniques.

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Food spoilage microorganisms

Edited by
Clive de W. Blackburn

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Food spoilage: a matter of perspective

Microbial metabolism of organic matter is a naturally occurring process in the environment that is essential for the recycling of nutrients. These activities are generally referred to as biodegradation; however, when these organic materials are important for the well-being of humans, such as foods, then microbial metabolism is considered to be spoilage (see Chapters 1 and 14). Indeed, when microbial activities are harnessed for the benefit of humans, they are given constructive epithets such as fermentation and biotransformation.

Food spoilage is therefore a social construct and can be defined as the process or change leading to a product becoming undesirable or unacceptable for human consumption. The manifestations of food spoilage are many and varied, and may be visual (e.g. discoloration, slime production, colony formation, breakdown of structure, blowing of container) or apparent by smell (e.g. off-odour) or taste (e.g. off-flavour, increase in acidity). Food spoilage may be caused by microbial, chemical or physical mechanisms. Although this compartmentalisation of the causes of food spoilage is convenient, there is really a continuum of causes and effects. However, by necessity this book is restricted to the microbial spoilage of foods with a focus on the major spoilage microorganisms and how they can be controlled.

Microbial food spoilage and safety

Microbial food safety and spoilage are often separated; indeed, the concept of this book was to treat food spoilage microorganisms in a similar way to that of foodborne pathogens in the book of the same name (Blackburn & McClure, 2002). From the perspective of the microbiologist this separation is sometimes blurred or more complex, as exemplified by the potential for mycotoxin production by contaminating moulds and the shattering of glass containers caused by the build-up of carbon dioxide from contaminating
yeasts. Perhaps more importantly in the eyes of the consumer and also the law, there is often no clear distinction. This has an associated impact on how the food industry perceives and manages food spoilage. Frequently the controls, preservation strategies and management procedures to ensure food safety are similar and overlap with those that control food spoilage. However, whereas safety is given priority, and rightly so, many food microbiologists spend much of their time ‘battling’ food spoilage microorganisms.

An expensive business

Although exact figures of the total economic losses due to food spoilage are unknown it is clear that it constitutes an enormous financial burden. It has been estimated that a quarter of the world’s food supply is lost through microbial activity alone (Huis in’t Veld, 1996). Worldwide losses of grain and legumes have been estimated to be at least 10% of production and for non-grain staples, vegetables and fruits, the loss is believed to be as high as 50% (WHO, 1995). Food losses begin on the farm and continue throughout post-harvest storage, distribution, processing, wholesaling, retailing and use in the home and in catering (Roller, 1999).

The competitive world economy means that no one can exist in isolation, and migration, overseas education and travel have resulted in tremendous demands for competitively priced food from different parts of the world (Morican, 1996). There is a two-way concern regarding safety and quality in importing and exporting countries that requires cooperation between developed and developing countries: the microbiological risks from products and/or ingredients from countries that have varying microbiological standards, and the dumping of substandard products in countries with insufficient monitoring systems (Morican, 1996).

There are a number of trends and drivers from all parts of the food supply chain that present increasing challenges to control microbial spoilage for all those involved in food production and manufacture. From a consumer perspective there are paradoxical drivers for less heavily preserved/processed foods and organic foods, against improved quality, guaranteed safety, longer shelf-lives and competitive pricing. This consumer pressure is passed along the supply chain and it requires those involved in the food industry to reduce operating costs, e.g. by having longer run times between scheduled cleaning and disinfection procedures; global sourcing of ingredients; optimising/reducing thermal processes. This places enormous challenges on controlling microorganisms.

The cost of microbiological mistakes range from having a high level of rework and/or product disposal to having to instigate a (trade or public) product recall. For example, in the USA between 1 October 1991 and 30 September 1992 there were 230 recalls, involving 569 foods and cosmetics, 25% of which were due to microbial contamination (Venugopal et al., 1996).
The usual suspects

The range of spoilage microorganisms is wide. The increasing variety of consumer products together with the need to preserve flavour and texture through minimum processing means that the susceptibility to spoilage has increased, as has the diversity of spoilage species.

Bacteria (Part V) are responsible for some of the most rapid and evident spoilage of proteinaceous foods such as meat, poultry, fish, shellfish, milk and some dairy products (Huis in’t Veld, 1996). Their range is huge and diverse, including the Gram-negative rods (e.g. *Pseudomonas*, *Shewanella*, Chapter 19), Gram-positive spore-formers (e.g. *Bacillus*, *Clostridium*, Chapter 21), lactic acid bacteria (Chapter 20) and other Gram-positive bacteria (*Brochothrix*, *Micrococcus*, Chapter 23) (Huis in’t Veld, 1996). In addition, the Enterobacteriaceae (Chapter 22) is a large group that as well as including spoilage bacteria also contains pathogens. Members of the group are often used as indicators of quality and safety.

The growth of yeasts and moulds (Parts III and IV) is generally slower than that of bacteria, but the wide variety of ecological niches they can exploit, the ability to utilise a variety of substrates, and tolerance of more extreme conditions than (vegetative) bacteria makes them formidable spoilage agents (Huis in’t Veld, 1996). With regard to the yeasts, species of *Zygosaccharomyces* and related genera are usually the yeasts that colonise and spoil high sugar and high salt products (Chapter 10). *Saccharomyces* are best known for their positive contributions to food and beverage production, but they also can have deleterious effects including circumstances when the same species spoil the very commodities they produce (Chapter 11). *Candida* species make up one-quarter of all known yeasts and their heterogeneity means that they are responsible for the spoilage of a wide range of foods (Chapter 12). In contrast the yeasts within the *Dekkera/Brettanomyces* genera are primarily spoilers of beverages, particularly alcoholic beverages, and they are a major concern to the wine industry (Chapter 13).

Although the yeast and mould forms of the fungi have been considered separately there is really a continuum, with some fungi exhibiting both single celled (yeast) and filamentous (mould) growth. However, the mould form has many specific characteristics that present specific problems with regards to food spoilage (Chapter 14). Although the range of moulds is immense there are a specific and rather limited number of genera and species that are spoilage hazards for each kind of food (Filtenborg *et al*., 1996). The Zygomycetes, popularly known as the ‘pin moulds’ are often seen as rapid growers following a ‘hit and run’ strategy for the foods that they spoil (Chapter 15). The Penicillia (Chapter 16) and Aspergilli (Chapter 17) are common spoilage moulds with the latter generally growing more rapidly and at higher temperatures or lower water activities than the former. Other types of moulds are significant in food spoilage (Chapter 18), but many moulds may be present on, or isolated from, foods in which they never or rarely cause spoilage (Chapter 18).
The need to compartmentalise is inherent in microbial classification and taxonomy, but the given name is often of only transient significance. New approaches in taxonomy utilising phylogenetic criteria (e.g. 16S rRNA and DNA sequence comparisons), while not significantly affecting foodborne pathogens, have had an impact on some food spoilage bacteria (Jay, 2003). These methods have been applied to some long-established genera of bacteria of importance in foods including: Bacillus, Clostridium, Flavobacterium, Micrococcus, Pseudomonas, Staphylococcus and Xanthomonas. The use of these molecular genetic methods allows the construction of bacterial taxonomy along phylogenetic rather than phenotypic lines (Jay, 2003). Molecular techniques have also brought greater understanding of the fungal genome and with it subsequent changes to fungal taxonomy (Chapter 17). Many species have a history of being established, dis-established, re-established or transferred to different genera, which has caused much confusion to researchers and industry professionals (Chapter 11).

New processes and food types have created niches for some ‘not-so-usual-suspects’, e.g. psychrotrophic clostridia in vacuum-packed and modified atmosphere packed chilled foods (Chapter 21). The reduction of preservatives as a result of consumer pressure has also caused the re-emergence of some spoilage organisms, e.g. certain moulds in bakery products (Chapter 18).

Food for thought

The factors that affect microbial spoilage of foods have been categorised into intrinsic parameters, extrinsic parameters, modes of processing and preservation and implicit parameters (Jay, 1992; referring to Mossel and Ingram, 1955). Intrinsic parameters are the physical, chemical and structural properties of the food itself (e.g. water activity, $a_w$, acidity, redox potential, available nutrients, natural antimicrobial substances). Extrinsic parameters are factors in the environment in which the food is stored (e.g. temperature, humidity and atmosphere composition). Modes of processing and preservation are the physical or chemical treatments that often result in changes in the characteristics of a food product, determining the associated microflora. Implicit parameters are the mutual influences, synergistic or antagonistic, among the primary selection of organisms resulting from the influence of the above-mentioned parameters (Huis in’t Veld, 1996).

Food spoilage is a complex process, and the type of product (intrinsic parameters) and the way it is packaged and stored (extrinsic parameters) select for the types of spoilage microorganism that can grow quickest under those conditions and hence lead to the spoilage of the product. In some cases (products) the interactive behaviour of microorganisms may contribute to their growth and/or spoilage activity. Examples include the competitive advantage of Pseudomonas spp. due to the production of iron-chelating siderophores (Chapter 19); the generation of substrates for spoilage reactions
by one organism from another organism (so-called metabiosis, Chapter 6); and the ‘switching on’ of spoilage metabolism through cell-to-cell communication (Gram et al., 2002). Because of this complexity, in addition to considering microbial spoilage from the perspective of the causative organisms (implicit parameters), there is also benefit from considering the food as the starting point (meat and meat products, Chapter 9; milk and dairy products, Chapter 7; and cereals and bakery products, Chapter 8).

**Understanding behaviour and control**

An understanding of microbial behaviour and ecology is fundamental in order to make the right decisions with regard to controlling microbial spoilage. It is vital to understand both the organisms associated with foods and those with the potential to spoil it. For example many moulds may be present on and isolated from foods in which they never or rarely cause spoilage. However, the presence of *Alternaria* might cause concern in a food commodity because of its production of a number of highly toxic mycotoxins, but its presence (without growth) in stored seed and brewing grains can be an indicator of freshness, since by the time it dies out seeds are becoming stale and are less effective at germinating (Chapter 18).

The implementation of the knowledge of specific spoilage organisms (Chapters 10–23) and the foods that they potentially spoil (Chapters 7–9) into control measures requires the use of systems, processes and practices. As with microbial safety, it is proposed that the use of a HACCP-based (hazard analysis critical control point) approach, supported by the necessary prerequisite programmes (e.g. good manufacturing practices) and even risk assessment, provides the best opportunity for controlling spoilage (Chapter 6). In turn, the translation of this knowledge into the necessary management systems often requires the use of tools and techniques. Methods for the detection, identification and quantification of both microbial spoilage and the causative microorganisms are needed (Chapters 1–3 and respective organism-based chapters). Although pathogen testing is beyond the scope of this book (see McMeekin, 2003), methods for indicator organisms are included. Knowing when it is appropriate to test, the appropriate microorganisms to look for and how to correctly interpret results are crucial in order to make the right management decisions. Analytical methods are integral to the procedures (e.g. challenge testing, shelf-life assessment) that are often necessary for the determination of the stability and shelf-life of foods (Chapter 5). These procedures also require knowledge of the relevant spoilage microorganisms and their spoilage capabilities in order to determine the spoilage end-point and understand the links with microbial numbers and metabolic activity. Both in conjunction with challenge testing and in relation to risk assessment and HACCP, the use of predictive microbiological models can be applied. Although these models have mainly focused on the growth, survival and
inactivation of pathogens, models for spoilage microorganisms are increasingly being developed (Chapter 4).

Food technologies play a pivotal role in improving the nutritional quality of food, ensuring its safety and preventing foodborne disease, but they also reduce losses due to spoilage or contamination and are thus vital in the prevention of malnutrition and starvation (WHO, 1995). However, the range and diversity of spoilage microorganisms mean that preservation boundaries often have to be set beyond those necessary to control pathogens. Most traditional food preservation processes have been developed empirically without a full understanding of the mechanisms of action of the antimicrobial agent used. With the move away from using high concentrations of individual food preservatives towards increasing reliance on sub-lethal levels of antimicrobial compounds or processes, there is a need to re-examine the basics (Roller, 1999). From a global perspective, the control of food spoilage is clearly still a huge challenge.

Structure of this book

The rationale behind this book, which is similar to that of the book Foodborne Pathogens (Blackburn and McClure, 2002), is that the control of microbial spoilage requires an understanding of a number of factors including the knowledge of possible hazards, their likely occurrence in different products, their physiological properties, and the availability and effectiveness of different preventative measures. The focus of this book is on the control of microbiial spoilage and its aim is to help provide the understanding necessary to do this. It has an applied approach and is intended both for the microbiologist and the non-specialist, particularly those whose role involves microbial quality in food processing operations.

The first part of the book looks at tools, techniques and methods for the detection and analysis of microbial food spoilage with a chapters focusing on analytical methods, predictive modelling and stability and shelf-life assessment. The second part tackles the management of microbial food spoilage with particular reference to some of the major food groups, where the types of spoilage, the causative microorganisms and methods for control are considered by product type. The following three parts are then dedicated to yeasts, moulds and bacteria in turn, and look in more detail at the major organisms of significance for food spoilage. In each chapter the taxonomy, spoilage characteristics, growth, survival and death characteristics, specific methods for detection, and control options are discussed.

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Part I

Detection and analysis of food spoilage
1

Quantitative detection and identification methods for microbial spoilage
D. I. Ellis and R. Goodacre, University of Manchester, UK

1.1 Introduction

Microbial metabolism of dead or decaying matter is a naturally occurring process in the environment, and is essential for the recycling of nutrients. Microbial metabolism of foodstuffs, however, that leaves them either unfit or unacceptable for human consumption is commonly termed microbial spoilage. It is to the obvious benefit of producers and processors of food to halt or delay this process for as long as possible. In the modern and highly automated food processing industry the ability to detect the onset or source of microbial spoilage is particularly advantageous as spoilage can lead to large-scale losses in economic terms and outbreaks of foodborne illness.

The microbial spoilage of foods has always been problematic and has been known for centuries, for example in the brewing industry where lactic acid bacteria can spoil beer by causing turbidity, acidity and unfavourable odours (Takahashi et al., 2000; Sakamoto and Konings, 2003; Suzuki et al., 2004). Indeed, as an ideal source of nutrients for bacteria and fungi all foodstuffs can be affected, including dairy products (Bleve et al., 2003; Gunasekera et al., 2003), eggs (Erickson and Jenkins, 1992; Deeming, 1996), fruit (Leverentz et al., 2003), vegetables (Jacxsens et al., 2001; Allende et al., 2004), juices (Casey and Dobson, 2004; Chang and Kang, 2004), fish (Gram and Dalgaard, 2002; Tryfinopoulou et al., 2002; Skjerdal et al., 2004), meat (Lebert et al., 2000; Jay et al., 2003) and poultry (Kalinowski and Tompkin, 1999; Ellis and Goodacre, 2001; Hinton et al., 2004). Therefore, as the potential scope of this particular area of food microbiology could take up a book in itself, this chapter will concentrate on microbial spoilage in meat, and in particular poultry, which is also an area in which the authors have practical experience.
In terms of food safety and foodborne illness, muscle foods (which include meat and poultry), as well as being a widely consumed and relatively inexpensive protein source, are an important foodstuff with both worldwide economic and public health significance (Altekruse et al., 1999; Mead et al., 1999; Vellinga and Van Loock, 2002). The health risks associated with the consumption of spoiled or contaminated meats, for example, are considerable and include debilitating and potentially lethal microbial diseases such as salmonellosis (Zhang-Barber et al., 1999; Schlundt, 2001; White et al., 2001), campylobacteriosis (Chan et al., 2001; Frost, 2001; Newell et al., 2001), and haemorrhagic colitis (Tarr et al., 1997; Cassin et al., 1998; Tuttle et al., 1999). Moreover, the global magnitude of the production, processing and retail industries also makes muscle foods extremely important in economic terms. Therefore, any advancement in the quantitative detection and identification methods for the microbiological contamination/spoilage of meats and poultry would be beneficial, in terms of both the health risks associated with food contamination and any reduction in large-scale losses due to bacterial spoilage. However, it must be stressed that any advancement in detection methods should ideally be concomitant with increased control of the microbial hazards at source if the health risks are to be reduced.

1.2 Microbial spoilage of meat and poultry

Meat and poultry are generally described as spoiled when undesirable changes have made them unacceptable to the consumer (Jackson et al., 1997). These organoleptic changes can include purely visible changes (such as discoloured meat), malodours or slime formation. It could be any one or combination of these changes, or indeed any other characteristic that makes the food undesirable for human consumption, that leads to the foodstuff being described as 'spoiled' (Jay, 1996; Jackson et al., 1997). It has been established that endogenous post-mortem enzymatic activity within muscle tissue, such as the activity of Ca\(^{2+}\) proteases (calpains), can contribute to some biochemical changes during storage (Koohmaraie, 1992, 1994, 1996; Alomirah et al., 1998; Schreurs, 2000). However, it has also been generally accepted and shown that detectable organoleptic spoilage of muscle foods is a result of the decomposition and the subsequent metabolite formation caused by the growth and catabolic activity of microorganisms (Stutz et al., 1991; Schmitt and Schmidtlorenz, 1992a,b; Kakouri and Nychas, 1994; Nychas and Tassou, 1997; Braun et al., 1999). These detectable organoleptic changes will vary, for example whether these changes are visible or cause a particular odour, according to the species of colonizing microflora, the general characteristics or type of meat/poultry, processing/production methods and storage conditions (Jackson et al., 1997; García-López et al., 1998).

Several researchers have described meat as the most perishable of all important foods (Jay, 1996; Stanbridge and Davies, 1998), with its nutrient-
Rich and moist surface being particularly conducive to the colonization and rapid growth of a wide range of spoilage bacteria. The colonization and growth on the meat surface have been widely researched and documented over several decades in some detail, with the initial (and obvious stage) being the attachment of bacterial cells to the meat surface. This has been described as a loose and reversible sorption (Marshall et al., 1971). The second and irreversible stage of attachment involves the production by the bacterium of a sticky polysaccharide extracellular layer, termed the glycocalyx (Costerson et al., 1981), which ultimately leads to a biofilm. Other factors may also play a role in bacterial attachment such as the population of microflora already present in the water film on the meat surface, how motile the species involved are, what growth phase they are in, and purely physical factors such as surface morphology and temperature (Jackson et al., 1997).

In storage conditions conducive to bacterial growth, such as a moist atmosphere and a temperature range between –1 and 25°C, a wide range of genera are responsible for organoleptic spoilage. Table 1.1 lists the genera most commonly found on meat and poultry and includes both spoilage and pathogenic bacteria. At this particular temperature range and in aerobic storage conditions it is the genus Pseudomonas (including P. fluorescens, P. lundensis, P. putida and P. fragi) which has been observed, in comparison to several other genera responsible for spoilage, to attach more rapidly to meat surfaces (Molin and Ternström, 1982; Jackson et al., 1997; García-López et al., 1998; Stanbridge and Davies, 1998). In aerobic refrigerated storage conditions, the other major spoilage bacteria include the genera Moraxella, Psychrobacter and Acinetobacter. It has also been observed that under these storage conditions, the dominant spoilage microflora are in general Gram-negative aerobic rods (motile and non-motile) and coccobacilli. However, the initial bacterial population of the meat/poultry may also contain Gram-positive genera such as micrococci and lactic acid bacteria, and under anaerobic (vacuum-packaged) storage conditions these can become the dominant spoilage microflora (Holzapfel, 1998; Stanbridge and Davies, 1998; Adams and Moss, 2000).

It has also been observed, following experiments in our own laboratories involving the investigation of bacterial diversity in meats, that the genera of spoilage organisms can vary significantly according to temperature and storage regime. These experiments were undertaken on samples of chicken breast fillets from three distinct temperature/storage treatments. The first of these was named ‘fresh’ and took place immediately following the purchase of the meat. For the second, named ‘spoiled’, samples were left on the bench to spoil at ambient temperature (21 ± 1°C) for 24h. The final regime, named ‘use by’, were packaged breast fillets stored in the refrigerator at 4°C until their use-by date. Bacterial isolates were obtained from each of these three storage regimes and identified using API strips; see Table 1.2 for details (Ellis, 2003).

In general, fresh meats have a pH range between 5.5 and 5.9 and contain sufficient levels of glucose, other simple carbohydrates and low molecular
weight compounds to support approximately $10^9$ colony-forming units per square centimetre (cfu cm$^{-2}$) of bacteria, and again, it is known that pseudomonads grow the fastest and utilize glucose at refrigeration temperatures (Gill and Newton, 1977; Seymour et al., 1994; Jay, 1996). The olfactory characteristics resulting from microbial spoilage are well known, for example at typically $10^7$ cfu cm$^{-2}$ a faint ‘dairy’ type aroma may become evident, while at a later stage ($\sim 10^8$ cfu cm$^{-2}$) the supply of simple carbohydrates and

<table>
<thead>
<tr>
<th>Genus</th>
<th>Gram reaction</th>
<th>Red meat*</th>
<th>Poultry*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter</td>
<td>–</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>–</td>
<td>XX</td>
<td>X</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Bacillus</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Brochothrix</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>–</td>
<td>–</td>
<td>XX</td>
</tr>
<tr>
<td>Carnobacterium</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Clostridium</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>+</td>
<td>X</td>
<td>XX</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>+</td>
<td>XX</td>
<td>X</td>
</tr>
<tr>
<td>Escherichia</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Hafnia</td>
<td>–</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Kocuria</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Kurthia</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Lactococcus</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Listeria</td>
<td>+</td>
<td>X</td>
<td>XX</td>
</tr>
<tr>
<td>Microbacterium</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Moraxella</td>
<td>–</td>
<td>XX</td>
<td>X</td>
</tr>
<tr>
<td>Paenibacillus</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pantoea</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pediococcus</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Proteus</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>–</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>Psychrobacter</td>
<td>–</td>
<td>XX</td>
<td>X</td>
</tr>
<tr>
<td>Salmonella</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Serratia</td>
<td>–</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Shewanella</td>
<td>–</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Vagococcus</td>
<td>+</td>
<td>–</td>
<td>XX</td>
</tr>
<tr>
<td>Weissella</td>
<td>+</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Yersinia</td>
<td>–</td>
<td>X</td>
<td>–</td>
</tr>
</tbody>
</table>

*XX = most frequently reported; X = known to occur, – = absent.

Table 1.1  Genera of bacteria most commonly found on red meat and poultry (Jay, 1996)
other low molecular weight compounds have been exhausted and recognizable
off-odours develop, which many consumers would recognize, and is termed
‘sensory’ spoilage (Jay, 1996; Jackson et al., 1997; Stanbridge and Davies,
1998); of course these exact values will vary depending on the individual.
The extent at which free amino acid utilization has occurred is the primary
cause of these changes in the olfactory characteristics of the meat and the
development of off-odours have been previously described in a variety of
terms such as dairy/buttery/fatty/cheesy at 10⁷ cfu cm⁻², then a sickly sweet/
fruity aroma at 10⁸ cfu cm⁻² and finally a putrid malodour at 10⁹ cfu cm⁻²
(Dainty et al., 1985; Adams and Moss, 2000). It is at the latter two levels of
microbial growth where spoilage would become distinctly evident to the
human senses.

Apart from olfactory characteristics, during microbial spoilage the meat
surface will also begin to feel sticky/tacky and this is as a result, and indicative,
of the early stages of slime formation which is directly attributable to bacterial
growth and polysaccharide synthesis, over time leading to the formation of
a layer on the meat surface (Ingram and Dainty, 1971; Jackson et al., 1997).
It is once surface levels of glucose have been depleted and the diffusion
gradient of this substance from the underlying tissue cannot meet microbial
demand, that the spoilage bacteria utilize other substrates sequentially, until
the metabolism of nitrogenous compounds leads to the formation and increase
in concentration of a variety of malodorous substances, including ammonia,
amines, dimethylsulphide and hydrogen sulphide (Jackson et al., 1997;
Stanbridge and Davies, 1998).

Table 1.2 Genera of isolates selected from samples of chicken breast fillets kept at
three storage/temperature regimes (unpublished data)

<table>
<thead>
<tr>
<th>Genera</th>
<th>Fresh</th>
<th>Spoiled</th>
<th>Use by date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bacillus</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Comamonas</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chryseomonas</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Serratia</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sphingobacterium</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Fresh, indicates poultry tested on the day of purchase. Spoiled, from poultry left to spoil at ambient
temperature (21±1°C) for 24h. Use by date, from poultry stored at 4 °C and tested on its use by date.
All experiments undertaken in triplicate. + indicates present, – indicates not present.
1.3 Microbial metabolites as possible markers of spoilage

Since the early 1970s, many researchers have investigated microbial metabolites as potential indicators of microbial spoilage and attempted to associate given metabolites with the spoilage status of meat, with the aim being to utilize this information to determine remaining shelf-life (Ingram and Dainty, 1971; Dainty et al., 1975, 1985, 1986, 1988; Edwards et al., 1985; De Castro et al., 1988; De Pablo et al., 1989; Drosinos and Board, 1994; Kakouri and Nychas, 1994; Seymour et al., 1994; Dainty, 1996; Nychas and Tassou, 1997; Alomirah et al., 1998; Nychas et al., 1998; Braun et al., 1999). However, while many investigations have studied the microbiological changes on the surface of meats in some detail (Jay, 1996; Nychas et al., 1998) it can be said that the physicochemical changes that take place during the colonization of spoilage bacteria have not been studied as equivalently. During the spoilage process the physicochemical changes take place within the aqueous phase of the meat, which contains a variety of low molecular weight compounds such as glucose, glucose-6-phosphate, lactic acid, pyruvate, gluconate, propionate, ethanol, acetate, amino acids, nucleotides, urea and water-soluble proteins (Drosinos and Board, 1994; Nychas et al., 1998). It is these substrates that are sequentially catabolized by the meat microflora, and the order in which this takes place has been studied in detail elsewhere (Drosinos and Board, 1994; Nychas et al., 1998).

Post-glucose utilization of amino acids by pseudomonads has most frequently been associated with spoilage in meats and organoleptic spoilage usually becomes evident when there is a significant decrease in surface levels of glucose. Further to this, at least one study has observed that glucose limitation instigated a change from a saccharolytic to an amino acid-degrading metabolism in some species of bacteria (Borch et al., 1991). Moreover, detailed studies into the metabolic action of pseudomonads have been undertaken within extracts of minced lamb (Drosinos and Board, 1994). These studies observed that the oxidation of glucose by pseudomonads led to a transient accumulation of D-gluconate and 6-phosphogluconate which coincided with the exponential growth curve of the bacteria.

As already stated, once bacteria have utilized surface levels of glucose they will then sequentially metabolize other substrates such as free amino acids. In order to achieve this, many bacteria secrete a variety of proteolytic enzymes, with Gram-negative bacteria at refrigeration temperatures on meat predominantly secreting aminopeptidases (Nychas et al., 1998). Several researchers have forwarded the idea that this presence of enzymes could be used to estimate meat quality in terms of bacterial numbers rapidly via the use of enzyme assays (De Castro et al., 1988; Braun et al., 1999). One of the by-products of amino-acid utilization is an increase in the concentration of ammonia, and the metabolism of free amino acids will still occur even when there are significant levels of glucose present deep with the tissue, making this predominantly a surface phenomenon. The increase in levels of ammonia is also concomitant with other products of amino acid utilization such as
amines, indole, scatole and sulphides, which lead to the well-recognized malodours characteristically associated with spoiled meat and an increase in pH (Dainty et al., 1986, 1988; Jay, 1996; Adams and Moss, 2000; Kumudavally et al., 2001).

1.4 Current detection methods

While the modern food industry is a predominantly large and highly automated working environment, the conventional approach to microbiological testing, on the whole, saw little progress over the latter part of the 20th century. Currently, it has been estimated that there exist in excess of 40 methods to identify and quantify bacterial spoilage in meats (Jay, 1996; Nychas et al., 1998; Betts, 1999). Therefore, there is much scope, indeed there is a specific requirement if the science is to keep up with the industry, to develop rapid and quantitative microbiological testing.

1.5 Identification methods

In the food industry at present the identification methods used are either immunological-based or nucleic acid-based. In the case of immunological techniques, these use antibodies which are raised to react to the surface antigens of specific bacteria known to be responsible for food spoilage and/or contamination (Jay, 1996; Betts, 1999). The ELISA test (enzyme-linked immunosorbent assay) is the most commonly used immunological method and is based on the use of an enzyme label. Those used at present are targeted at the detection of foodborne pathogens such as *Escherichia coli* O157:H7, toxins produced by *Staphylococcus aureus* and proteases from the food spoilage genus *Pseudomonas* (Jabbar and Joishy, 1999). Nucleic acid-based procedures use probes in the form of small segments of complementary single-stranded nucleic acid which are then applied to detect specific gene sequences (either DNA or RNA) in test samples and thereby identify accurately specific microorganisms (Venkitanarayanan et al., 1996; Alexandre et al., 2001).

The most well-known and widely used nucleic acid detection method at the moment is of course the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). PCR allows for rapid and selective identification and/or detection of microorganisms by amplifying specific gene fragments and then detecting the amplicons by gel electrophoresis (Gutierrez et al., 1998; Scheu et al., 1998; Yost and Nattress, 2000; Cloak et al., 2001). Therefore, as with nucleic acid probes, the DNA of the microorganism being tested must be known prior to analysis. However, as with the majority of methods, PCR does have its limitations, such as the fact that any intact nucleic acid sequences
present within a sample will be amplified, not just those of the target microorganism. This factor can lead to false positive results being obtained owing to the presence of DNA from non-viable microorganisms.

Problems may also arise from the presence of PCR inhibitors such as those reported to be present in the matrix of cheese products (Jay, 1996; Scheu et al., 1998). While the degree of PCR inhibition is dependent upon the type of food sampled, and although procedures exist (i.e. dilution of samples) to circumvent this, the circumvention procedures themselves can also decrease the sensitivity of the PCR test itself (Jay, 1996; Scheu et al., 1998). It must also be stated that although PCR is one of the most rapid procedures available at present for the detection of pathogens in foods, it is still time consuming, particularly in terms of large-scale testing and particularly when the, what has been termed, ‘tedious and exacting nature’ of the reaction set-up is taken into account (Barbour and Tice, 1997).

1.6 Quantitative methods

The current quantitative methods available for the enumeration of microorganisms in foods are based on the measurement of ATP bioluminescence, electrical phenomena or microscopic methods. In the case of the measurement of ATP bioluminescence, these methods act by quantifying the levels of ATP in bacterial cells in culture in order that the number of cells present in that culture may be calculated (Siragusa et al., 1996; de Boer and Beumer, 1999; Champiat et al., 2001; D’Souza, 2001). One major drawback with this type of method is the obvious fact that ATP is the major source of energy for cellular reactions in all living cells and therefore any food sample will contain large amounts of ATP, necessitating the separation of microbial ATP from that within the sample itself. It could therefore be considered to be a method more ideally suited to quantifying the levels of organic contamination on machinery and preparation surfaces within the food production industry, and indeed this method has found much more practical application in this regard.

The methods in use for the measurement of electrical phenomena are based on the detection of levels of electrical current that occur during microbial growth. These methods take advantage of the knowledge that as bacteria metabolise uncharged particles in any medium, these particles become charged, thereby increasing the conductivity of that medium. The media used, sometimes termed impedance media, can be general or selective for specific bacterial genera, yeasts and moulds or to the foodstuff being analysed such as cheeses. At present, commercially available instruments for the measurement of electrical phenomena include Bactrac, Rabit, the Malthus Analyser and the Bactometer (Jay, 1996; Betts, 1999).

With microscopy, highly developed techniques are applied to the quantification of microorganisms whereby the microorganisms are stained
using fluorescent dyes and then viewed with an epifluorescent microscope. Initially problems were encountered as both viable and non-viable microorganisms were stained with the dyes used for this technique, but these problems were overcome with the introduction of the direct epifluorescent filter technique (DEFT). However, even this technique has been described as time-consuming and laborious (Shaw et al., 1987; Restaino et al., 1996; Wang and Sharpe, 1998; Pyle et al., 1999). That being said, the process has been aided considerably with the development of fully automated systems as well as the use of flow cytometry.

Rattanasomboon and co-workers, for example, undertook a comparative study of the common meat spoilage organism *Brochothrix thermosphacta*, which, owing to changes in the morphology of this bacterium during growth, can lead to false estimates in numbers with some enumeration techniques (Rattanasomboon et al., 1999). Using turbidimetrics as the standard for quantification they compared plate counts, flow cytometry and manual (microscope) counts in terms of sensitivity of analysis, growth rates and lag times. It was shown that flow cytometry was an efficient and reliable method for enumeration of the pleomorphic bacterium *B. thermosphacta*. However, the technique can still not be termed rapid, as the spoilage organisms need to be separated from the meat surface before analysis and with some organisms forming a glycocalyx layer this can obviously be difficult.

### 1.7 Emerging techniques

Several different types of analytical approaches to the rapid detection of microbial spoilage in meats have been attempted since 1995 with varying degrees of success, and these could all be termed ‘biosensors’ (Patel, 2002). These include amperometric detection methods, which utilized enzymatic reactor systems with amperometric electrodes in order to sense diamine levels and thereby determine the quality of chicken (Yano et al., 1996; Okuma et al., 2000; Suzuki et al., 2001). Suzuki and co-workers (2001) demonstrated that it was possible to obtain accurate results within 5 minutes, although this was preceded by a 10 minute sample preparation step for the enzyme reactor system.

Other techniques have concentrated on the recognition and measurement of odours, such as electronic noses, which were first developed in the 1980s. These comprise an array of electronic chemical sensors with partial specificity linked to a pattern recognition system that is capable of recognizing simple or complex odours (Gardner and Bartlett, 1994, 1999; Craven et al., 1996). These sensor arrays utilize a variety of technologies including microbalances, organic polymers and metal oxides (Harper, 2001). The electronic nose approach, which in a sense attempts to mimic the human olfactory interpretation of microbial spoilage by the detection of microbial volatiles, has been demonstrated to have some success in terms of the analysis of meat and
Fish (Di Natale et al., 1997, 2001; Schaller et al., 1998; Ziegler et al., 1998; Haugen, 2001) and has also been applied to shelf-life estimation of tomatoes and apples (Berna et al., 2004; Echeverría et al., 2004).

However, electronic nose systems, while available commercially and at the same time still within the developmental stage, do have a number of recognized limitations. These include instrumental drift, loss of sensitivity in humid conditions (and many food production facilities have a high humidity), inability to provide absolute calibration, short sensor lifespan and inability to provide quantitative data for aroma differences (Harper, 2001). However, despite these limitations, there remains a significant amount of research activity involving electronic nose systems and it is anticipated that after addressing these limitations, such olfactory-based techniques will find several applications within the food production industry.

It is known for example that the machine drift problems associated with the chemical sensors can be surmounted via the application of mathematical transformations which have been successfully applied for other analytical techniques (Goodacre et al., 1996a, 1997). Further, the application of headspace analysis by a mass spectrometry detector may also significantly improve the levels of detection. One recent study, for example, has applied the recently developed proton transfer reaction mass spectrometry system to the detection of volatile organic compounds (VOCs) from meat spoilage. It was demonstrated that the concentration of the VOCs increased linearly with the bacterial numbers and that the bacterial numbers from these headspace measurements could be determined in minutes (Mayr et al., 2003).

Fourier transform infrared (FT-IR) spectroscopy is a rapid, high-throughput, non-destructive analytical technique, which, although well established, is continually developing and being used for an ever-increasing range of applications in both scientific and commercial settings. FT-IR involves the observation of molecules that are excited by an infrared beam, resulting in an infrared absorbance spectrum which represents a ‘fingerprint’ characteristic of any chemical or biochemical substance. Its major advantages when compared with other techniques are its rapidity, as an infrared spectrum can be collected in seconds, and the fact that it requires minimum sample preparation and background training (Dunn and Ellis, 2005).

Although FT-IR has been demonstrated to be a particularly useful technique for the discrimination of axenically cultured bacteria (Goodacre et al., 1996b, 1998), for several years infrared spectroscopy in general has been viewed as a useful tool for the analysis of foodstuffs. While initial interest was to some extent reserved to near infrared spectroscopy (NIR) (due in part to simplicity of instrumentation, portability and affordability) (Monin, 1998; Ru and Glatz, 2000; Murray et al., 2001) it is becoming increasingly evident that mid-infrared spectroscopy, which provides for a more information-rich analysis, has much promise for future industrial applications in the food and related industries (Riquet et al., 1998; Mura et al., 2001; Yang et al., 2001; Safa and Abbes, 2002). Potential food-related applications of FT-IR have included...
those in product authenticity and adulteration, such as studies into the classification and determination of key quality parameters of edible oils (Dahlberg et al., 1997; Cooke and Billingham, 1999) and the rapid determination of total fat content in a variety of foods (Mossoba et al., 2001). Analyses into sugars in milk (Kameoka et al., 1998), identification of modified starches (Dolmatova et al., 1998) and rapid identification of foodborne fungi, have also been undertaken (Kummerle et al., 1998; Kos et al., 2002).

In the case of meat products, the infrared analysis of muscle foods has been primarily concerned with studies related to authenticity and adulteration issues, such as the discrimination between beef, ox liver and pork (Al-Jowder et al., 1997, 1999), detection of adulteration of beef with offal (Al-Jowder et al., 2002) and attempts at identification of species such as pork, chicken and turkey, with varying degrees of success (Rannou and Downey, 1997; Downey et al., 2000). The assessment of processed meat quality parameters, such as the determination of protein:lipid ratios, has also been undertaken (Murcia et al., 1994). However, it is notable that none of the above studies has attempted to detect bacterial spoilage in meats. It is also notable that studies into the discrimination of meats along the lines of those already mentioned above have been attempted using NIR (Fumiere et al., 2000; Ru and Glatz, 2000; Abeni and Bergoglio, 2001) rather than mid-IR.

It is only recently that FT-IR has been successfully used to detect microbial spoilage in both poultry (Ellis et al., 2002) and beef (Ellis et al., 2004) and attempts have been made to replicate these experiments using NIR (Lin et al., 2004). With the background knowledge that spoilage is a result of decomposition and the formation of metabolites caused by the growth and enzymatic activity of microorganisms (see above), FT-IR was used to exploit this knowledge and measure biochemical changes within the meat substrate, enhancing and accelerating the detection of microbial spoilage. This was achieved by comminuting meat samples and taking hourly measurements for 24 h using attenuated total reflectance (ATR). This process is shown in Fig. 1.1. Quantitative interpretation of infrared spectra was undertaken using partial least squares (PLS) regression, which illustrated that accurate estimates of bacterial loads could be calculated directly from the surface of the meat in 60 s. Machine learning methods were also employed, such as genetic algorithms (GAs) (Mitchell, 1997; Broadhurst et al., 1997; McGovern et al., 2002; Johnson et al., 2003) and genetic programming (GP) (Koza, 1992; Banzhaf et al., 1998; Johnson et al., 2000; Goodacre, 2003; Vaidyanathan et al., 2003), and GP was used to derive rules which showed that at levels of $10^7 \text{ cfu g}^{-1}$, the predominant biochemical indicator of microbial spoilage was the onset of proteolysis (Ellis and Goodacre, 2001; Ellis et al., 2002).

The experimental methods applied to poultry were later replicated using beef as the foodstuff of interest (Ellis et al., 2004). The aim of this particular study was to build upon the previous experiments on poultry and ascertain if the technique could be successfully applied to another muscle food with distinctly different microbial spoilage processes (Doyle et al., 1997; Adams...
Fig. 1.1 Diagram showing how a meat sample is analysed by FT-IR using attenuated total reflectance (ATR) (HATR = horizontal ATR).
and Moss, 2000). It was again demonstrated that FT-IR spectroscopy, in combination with PLS regression and evolutionary computational-based machine learning methods, was able to correlate bacterial spoilage processes with infrared spectra. While the results from the experiments on beef were not as accurate as those on poultry, the levels of detection were significantly lower at $10^5 \text{cfu g}^{-1}$ (Ellis et al., 2004). As with the experiments on poultry, the machine learning methods were also able to elucidate the wavenumbers of interest directly related to the spoilage process which were again determined to be a result of the onset of proteolysis.

A recent study employing NIR (Lin et al., 2004), using the same experimental protocol as that of Ellis and co-workers (although measurements were taken at less frequent time points), has shown that this technique is also able to quantify the bacterial loads on poultry tissue. However, this study was not able to elucidate the specific wavenumbers of interest that were directly related to the spoilage process. In comparison, the study on poultry by Ellis and co-workers, using evolutionary computation, was able to determine that bacterial levels of $10^7 \text{cfu g}^{-1}$ could be correlated to a simple ratio of two wavenumbers, associated with amides and amines (Ellis et al., 2002). These studies demonstrate that vibrational spectroscopy is capable of rapid and quantitative detection of microbial spoilage in meats and appears to be a significant improvement, particularly in terms of speed, on current methods used within the industry. However, in comparing the two vibrational methods (FT-IR and NIR) it is also readily apparent that there is also a need for these spectroscopic approaches to be combined with modern machine learning methods such as GAs and GP (Goldberg, 1989; Kell et al., 2001; McGovern et al., 2002; Goodacre, 2003; Johnson et al., 2003; Kell, 2004).

1.8 Data analysis

Many methods produce copious amounts of data and something has to be done in order to analyse them. A typical infrared experiment will generate data on the absorbance at hundreds or thousands of wavenumbers for each sample; therefore, simple visualization is not possible and alternative strategies are needed. Indeed, modern analytical methods can readily be applied to biological systems but it is the integrative analysis of this interdisciplinary science that will probably lead to success (Fig. 1.2).

Multivariate data (such as an FT–IR spectrum) consist of the results of observations of many different characters (IR absorbances) for a number of individuals (objects). Each character (or variable) may be regarded as constituting a different dimension, such that if there are $n$ variables each object may be said to reside at a unique position in an abstract entity referred to as $n$-dimensional hyperspace. This hyperspace is necessarily difficult to visualize, and the underlying theme of multivariate analysis (MVA) is thus simplification (Chatfield and Collins, 1980) or dimensionality reduction,
which usually means that we want to summarize a large body of data by means of \textit{relatively} few parameters, preferably the two or three that lend themselves to graphical display, with minimal loss of information.

The analysis of multivariate data by chemometrics is split loosely at four levels (Goodacre \textit{et al.}, 2004): (1) clustering algorithms, (2) identification strategies, (3) quantification methods, and (4) inductive/mining algorithms. Each of these chemometric methods will be briefly detailed below and see Table 1.3.

### 1.8.1 Clustering algorithms

These algorithms are based on \textit{unsupervised} learning (Duda and Hart, 1973) and seek to answer the question ‘How similar to one another are these samples based on the infrared fingerprints I have collected?’ Typically, principal components analyses (PCA) and hierarchical cluster analysis (HCA) are used, and in post-genomics such methods are sometimes referred to as ‘guilt-by-association’ (Altshuler \textit{et al.}, 2000). In addition a neural network-based

<table>
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<th>Learning type</th>
<th>Strategies</th>
<th>Typical algorithms*</th>
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<tr>
<td>Unsupervised</td>
<td>clustering algorithms</td>
<td>PCA, HCA, SOM</td>
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<td></td>
<td>identification methods</td>
<td>ANN, DA</td>
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<tr>
<td>Supervised</td>
<td>quantification methods</td>
<td>PLS, PCR, MLR</td>
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<td></td>
<td>inductive/mining algorithms</td>
<td>GA, GP</td>
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</table>

*PCA = principal components analysis, HCA = hierarchical cluster analysis, SOM = self-organizing map, ANN = artificial neural network, DA = discriminant algorithm, PLS = partial least squares, PCR = principal components regression, MLR = multiple linear regression, GA = genetic algorithm, GP = genetic programme.
method called self-organizing (feature) maps (SOMs), or Kohonen neural networks (Kohonen, 1989), can also be used and this is currently in vogue within the transcriptomics community. Clustering algorithms can be used to identify unknown samples using a process known as ‘operational fingerprinting’ (Meuzelaar et al., 1982), where samples of unknown origin cluster with those of known origin. However, much more powerful algorithms exist and these are detailed below.

1.8.2 Identification strategies
These algorithms are based on supervised learning (Beebe et al., 1998) and seek to give answers of biological interest which have much-lower dimensionality, such as ‘Based on the infrared fingerprint of this new sample I have just collected, which class in my database does it (most likely) belong to?’ One of the most popular methods for doing this has been artificial neural networks (ANNs) (Wasserman, 1989; Ripley, 1996), which are able to map non-linear relationships in data; however, owing to their computational expense and ‘black box’ nature, these multilayer perceptrons are losing popularity. Alternative linear mapping approaches exist and these are based on discriminant algorithms (DAs) (Manly, 1994), the most popular of which are Fisher’s linear discriminant algorithm (LDA), discriminant function analysis (DFA; also known as CVA (canonical variates analysis)) and partial least squares discriminant analysis (PLS-DA).

Supervised learning is fundamentally different from unsupervised learning. The latter uses only the data generated from the experiment (e.g. infrared measurements) and uses multivariate algorithms that seek ‘clusters’ in the data (Everitt, 1993), which allow the investigator to group objects together on the basis of their perceived closeness in the n-dimensional hyperspace referred to above. By contrast, supervised learning uses both the data generated from the experiment and some knowledge about the samples analysed (e.g. the identity of the spoilage organism being analysed). These data together are used to teach (hence supervise) the algorithm. This is usually an iterative process where the system is shown the experimental data and asked to predict the identity of the sample; the errors in the predictions are then used to ‘tune’ the learning algorithm.

1.8.3 Quantification methods
These are also based on supervised learning and, as the name suggests, attempt to generate a quantitative model that answer questions such as ‘what are the levels of these metabolites in my biological sample?’ or, perhaps more pertinent here, ‘what is the total viable biomass on this meat surface?’ Regression-based methods are very popular algorithms and in particular partial least squares (Martens and Næs, 1989). Other linear regression methods that are used include principal components regression (PCR) and multiple linear regression (MLR) (Martens and Næs, 1989).
1.8.4 Inductive algorithms

These data mining algorithms are also based on *supervised* learning but, in addition to being based on either identification or quantification, seek to answer the question ‘what have I measured in my infrared fingerprint that makes samples in this class different from samples in that other class?’ or ‘what infrared vibrations are correlated with the level of microbial spoilage organisms on this piece of meat?’ Typically the evolutionary computational-based methods (Bäck *et al*., 1997) of GAs (Goldberg, 1989; Holland, 1992) and GP (Banzhaf *et al*., 1998; Koza *et al*., 1999) are employed for so-called inductive reasoning or hypothesis generation (Kell, 2004; Kell and Oliver, 2004). In addition, multivariate decision trees (Brodley and Utgoff, 1995) such as classification and regression trees (CART) are employed (Breiman *et al*., 1984).

As is clear from the above, each of the above algorithms has its own specific use and therefore the choice of the algorithm depends on the question that needs to be answered. Most researchers are happy with using cluster analyses since the mathematics is relatively easy to compute and PCA has after all been around for over 100 years (Pearson, 1901) so is somewhat of a comfort blanket to some! However, supervised algorithms are much more powerful as they can be trained to discriminate when clustering fails; and of course PCA should never be used as a discriminatory tool as it was never designed for such a purpose. Yet despite the power of supervised methods, they do have to be treated with caution; the adage ‘garbage in – garbage out’ springs to mind (Zupan and Gasteiger, 1993). Indeed, an excellent example of this was discussed some time ago on the BBC television programme *Horizon*. A neural network was trained to attempt to distinguish tanks from trees. Pictures were taken of rustic forest scenes lacking military hardware and of similar, but perhaps less bucolic, landscapes that also contained more-or-less camouflaged battle tanks. A neural network was trained with these input data and was able to differentiate between tanks and trees. However, when a new set of pictures was analysed by the network, it failed to distinguish the tanks from the trees. Close inspection showed that the first set of pictures containing tanks had been taken on cloudy days while those pastoral scenes containing no tanks were obtained on sunny days. The neural network had thus learned to recognize the weather! Data modellers should thus beware and always use good modelling practice (Kell and Sonnleitner, 1995).

1.9 Conclusions

Current methods for the rapid detection of spoilage in meats are inadequate and all have the same recurring theme in that they are time consuming and labour intensive and therefore give retrospective information. The processes involved in the microbial spoilage of meats are well established and for three decades microbial metabolites have been forwarded as potential indicators
of organoleptic spoilage and remaining shelf-life. Despite this knowledge most research has been aimed at single markers rather than multiple ones, or looking at the system as a whole. The ability to correlate multiple biochemical change with microbial biomass is a complex problem, and perhaps only very recently surmountable with the advent of suitable chemometric methods.

With continuous advances in analytical instrumentation coupled with the realization that miniaturization instrumentation is assuming increasing importance (as computer processing speeds increase and as our understanding of complex multivariate spectroscopic data and their machine learning interpretation deepens), it will not be long before the so-called ‘rapid’ detection methods used at present are replaced by those that are truly rapid and detect quantitatively microbial spoilage in meats within seconds as opposed to hours. It is also considered that as microbial spoilage of meats is somewhat complex, these detection methods could be readily applied to a whole range of other foodstuffs where microbial spoilage processes are less multifarious. Additionally, with mathematical models that take account of environmental and physical factors such as temperature and humidity then these ‘rapid’ methods should also have the ability to accurately predict remaining shelf-life and find inclusion in HACCP systems within the food production/processing industries.

1.10 Acknowledgements

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1.11 References


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2

Detection, identification and enumeration methods for spoilage yeasts
C. P. Kurtzman, US Department of Agriculture, USA

2.1 Introduction

Microbiological spoilage of foods and beverages is caused by a wide variety of bacteria, molds and yeasts. Yeast spoilage is favored in products with low pH, generally 5.5 or lower, and by the presence of sugars, organic acids and other easily metabolized carbon sources. Many high-sugar products are also susceptible to spoilage by yeasts. Yeast spoilage is often manifested by growth on the surface of products such as cheeses and meats and by fermentation of sugars in liquid and semi-liquid products. Pitt and Hocking (1997) stated that while many yeasts occur in foods, only about ten species are the primary spoilage organisms. With an ever-increasing variety of consumer products and the need to preserve flavor and texture through minimum processing, susceptibility to spoilage has increased, thereby increasing the number of spoilage species. Additionally, molecular identification methods are showing that there is greater diversity of spoilage yeasts than previously recognized from phenotypic identification methods.

In this chapter, an overview is provided of the yeasts that cause spoilage, methods for their enumeration, isolation and identification, and a consideration of methods for the control of spoilage in products. Subsequent chapters will focus on particular groups of yeasts that cause spoilage, thus providing greater detail for each of these groups.

2.2 Relationship of yeasts with other fungi

Food and beverage spoilage yeasts include a relatively large number of
species representing both ascomycetes and basidiomycetes. Recent comparisons of gene sequences have provided an understanding of the phylogenetic relationships of yeasts with other fungi that was not possible to resolve from analyses of morphology and physiology. An additional benefit of molecular studies is that gene sequence determinations also provide a rapid, accurate means for identification of individual species, either through direct comparisons of sequences or from development of molecular detection methods based on the sequences.

The relationship of yeasts with other fungi has been uncertain. Some have viewed the yeasts as primitive fungi, whereas others perceived them to be reduced forms of more evolved taxa (Cain, 1972; Redhead and Malloch, 1977). Phylogenetic analyses of rDNA sequences demonstrated that the ascomycetous yeasts comprise a clade that is a sister group to the ‘filamentous’ ascomycetes (euascomycetes) (Fig. 2.1). Included in the yeast clade are such genera as Ascoidea, Cephaloascus, Eremothecium (synonym Ashbya) and Geotrichum, which were earlier believed to be euascomycetes because of their often filamentous appearance. Interestingly, Schizosaccharomyces, Taphrina, Protomyces, Saitoella, Pneumocystis and Neolecta, a mushroom-
like fungus, form a separate clade that is basal to the yeast–euascomycete branch (Hausner et al., 1992; Hendriks et al., 1992; Kurtzman, 1993b, 2003; Kurtzman and Robnett 1994, 1995, 1998; Kurtzman and Sugiyama, 2001; Landvik, 1996; Nishida and Sugiyama, 1993; Sjamsuridzal et al., 1997; Sugiyama, 1998; Wilmotte et al., 1993). Nishida and Sugiyama (1994) have informally termed the basal ascomycete clade the ‘archiascomycetes’. Some of the confusion on understanding relationships among genera arose because of growth characteristics. Certain species of Ascoidea and Eremothecium show no typical budding, whereas budding is common among the so-called black yeasts in the genera Aureobasidium and Phialophora, as well as in certain other dimorphic fungi. Similarly, vegetative reproduction by fission is shared by Dipodascus and Galactomyces, members of the yeast clade, as well as by the distantly related genus Schizosaccharomyces. Consequently, ascomycetous yeasts cannot be recognized solely on the basis of presence or absence of budding, but with a few exceptions, they can be separated phenotypically from euascomycetes by the presence of budding or fission and the formation of sexual states unenclosed in a fruiting body. Currently accepted ascomycetous yeast genera are listed in Table 2.1.

Because ascomycetous yeasts were widely encountered, recognition that some yeasts are basidiomycetes was slow to happen. The presence of ballistoconidia in Sporobolomyces led Kluyver and van Niel (1924, 1927) to suggest that some yeasts might be basidiomycetes. A clear connection was provided by Nyland’s (1949) description of the teliosporic genus Sporidiobolus, which was followed by Banno’s (1967) description of a teliosporic life cycle in the red yeast Rhodosporidium toruloides. These discoveries were followed by descriptions of several other teleomorphic genera including Filobasidium (Olive, 1968), Leucosporidium (Fell et al., 1969), Filobasidiella (Kwon-Chung, 1975), Cystofilobasidium (Oberwinkler et al., 1983) and Bulleromyces (Boekhout et al., 1991). In contrast to ascomycetous yeasts, which represent a single clade, basidiomycetous yeasts occur in all three large phylogenetically resolved clades of the Basidiomycota, i.e. the classes Hymenomycetes, Uredinomycetes and Ustilagenomycetes (Swann and Taylor, 1995) (Fig. 2.1). Yeasts are found within four major clades of the Hymenomycetes: Tremellales (jelly fungi), Trichosporonales, Filobasidiales and Cystofilobasidiales. The Uredinomycetes, most commonly known for the rust fungi, include four major clades of yeasts and related genera: Agaracostilbales, Microbotryales, Sporidiobolales and the Naohidea clade. Of the Ustilaginiales, the majority are plant and fungal parasites, with the smuts as well-known examples. Sampaio (2004) reported three major groups in the Ustilaginiales: Entorrhizomycetidae, Exobasidiomycetidae and Ustilaginomycetidae. Yeasts are found in the latter two subclasses. Currently accepted basidiomycetous yeast genera are given in Table 2.2. As with the ascomycetes, basidiomycetous yeasts can be recognized from a combination of budding or fission and sexual states unenclosed in fruiting bodies.

Much of what is known about the molecular phylogeny of yeasts is based
### Table 2.1 Classes, orders and families of yeasts and yeastlike genera of the Ascomycota

<table>
<thead>
<tr>
<th>Class</th>
<th>Order</th>
<th>Family</th>
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<tr>
<td><strong>Neolectomycetes</strong></td>
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<td>Neolecetales Landvik, O. E. Eriksson, Gargas &amp; P. Gustafsson</td>
<td>Neolectaceae Redhead</td>
<td>Neolecta Spegazzini (T)</td>
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<td><strong>Pneumocystidomycetes</strong></td>
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<td><strong>Schizosaccharomycetes</strong></td>
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<tr>
<td>Schizosaccharomycetales Prillinger, Dörfler, Laaser, Eckerlein &amp; Lehle ex Kurtzman</td>
<td>Schizosaccharomycetaceae Beijerinck ex Klöcker</td>
<td>Schizosaccharomyces Lindner (T)</td>
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<td><strong>Taphrinomycetes</strong></td>
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<td>Taphrinales Gäumann &amp; C. W. Dodge</td>
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<td>Protomyces Unger (T)</td>
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<td>Protopancyopsis Magnus (T)</td>
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<td>Lalaria R. T. Moore (A)</td>
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<td><strong>Saccharomycetes</strong></td>
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<td>Galactomyces Redhead &amp; Malloch (T)</td>
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<td>Dipodascopsis Batra &amp; Millner (T)</td>
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<td><em>Pachysolen</em> Boidin &amp; Adzet (T)</td>
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<td><em>Schizoblastosporion</em> Ciferri (A)</td>
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<td><em>Starmerella</em> Rosa &amp; Lachance (T)</td>
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<td><em>Starmera</em> Y. Yamada, Higashi, S. Ando &amp; Mikata (T)</td>
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<td><em>Stephanosascus</em> M. Th. Smith, van der Walt &amp; Johannsen (T)</td>
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Detection, identification and enumeration methods for yeasts

Table 2.1  Continued

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<tr>
<td>Sympodiomyces</td>
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<td>Zygoascus</td>
<td>M. Th. Smith (T)</td>
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1 (A) = anamorphic genus, (T) = teleomorphic genus.  
2 Anamorphic and teleomorphic genera are placed together in the same family when relationships are known. For many anamorphic and teleomorphic genera, phylogenetic relationships are unclear and the genera are placed in Saccharomycetales *incertae sedis* until family relationships become known.

Table 2.2  Classes and orders of yeasts and yeast-like genera of the Basidiomycota

<table>
<thead>
<tr>
<th>Class</th>
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<tr>
<td>Hymenomycetes</td>
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<td>Cystofilobasidiales</td>
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<td>Guehomyces</td>
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<td>Iteronilia</td>
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<tr>
<td>Mrakia Y. Yamada &amp; Komagata</td>
<td>(T)</td>
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<tr>
<td>Phaffia Miller, Yoneyama &amp; Soneda</td>
<td>(A)</td>
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<tr>
<td>Tausonia Bab’eva</td>
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<tr>
<td>Udeniomyces Nakase &amp; Takematsu</td>
<td>(A)</td>
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<td>Xanthophyllomyces</td>
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<td>Filobasidiales Julich</td>
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<tr>
<td>Cryptococcus</td>
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<tr>
<td>Filobasidium</td>
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<td>Trichsporonales</td>
<td>Boekhout &amp; Fell</td>
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<td>Dioszegia Zsolt emend. Takashima, Deak &amp; Nakase</td>
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<td>Fellomyces Y. Yamada &amp; Banno</td>
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**Microbotryales**

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**Naohidea Clade**

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**Sporidiobolales** *Sampaio, Weiss & Bauer*

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**Ustilaginomycetes**

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1 (A) = anamorphic genus, (T) = teleomorphic genus.
2 Some genera, such as the anamorphic genus *Cryptococcus*, are presently polyphyletic as defined, and members of the genus are found in more than one teleomorphic order.
3 From Kurtzman and Fell (2005).

On single gene analyses, usually rDNA. Comparisons with other genes have shown no major conflicts with phylogenies derived from rDNA. However, for single gene analyses, basal branches in phylogenetic trees are weak, leading to uncertainty about relations between clades. Kurtzman and Robnett (2003) analyzed species in the ‘Saccharomyces complex’ from a concatenation of seven genes, resulting in generally strong basal support for clades that were interpreted as genera, but interclade support was often weak. Rokas *et al.* (2003) compared 106 orthologous genes from the seven presently accepted species of *Saccharomyces* and concluded that a
concatenation of a minimum of any 20 genes gave well-supported trees that were comparable to a dataset comprising 106 genes. Consequently, these studies clearly illustrate that a large number of genes are required for reconstructing robust species phylogenies and understanding the boundaries of the genera to which they are assigned.

2.3 Products affected by yeasts

Yeasts affect a wide variety of products and show some degree of specialization. Products with high sugar content or low pH, such as soft drinks, syrups and salad dressings, are often spoiled by species of *Zygosaccharomyces* and *Torulaspora* (Chapter 10), whereas beer and wine are susceptible to *Brettanomyces*, *Saccharomyces* and a number of other genera (Chapters 11 and 13). Spoiled meats and cheeses often have high counts of *Debaryomyces*, *Yarrowia* and *Rhodotorula*. In addition to information provided in the following chapters, Fleet (1992) has given comprehensive lists of yeast species found in various foods and beverages. In view of this diversity of taxa, correct identification of species is often a challenge.

2.4 Detection, enumeration, isolation

The methods presented here can be used for quality control of ingredients, newly manufactured products and the manufacturing environment as well as for spoiled products. An initial question when examining spoiled products is whether the changes seen result from microbial growth, unstable formulations or extreme storage conditions. For heavily spoiled products, the spoilage organisms can often be detected by bright field light microscopy at a magnification of 400–500. Yeasts, as well as bacteria, are readily stained for microscopic observation by using crystal violet. A small drop of 0.5% crystal violet may be mixed directly with the product on a microscope slide or mixed with a drop of product that has been diluted 1:10 with distilled water. Since there are occasions when viable microorganisms are not recovered from spoiled product, microscopic detection provides a clue to the identity of the spoilage organism.

A variety of media have been proposed for isolation of yeasts from spoiled products. Pitt and Hocking (1997) recommended dichloran rose bengal chloramphenicol (DRBC) medium for both molds and yeasts. The advantage of this medium is that the spreading growth of mold colonies is restricted, allowing more accurate colony counts on crowded plates. Plates with this medium must be incubated in the dark to prevent formation of photo-induced inhibitors. Most spoilage yeasts usually grow well at 25°C on standard YM agar (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 20 g agar,
1 liter water, pH 5 to 7); consequently, this medium can be used for detection as well as enumeration by standard plate count methods. A sample diluent of 0.1% peptone water is effective. Spread plates, rather than pour plates, are recommended for both isolation and enumeration because the increased aeration of surface growth favors recovery and subsequent growth of the yeast cells. Because of its near neutral pH, YM agar also favors growth of bacteria, which can be inhibited either by adjusting the pH to 4.5 with hydrochloric acid or by adding antibiotics. Use of antibiotics is preferred over pH adjustment, and the choice of antibiotics varies somewhat. Tetracycline (30 mg/l) or a combination of chlortetracycline and chloramphenicol, each at a concentration of 100 mg/l, are often effective (Koburger and Marth, 1984; Kurtzman and Smittle, 1984).

Other plating media typically used by food microbiologists include malt extract agar (MEA) and tryptone glucose yeast extract agar (TGY), the latter often in preference to YM agar. For rapid, presumptive detection of preservative-resistant species such as *Z. bailii* and *Z. bisporus*, two media routinely used are acidified MEA (MAA, i.e. malt acetic agar) and acidified TGY (TGYA) (Pitt and Hocking, 1997). In both cases, the basal medium (MEA or TGY) is supplemented with 0.5% acetic acid. Yeast colonies are usually visible within 3–7 days after sample plating, and more than one yeast species can be present in spoiled products, which may not be initially evident because of similarities in colony appearance. For *Zygosaccharomyces* (e.g., *Z. bailii*) and certain other species, Hocking (1996) reported that the time required to detect their presence in products could be reduced by incubating agar plates at 30°C, instead of at 25°C. Because some species are osmophilic (e.g. *Z. bailii*, *Z. bisporus*, *Z. rouxii*), diagnostic plating can include use of a high osmotic medium comprising YM or TGY agars supplemented with 40% glucose. If these media are used, the sample diluent should be high osmotic as well, e.g. 0.1% peptone water with 40% glucose.

A number of specialized detection media have been developed for species of *Zygosaccharomyces* and other yeasts with reports of varying degrees of effectiveness (Beuchat and Cousin, 2001; Beuchat et al., 2001; Loureiro and Malfeito-Ferreira, 2003; Pitt and Hocking, 1997). Among these is lysine agar, which is used to selectively distinguish non-*Saccharomyces* species when they occur with *Saccharomyces cerevisiae*, because the latter species cannot utilize lysine as a nitrogen source (Heard and Fleet, 1986). Psychrophilic species of *Mrakia* and *Rhodosporidium* occasionally have been isolated from refrigerated condiments, so isolation plates of these food products may need to be incubated at 5–10°C for several weeks if low-temperature spoilage is occurring. Additional, more specialized protocols are given in later chapters of this book.

Another concern in microbiological analysis of foods is the presence of yeast cells with sublethal injuries. These cells may have been damaged in processing, but might eventually recover to spoil the product. If cells with sublethal injuries are present during initial quality control analysis, will they
Detection, identification and enumeration methods for yeasts

be detected on standard plating media? The literature, which has been reviewed by Fleet (1990), is somewhat conflicting. Beuchat et al. (2001) showed that plating media do affect detection of yeasts isolated from desiccated products, suggesting that cells with sublethal injuries may recover on some media but not on others.

Infections arising from pathogenic yeasts that originate in spoiled foods appear not to be a problem, but if there are concerns, isolation plates can be incubated at 35–37°C to favor these species. The fact should not be ignored that such opportunistic human pathogens as Debaryomyces hansenii, Candida zeylanoides and Yarrowia (Candida) lipolytica are common food spoilage organisms (Fleet, 1992). Another aspect of human health is allergies to yeasts but, again, there are no data to suggest that spoilage yeasts are associated with this problem.

Once spoilage yeasts are isolated and purified, they can be maintained by growing on YM or 5% ME agar at 25°C for 2–3 days followed by storage for several months in a refrigerator before the need to retransfer. Exceptions are species of Dekkera (anamorph Brettanomyces), which often die in culture after a week or two because of accumulation of acetic acid.

A rapid means for temporary preservation is to suspend the yeast cells in 10% glycerol and freeze at –80°C. Long-term storage should be by lyophilization or freezing in the vapor phase of liquid nitrogen.

As seen in the preceding discussion, the primary means for detecting spoilage organisms is the plating of samples and then waiting for the spoilage organisms to appear. Because of the time required for this approach, alternate technologies have been developed to detect spoilage organisms in far less time. One of these, electrical impedance, is based on the observation that microorganisms change the electrical properties of a substrate by their growth. Henschke and Thomas (1988) used electrical impedance to detect wine spoilage yeasts and discussed some of the problems associated with its use. Measurement of ATP by bioluminescence is another means for estimating the concentration of microorganisms in foods and beverages because all living cells have ATP. This method has shown promise for detection of spoilage organisms in beer (Miller and Galston, 1989), but it cannot be used to identify individual species. However, another possibility for beverages is to pass the product through a membrane filter, stain the cells and do a direct count microscopically. Certain dyes reliably differentiate between living and dead cells (Betts et al., 1989). In addition to the preceding, several DNA-based methods have been developed for rapid detection and quantitation of spoilage species and these are discussed in Section 2.7.

2.5 Identification by conventional methods

In 5–10 years, we will probably regard molecular-based methods as conventional for yeast identification, but at this time (2006) conventional
methods include determining cell morphology, formation of a sexual state, sugar fermentation and growth on various carbon and nitrogen compounds. A recurring question is how accurate are species identifications from conventional methods? Yeast species described before ca. 1980 were recognized from conventional phenotypic methods and more than half are valid when compared by molecular methods (Kurtzman and Fell, 1998). In a recent yeast identification workshop, about three-quarters of the ‘unknowns’ were correctly identified by the participants when using conventional methods. Consequently, phenotypic methods are still useful, but they are labor intensive and often slow. The culture media and methods for their preparation, as well as methods for preparing and conducting fermentation and assimilation tests, were given in detail by Yarrow (1998), and this work should be consulted before undertaking phenotypic characterization, which, briefly summarized, includes the following:

- Cell division. Presence of budding or fission and morphology of the cells.
- Presence or absence of hyphae and pseudohyphae.
- Presence or absence of a sexual state.
- Ability to ferment sugars.
- Growth on specified carbon and nitrogen compounds and on other diagnostic media.

2.5.1 Vegetative growth
Freshly grown cultures, e.g. 1–3 days, 25°C, YM agar, should be examined microscopically to determine the nature of cell division since this will provide essential initial guidance to identification. For ascomycetous yeasts, budding may be: (1) multilateral, in which buds are not restricted to particular locations on the cell; (2) bipolar, in which budding occurs successively on a relatively broad base at the poles of the cell, or; (3) by fission, in which a crosswall bisects the cell into two halves, prompting separation (Fig. 2.2). Species of *Saccharomyces*, *Kluyveromyces*, *Zygosaccharomyces* and *Pichia*, for example, divide by multilateral budding, whereas *Hanseniaspora*, *Saccharomycodes*, *Nadsonia* and *Wickerhamia* show bipolar budding. *Schizosaccharomyces* divides exclusively by fission, as is the case for species assigned to *Geotrichum*, *Dipodascus* and *Galactomyces*, even though the latter three genera are only distantly related to *Schizosaccharomyces*.

Basidiomycetes also typically show several types of budding. Many divide by polar budding, which is on a narrow base at or near the poles of the cell. The genus *Malassezia*, which is unlikely to be found in foods, exhibits polar budding on a broad base. Additionally, some basidiomycetes show typical multilateral budding whereas others, such as *Trichosporon*, reproduce by both budding and fission. When young (24–48 h), rapidly dividing cultures are examined, the type of budding is usually easily recognized. Cells that
form by fission soon round at the site of division, so it may not be apparent how they formed.

In the past, taxonomic importance was accorded to the presence or absence of pseudohyphae and true hyphae. Pseudohyphae are filaments formed by budding and the cells are generally elongated. The point of attachment between cells is usually noticeably constricted (Fig. 2.2e). True hyphae have distinctly septate crosswalls (Fig. 2.2f). The genus *Candida* and its synonym *Torulopsis* were separated by the criterion of pseudohypha formation, but it was recognized that some species classified as *Torulopsis* developed pseudohyphae whereas not all strains of a species of *Candida* formed this type of mycelium. Similarly, formation of true hyphae can be strain variable. The search for pseudohyphae and true hyphae is usually done on a Dalmau plate, which is a Petri plate containing either yeast morphology agar or corn meal agar. A microscope coverglass is placed over the point of inoculation, and the resulting reduced oxygen under the coverglass favors formation of hyphae and pseudohyphae. Dalmau plates are usually examined 7–10 days after inoculation, but longer incubation periods are sometimes useful.

### 2.5.2 Sexual states

The formation of a sexual state may be preceded by conjugation. Conjugation can occur between independent cells or between a cell and its bud (Fig. 2.3). If a bud serves as a conjugant, the neck often slightly elongates and the wall of the bud may appear thinner when viewed under the microscope. Species with conjugation between a cell and its bud are almost certainly homothallic. Conjugation between independent cells signals homothallism as well, but cultures of diploid, heterothallic species may produce ascospores that germinate prior to observation, providing mating types that undergo conjugation. Unconjugated asci are formed by both homothallic as well as heterothallic diploid species. Failure of a species to form a sexual state may indicate that

![Fig. 2.2 Vegetative reproduction: (a) multilateral budding; (b) bipolar budding; (c) polar budding on a broad base (left) and on a narrow base (right); (d) fission; (e) pseudohyphae; (f) true hyphae.](image-url)
necessary conditions have not been met or that the culture represents a mating type.

The morphology of the sexual state has often been used to define genera, but recent molecular comparisons have shown that some phylogenetically divergent groups have similar teleomorphic states. For example, among the ascomycetes, hat-shaped ascospores are common to the major clades of the polyphyletic genus *Pichia* as well as to some other genera. In contrast, species of the genus *Debaryomyces* show a surprising variety of ascospore shapes, which include spherical with a roughened surface, ellipsoidal with ridges, and lenticular with a smooth surface. However, in certain cases, morphology may be unique to phylogenetically defined groups. *Cephaloascus* has clusters of asci borne on branched ascophores, species of *Metschnikowia* have needle-shaped ascospores, and *Zygosaccharomyces* species generally produce conjugated asci that are ‘dumbbell’ shaped. The range of ascospore shapes commonly encountered is illustrated in Fig. 2.4. Asci may be persistent at maturity or become deliquescent and release the ascospores. Ascus persistence and deliquescence were previously used to define genera, but it is now recognized that these states may be species-specific or even strain-specific for some species. Consequently, the diagnostic value of sexual states varies, but some have sufficient unique features to be useful for identification.

Basidiomycete sexual states among yeasts are characterized by the basidium developing from a teliospore, typical of the rust and smut fungi, or arising

![Fig. 2.3](image1) Types of cellular conjugation: (a) conjugation between independent cells; (b) conjugation between a cell and its bud, which has slightly elongated.

![Fig. 2.4](image2) Common ascospore shapes and typical genera: (a) hat-shaped, *Pichia*; (b) spherical and smooth, *Saccharomyces*; (c) Saturn-shaped and smooth, *Saturnispora*. (d) spherical and rough, *Debaryomyces*; (e) Saturn-shaped and rough, *Debaryomyces* (*Schwanniomyces*); (f) elongated with a whip-like appendage, *Eremothecium*; (g) needle-shaped, *Metschnikowia*. 
directly from hyphae, typical of some of the mushrooms such as *Tremella* (Fig. 2.5). The range in types of basidia is shown by the various authors of *The Yeasts, A Taxonomic Study* (Kurtzman and Fell, 1998). Some species forcibly eject basidiospores from the basidia whereas others do not. If basidiospore formation is abundant, ejected spores will form a visible layer on the lid of an inverted Petri plate culture. Additionally, some species produce ballistoconidia, and these asexually produced cells, as the name implies, are also forcibly ejected much like the meiotically produced basidiospores.

Conditions for formation of sexual states vary considerably. Commonly used agar media include YM, 5% ME, McClary’s acetate, V-8 juice, either full strength or diluted, corn meal and even water agar (Yarrow, 1998). Cultures should be incubated at 15 and 25°C. Some basidiomycetes may even require 5–10°C. Times for formation of ascospores or basidiospores vary considerably. Ascospores may form within a few days to 3 weeks, but examining cultures for up to 2 months is often prudent, especially for those incubated at lower temperatures. Basidiomycetes may take even longer to form sexual states, and when teliospores are formed, special conditions, such as incubation on water agar in a refrigerator, may be needed to induce germination. Detection of ascospores and basidiospores is generally done using bright field microscopy at a magnification of 400–500. Phase contrast is sometimes helpful, as is oil immersion, because the additional magnification and resolution are useful to determine if an object is an inclusion or an ascospore with definite walls. Ascospore staining with malachite green or other dye is usually not particularly helpful.

### 2.5.3 Fermentation and growth tests

The fermentation, assimilation and other growth tests common to yeast taxonomy are often used to identify species even if morphological features are not examined. The traditional way of conducting these tests is in liquid media in test-tubes. Shaking the tubes facilitates growth, and the reactions

![Fig. 2.5 Basidiomycete sexual states: (a) teliospore with basidium and basidiospores; (b) basidium with basidiospores arising directly from a hypha.](image)
Food spoilage microorganisms are usually monitored over a 3–4 week incubation period. Alternative methods include agar media, which should also be monitored as long as possible because growth on some compounds may not develop until after 1–2 weeks of incubation. Once data are collected, a physiological key to species can be used, such as the one presented in the 4th edition of *The Yeasts, A Taxonomic Study* (Kurtzman and Fell, 1998). It should be emphasized that results from various test methods may vary a bit, which can lead to erroneous identifications. Because many species have similar physiological profiles, knowledge of the microscopic appearance of vegetative and sexual states may be critical for correct identification.

A number of rapid physiological tests have been developed that rely on either a relatively few specially selected diagnostic reactions and/or are based on rapid plate assays (Deák, 2003). Deák and Beuchat (1987) developed a diagnostic key for food yeasts that was based on 15 physiological tests, but the key was reported unreliable when tested by others (Rohm and Lechner, 1990). The API test kits for medically important yeasts have been used by some, but the tests included are often not resolving for food spoilage yeasts. Heard and Fleet (1990) developed a microtiter tray procedure that utilized a relatively large number of growth tests, potentially increasing the reliability of the identifications. However, if there are a certain percentage of misidentifications when using standard tests, results from rapid tests should be expected to be no better.

### 2.6 Molecular methods for species identification – an overview

The transition from phenotypic identification of yeasts to molecular identification began with determination of the mol% guanine + cytosine ratios of nuclear DNA. These analyses demonstrated that ascomycetous yeasts range from *ca.* 28 to 50 mol% G + C, whereas basidiomycetous yeasts range from *ca.* 50 to 70 mol% G + C. Depending on the analytical methods used, strains differing by 1–2 mol% are recognized as separate species (Kurtzman and Phaff, 1987; Price *et al.*, 1978).

The need for quantitative molecular assessment of genetic similarity between strains and species was satisfied, in part, by the technique of nuclear DNA reassociation or hybridization. DNA from the species pair of interest is sheared, mixed and made single-stranded, and the degree of relatedness determined from the extent of reassociation. Many different methods are used to measure this process, which can be done spectrophotometrically or through use of radioisotopes or other markers (Kurtzman, 1993a). Measurements of DNA complementarity are commonly expressed as percentage relatedness. This usage can be misleading because DNA strands must show at least 75–80% base sequence similarity before duplexing can occur and a reading is registered on the scale of percentage relatedness (Bonner *et al.*, 1973; Britten *et al.*, 1978).
1974). Experimental conditions can greatly influence extent of duplex formation, but when measured under optimum conditions, different methods of assessing DNA relatedness do give essentially the same result (Kurtzman, 1993a). Percentage DNA relatedness provides an approximation of overall genome similarity between two organisms, but the technique does not detect single gene differences or exact multiples of ploidy, although aneuploidy can sometimes be detected (Vaughan-Martini and Kurtzman, 1985).

A major question has been how to interpret DNA reassociation data. On the basis of shared phenotype, strains that showed 80% or greater nuclear DNA relatedness were believed to represent members of the same yeast species (Martini and Phaff, 1973; Price et al., 1978). This issue was also examined on the basis of the biological species concept (e.g. Dobzhansky, 1976), asking what is the fertility between strains showing varying degrees of DNA relatedness (Kurtzman, 1984a, b, 1987; Kurtzman et al., 1980a, b). In one of these studies, the heterothallic species *Pichia amylophila* and *P. mississippiensis*, which showed 25% DNA relatedness, gave abundant interspecific mating, but ascus formation was limited and no ascospores were formed. Similar results were found for crosses between *Pichia americana* and *P. bimundalis* (21% DNA relatedness) and between *Pichia alni* and *P. canadensis* (*Hansenula wingei*), the latter pair showing just 6% DNA relatedness. The varieties of *Issatchenkia scutulata*, which exhibit 25% DNA relatedness, behaved somewhat differently. Crosses between var. *scutulata* and var. *exigua* gave extent of mating and ascospore formation comparable to intravarietal crosses. Ascospore viability from these intervarietal crosses was about 5%, but sib-matings of the progeny had 17% ascospore viability. However, backcrosses to the parentals gave poor ascosporulation and very low viability, which suggest that these two varieties represent separate species. *Williopsis saturnus* is a homothallic species with five varieties that range in DNA relatedness from 37% to 79% (Kurtzman, 1987). Intervarietal fertility is reduced and varies depending on the strains crossed. Consequently, the preceding studies show that mating among heterothallic as well as homothallic taxa can occur over a wide range of DNA relatedness values, but that highly fertile crosses, which demonstrate conspecificity, seem to exhibit 70–80% or greater DNA relatedness. Because species barriers are complex and involve a number of factors, the numerical range of 70–100% DNA relatedness as indicative of conspecificity should be viewed as a prediction.

Nuclear DNA reassociation studies have had a marked impact on recognizing yeast species, but the method is time consuming and the extent of genetic resolution goes no further than that of closely related species. Gene sequencing offers a rapid method for recognizing species and resolution is not limited to closely related taxa. Peterson and Kurtzman (1991) determined that domain 2 of large subunit (26S) ribosomal RNA (rRNA) was sufficiently variable to resolve individual species. Kurtzman and Robnett (1998) expanded the preceding work by sequencing both domains 1 and 2 (ca. 600 nucleotides) of
26S ribosomal DNA (rDNA) for all known ascomycetous yeasts, thus providing a universally available database for rapid identification of known species, the detection of new species, and initial phylogenetic placement of the species. Fell et al. (2000) published the D1/D2 sequences of known basidiomycetous yeasts, thus completing the database for all known yeasts. Resolution provided by the D1/D2 domain was estimated from comparisons of taxa determined to be closely related from genetic crosses and from DNA reassociation. In general, strains of a species show no more than 0–3 nucleotide differences (0–0.5%), and strains showing six or more noncontiguous substitutions (1%) are separate species. Strains with intermediate nucleotide substitutions are also likely to be separate species. One impact of the D1/D2 database has been to permit detection of a large number of new species, which has resulted in a near doubling of known species since publication of the 4th edition of The Yeasts, A Taxonomic Study (Kurtzman and Fell, 1998). Another use is that the non-taxonomist can now quickly and accurately identify most known species, as well as recognize new species, by sequencing ca. 600 nucleotides and doing a Blast Search in GenBank. In essence, the D1/D2 database provides a ‘bar code’ system for yeast identification.

The internal transcribed spacer regions ITS1 and ITS2, which are separated by the 5.8S gene of rDNA, are also highly substituted and often used for species identification, but for many species, ITS sequences give no greater resolution than that obtained from 26S domains D1/D2 (James et al., 1996; Kurtzman and Robnett, 2003). However, Fell and Blatt (1999) were able to resolve cryptic species in the Xanthophyllomyces dendrorhous species complex that had been unresolved from D1/D2 sequence analysis, and Scorzetti et al. (2002) reported ITS sequences to provide somewhat greater resolution among many basidiomycetous species than was found for D1/D2, although, a few species were less well resolved by ITS than by D1/D2. Consequently, it appears useful to sequence both D1/D2 and ITS when comparing closely related species. The intergenic spacer region (IGS) of rDNA tends to be highly substituted and sequences of this region have been used with good success to separate closely related lineages of Cryptococcus (Diaz et al., 2000; Fan et al., 1995), Xanthophyllomyces (Fell and Blatt, 1999), Mrakia (Diaz and Fell, 2000) and Saccharomyces (Kurtzman et al., unpublished). Because of the occurrence of repetitive sequences and homopolymeric regions, IGS tends to be difficult to sequence for some species. Small subunit (18S) rDNA, which has been extremely important in broad-based phylogenetic analyses, is generally too conserved to allow separation of individual species (James et al., 1996; Kurtzman and Robnett, 2003).

The focus of the discussion on species identification from gene sequences has been on rDNA. A major advantage of rDNA is that it is present in all living organisms, has a common origin, occurs as multiple copies and is easy to sequence because primer pairs for conserved regions can generally be used for all organisms. However, gene sequences other than those of the rDNA repeat have been used for separation of species from many kinds of
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fungi (Geiser et al., 1998; O’Donnell et al., 2000), including the yeasts. Belloch et al. (2000) demonstrated the utility of cytochrome oxidase II for resolution of Kluyveromyces species, Daniel et al. (2001) successfully used actin-1 for species of Candida, and Kurtzman and Robnett (2003) showed the usefulness of elongation factor 1-α and RNA polymerase II for resolution of Saccharomyces species. At present, the main impediment to widespread use of other gene sequences is developing sequencing primers that are effective for essentially all species, and construction of databases that include sequences from all known species. Daniel et al. (2001) and Daniel and Meyer (2003) have made considerable progress in development of an actin sequence database for species identification, although no primer set has been effective for all species, thus requiring additional primers to obtain these sequences. The need for multiple primers seems to be a problem common to sequencing of protein-encoding genes because of frequent nucleotide substitutions throughout the gene sequences. Resolution of taxa from actin is somewhat greater than from D1/D2 but, not surprisingly, clear separation of closely related species is not always certain.

Despite the convenience and utility, separation of species using single gene sequences is not always reliable. Different lineages may vary in their rates of nucleotide substitution for the diagnostic gene being used, thus confusing interpretation of genetic separation, and hybrids are common and appear to be part of the speciation process. For example, Vaughan-Martini and Kurtzman (1985) proposed from DNA reassociation studies that Saccharomyces pastorianus is a natural hybrid of S. cerevisiae and S. bayanus. Peterson and Kurtzman (1991) confirmed the proposal by showing that the D2 domain rRNA sequence of S. pastorianus is identical to that of S. bayanus, but divergent from S. cerevisiae. The three varieties of Candida shehatae may also represent hybrids, or they are examples of a lineage with a slow rate of nucleotide substitution in the rDNA. From DNA reassociation, the varieties show ca. 50% relatedness, but they have essentially identical domain 2 large subunit sequences (Kurtzman, 1990). Groth et al. (1999) discovered a natural chimeric isolate of Saccharomyces with genetic material from three species, and Nilsson-Tillgren et al. (1981) presented evidence that several natural and industrial yeast strains are hybrids. Kurtzman et al. (2005) reported that Kazachstania heterogenica appears to be a natural hybrid that shares an RNA polymerase II gene with K. pintolopesii. In an additional study, Lachance et al. (2003) found strains of Clavispora lusitaniae that are highly polymorphic in the D1/D2 domain, but that produce ascospores on mating. Detection of unexpected divergence in a gene sequence should be possible from its lack of congruence with other gene sequences. Single gene sequences are extremely useful for rapid species identification, but from the foregoing examples, some caution in interpretation of species identity is required.
2.7 Rapid molecular methods for species identification and quantitation

2.7.1 Species identification

Rapid methods commonly used for species identification include species-specific primer pairs and probes, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP) and karyotyping. Species-specific primers are effective when used for polymerase chain reaction (PCR)-based identifications involving a small number of species or when a particular species is the subject of the search (Chapman *et al.*, 2003; Fell, 1993; Mannarelli and Kurtzman, 1998). Otherwise, there is the likelihood that PCR mixtures containing large numbers of species-specific primer pairs will lead to uncertain banding patterns. Microsatellite-primed RAPDs (Gadanho *et al.*, 2003) and AFLP fingerprints (de Barros Lopes *et al.*, 1999) have been effectively used in some laboratories. One concern in using these latter two techniques is reproducibility between laboratories because small differences in PCR conditions may impact the species-specific patterns that serve as reference. Karyotyping with pulse field gel electrophoresis and RAPD on mitochondrial DNA can serve in the initial characterization and identification of yeast species. However, the interpretation of the chromosome band patterns and mitochondrial restriction fragments for taxonomic purposes is complicated by the high degree of polymorphisms, such as chromosomal rearrangements within some yeast taxa (Špírek *et al.*, 2003). However, these polymorphisms may be useful for detecting particular lineages.

High-throughput probe hybridization methods for detection of multiple species in samples or groups of samples offer the flexibility and speed needed by diagnostic laboratories. One such method (Diaz and Fell 2004; Page and Kurtzman 2005) is an adaptation of the Luminex xMAP technology (Luminex Corp), which consists of a combination of 100 different sets of fluorescent beads covalently bound to species-specific capture probes. Upon using either hybridization or the primer extension method, the beads bearing the target amplicons are classified in a flow cytometer by their spectral addresses with a 635nm laser. The hybridized, biotinylated amplicon is quantitated by fluorescence detection with a 532nm laser. The multiplex assay is specific and fast; species that differ by one nucleotide can often be discriminated and the assay can be performed, after amplification, in less than 50 min in a 96-well format with as many as 100 different species-specific probes per well. The advantage of this method for the food microbiology laboratory is that multiple species can be quickly identified from multiple samples.

2.7.2 Species identification and quantitation in foods and beverages

The molecular detection methods discussed in earlier sections are not designed to quantitate populations in spoiled products, although the Luminex system
has this capability, it has not yet been developed for yeasts. A number of factors affect detection and quantitation and include: (1) cellular copy number of the gene to be used, (2) whether the gene is sufficiently conserved to be PCR amplified by ‘universal’ primers that will detect all species of interest, (3) efficiency of DNA extraction from cells in the product, (4) efficiency of DNA recovery from the product, (5) product ingredients that may interfere with DNA recovery or PCR amplification, and (6) level of cell population detectable.

Denaturing gradient gel electrophoresis (DGGE) is a promising technique that has been used for species identification and quantitation of yeast populations in foods and beverages. The technique is based on separation of DNA fragments of differing nucleotide sequences (e.g. species-specific) through decreased electrophoretic mobility of partially melted double-stranded DNA amplicons in a polyacrylamide gel containing a linear gradient of DNA denaturants (a mixture of urea and formamide). A related technique is temperature gradient gel electrophoresis (TGGE), in which the gel gradient of DGGE is replaced by a temperature gradient (Muyzer and Smalla, 1998). Recent applications of DGGE include identification and population dynamics of yeasts in sourdough bread (Meroth et al., 2003), in coffee fermentations (Masoud et al., 2004) and on wine grapes (Prakitchaiwattana et al., 2004). Levels of detection are often around $10^3 \text{cfu ml}^{-1}$, but $10^2 \text{cfu ml}^{-1}$ have been reported, which compares favorably with standard plate count methods. Prakitchaiwattana et al. (2004) provided information on mixed species populations, noting that when the ratio of species is not greater than 10–100-fold, detection of individual species was possible, but if the ratio exceeds 100-fold, the low population species will not be detected. Masoud et al. (2004) and Prakitchaiwattana et al. (2004) reported detection of species by DGGE that were not recovered by plating, suggesting that some yeasts may establish significant populations in a product and then die. Yet another rapid, quantitative molecular method is real-time PCR. This technique is becoming widely employed in food and beverage analyses and has been used to detect and quantitate spoilage yeasts in orange juice (Casey and Dobson, 2004) as well as in wine fermentations (Cocolin et al., 2001).

Peptide nucleic acid (PNA) probes offer another means for detection and quantitation of species in products through fluorescence in situ hybridization (FISH). PNA probes have a peptide backbone to which are attached nucleotides complementary to a species-specific target sequence, and a fluorescent label is added for detection by fluorescence microscopy (Stender et al., 2001). If probes are complementary to rRNA, the whole cell of the target species will ‘glow’ when visualized, which will also allow quantitation by cell counts. An advantage is that a product can be diluted and directly probed. One disadvantage is that probes must be developed for each species of interest. PNA technology has been effective for detection of Dekkera (Brettanomyces) bruxellensis in spoiled wine (Stender et al., 2001) and for detection of Candida albicans in blood samples (Rigby et al., 2002).
In summary, rapid detection, accurate identification and quantitation of yeasts is now possible through use of a variety of molecular methods. Increased application of these methods will bring a greater degree of clarity to studies in food microbiology, which previously was not possible when yeasts were identified from phenotype.

2.8 Future trends

2.8.1 Identification of spoilage yeasts
At present, most food microbiology laboratories identify spoilage yeasts using phenotype-based methods, and these methods are laborious and time consuming with often uncertain results. In contrast, molecular methods offer a fast, accurate means for identification and are well suited to recognizing new spoilage species. Currently, the most reliable molecular method is sequencing diagnostic nuclear- and mitochondrial-encoded genes, but the process of gene sequencing is generally not easily usable in most diagnostic laboratories. Consequently, rapid, easy-to-use molecular identification systems need to be developed. Some of these systems are nearing commercialization and include diagnostic probes and chip-based systems developed from species-specific gene sequences. Through the use of PCR-based typing techniques, such as microsatellite PCR fingerprinting (van der Vossen and Hofstra, 1996), AFLP typing (Vos et al., 1995) and RAPD analysis (Williams et al., 1990), the opportunity now exists for differentiating individual spoilage strains, either of the same or different species, and identifying their sources of origin (Baleiras Couto et al., 1996). However, these typing techniques are likely to be soon replaced by probes, chips and other such methods. Another pressing need is quantitation of spoilage organisms in products. This is essential for initial quality control as well as for examination of spoiled products. DGGE and real-time PCR offer possibilities for this requirement.

2.8.2 New methods for food preservation
One of the greatest challenges facing food producers is development of more effective preservation methods. Some of these new methods are presented in Chapter 10, and others have been discussed by Gould (1996) and Raso and Barbosa-Canovas (2003). Multi-parameter modeling represents a new approach for development of effective preservation methods (e.g. Reyns et al., 2000), and Brul et al. (2002) discussed a genomics approach in which the effect of preservation processes on food spoilage yeasts can be followed by measuring gene expression using DNA micro-array technology. These approaches, along with additional studies of yeast physiology, will markedly lessen the threat of food and beverage spoilage.
2.9 Sources of further information and advice

The following references are especially noted and provide extensive information on yeasts causing food spoilage, methods for their isolation and information on identification and taxonomy.

Spoilage yeasts
Yeasts as spoilage organisms (Walker and Ayres, 1970).

Yeast taxonomy and systematics
*Yeasts: Characteristics and Identification* (Barnett et al., 2000).

Species identification by sequencing
Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences (Kurtzman and Robnett, 1998).
Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis (Fell et al., 2000).

Detection of species and populations by denaturing gradient gel electrophoresis (DGGE) and real-time PCR
Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes (Prakitchaiwattana et al., 2004).
Direct identification of the indigenous year in commercial wine fermentations (Cocolin et al., 2001).

2.10 References

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KURTZMAN C P (1984b), ‘Resolution of varietal relationships within the species Hansenula


3

Detection, enumeration and identification methods for spoilage molds

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3.1 Introduction

Molds are filamentous fungi that are involved in food spoilage, fermentation of plant and animal foods, and production of mycotoxins by select species. Mold growth in foods can be controlled by preventing spores from entering foods, adjusting storage temperatures and humidity, altering oxygen levels, using chemical preservatives and related measures; however, these methods do not prevent molds from altering food quality and safety in every situation. Therefore, it is necessary to detect molds in food and in the processing, packaging and storage environments. The early methods to enumerate and identify molds in foods were based on bacterial detection where the plates were simply incubated for longer times and filamentous colonies were examined microscopically by using water mount slides (Tanner, 1919). When research in the 1930s for the detection of molds in dairy products was done, microbiological media were acidified or antibiotics were added to prevent bacteria from growing and, thereby, allow molds and yeasts to grow (Koburger, 1976); however, no specific medium was best and potato dextrose agar acidified to pH 3.5 with tartaric acid became widely used for enumeration of yeasts and molds. Acidified media were replaced by media with antibiotics, chemicals or dyes in an effort to inhibit bacteria and allow yeasts and molds to grow (Koburger, 1976).

By the 1970s, methods designed specifically for mold isolation, enumeration and identification from foods were researched and evaluated by food microbiologists and mycologists worldwide through collaborative studies, and, eventually, these mycologists formed the International Commission on Food Mycology (ICFM) in 1990 (http://www.foodmycology.org). There were
five international workshops under the guidance of this group whose intent is to standardize and promote the international adoption of ‘methods for mycological examination of foods’.

Methods used to detect and enumerate molds in foods are listed in Table 3.1. There is a need to develop more rapid methods for mold detection that could be used for on-line applications in real-time quality assurance during food processing. Also, new methods are needed to rapidly assess the quality of raw ingredients, to determine if mycotoxin-producing molds are present, and to determine if molds involved in occupational-related allergenic reactions (Gravesen et al., 1994; Lehrer and O’Neil, 1992) are present before foods are processed. New methods that save time, labor, materials and costs are needed to replace current methods. Although rapid detection methods for molds lag behind methods developed to detect bacteria, new areas that are being researched or that have been introduced recently include immunological, molecular, flow cytometric, near infrared spectroscopic and electronic nose techniques.

3.2 Current enumeration methods and their limitations

3.2.1 Plating methods

The current methods recommended for enumeration of molds in foods are still the classical plating methods presented in Table 3.1. There are two major types of plating: direct for internal mold contamination and dilution plating for total mold count (Fig. 3.1). In the direct method, intact foods such as grains, nuts or seeds are decontaminated to remove surface molds because the intent is to determine whether molds have invaded the food particles and could outgrow once proper conditions are obtained. Various methods, which use either chlorine or ethanol in different concentrations, have been used to decontaminate the surface of foods. The use of a 2 min rinse with 0.4% chlorine or 10% household bleach, which contains about 4% chlorine, has been recommended (Pitt and Hocking, 1997; Samson et al., 2002b; Tournas et al., 2001). After discarding the chlorine, the food particles are rinsed with sterile water and a minimum of 50 (Pitt and Hocking, 1997; Tournas et al., 2001) to 100 (Samson et al., 2002b) particles are placed onto the appropriate medium with 5–20/each plate depending on the size of the food particles. These are incubated upright, preferably in plastic bags that are perforated to allow forced air to enter and prevent the accumulation of CO₂ (Samson et al., 2002b).

Some of the limitations to this direct plating method include the inability to decontaminate heavily contaminated surfaces; organic matter can denature chlorine and reduce the ability to kill surface molds; food particles may germinate on the agar and prevent molds from growing; and too many particles/plate may interfere with mold outgrowth. Pitt and Hocking (1997) suggested that the use of 70% ethanol for 2 min before using 0.4% chlorine for 2 min
<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscopic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bright field</td>
<td>Determine mold identity by microscopic structures</td>
<td>Pitt and Hocking (1997)</td>
</tr>
<tr>
<td></td>
<td>Count mold filaments or spores</td>
<td>Harrigan (1998)</td>
</tr>
<tr>
<td>Direct epifluorescent filter technique (DEFT)</td>
<td>Count mold filaments or spores that fluoresce under ultraviolet light</td>
<td></td>
</tr>
<tr>
<td><strong>Plating</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct</td>
<td>Surface decontaminate intact seeds, nuts or grains; place 5–10 particles on agar; and after incubation count molds that grow from internal contamination</td>
<td>Pitt and Hocking (1997)</td>
</tr>
<tr>
<td>Dilution plating</td>
<td>Count colonies based on number per gram of food that grow on media after a given time and temperature of incubation</td>
<td>Samson et al. (2002b)</td>
</tr>
<tr>
<td><strong>Most probable number (MPN)</strong></td>
<td>Dilution to extinction using multiple plates and probability tables</td>
<td>Hastings et al. (1986)</td>
</tr>
<tr>
<td><strong>Hydrophobic grid membrane filter (HGMF)</strong></td>
<td>Capture of molds on 0.45µm filter; count colonies by MPN</td>
<td>Downes and Ito (2001)</td>
</tr>
<tr>
<td><strong>Metabolic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Amount of ATP corresponds to number of viable cells or spores</td>
<td>Downes and Ito (2001)</td>
</tr>
<tr>
<td>Impedance</td>
<td>Cells produce metabolites that change impedance of electrical current and values can correlate to cell numbers</td>
<td>Samson et al. (2002b)</td>
</tr>
<tr>
<td>Metabolite produced</td>
<td>Detect acids, volatiles or other metabolites produced as molds grow</td>
<td></td>
</tr>
<tr>
<td><strong>Cellular components</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitin</td>
<td>Cellular component of many molds</td>
<td>Cousin (1996)</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>Major sterol in membranes of fungi</td>
<td>Gourama and Bullerman (1995b)</td>
</tr>
</tbody>
</table>
may be needed for commodities that are heavily contaminated with *Aspergillus* and *Penicillium* species. Andrews et al. (1997) reported that this prerinse with chlorine was effective for cereal grains with greater than $10^4$ mold spores/g; however, the ethanol was absorbed by some grains and killed molds such as *Alternaria* species. Rabie et al. (1997) used 80% ethanol and 3.5% hypochlorite to decontaminate barley kernels and found that disinfection with chlorine resulted in higher counts of some mold species belonging to *Aspergillus*, *Eurotium* and *Penicillium*; however, the ethanol disinfection resulted in higher counts of other *Aspergillus* species as well as species of *Mucor*, *Phoma* and *Rhizopus*. Hence, there may be no one disinfection procedure that may work to enumerate all molds that invade all food particles.

In dilution plating, a sample is weighed, blended with diluent and plated onto agar that is incubated at a set temperature for 5 days. A general scheme for dilution plating of foods for molds is presented in Fig. 3.1. Generally, both molds and yeasts have been enumerated simultaneously because molds can be distinguished from yeasts by their filamentous nature. A sample size from 5 to 400g is used depending on the homogeneity of the food and is blended in 0.1% peptone to make a 1 to 10 dilution (Pitt and Hocking, 1997; Samson et al., 2002b). If a low mold count is expected, then Samson et al. (2002b) recommend a 1:5 dilution be used in 0.1% peptone. A Stomacher for 2 min is preferred for homogenizing the sample and diluent; however, a blender may be needed for hard foods (grains, nuts, dried produce) where a 1 min blending time is recommended to prevent excessive fragmentation of
mycelia (Pitt and Hocking, 1997; Samson et al., 2002b). Each mycelial fragment that has an intact nucleus and spores of molds has the potential to develop into colonies. Hard samples should be soaked in 0.1% peptone diluent for 30 min (Samson et al., 2002b) or up to 3 h for extremely hard samples (Pitt and Hocking, 1997) to help release internal molds before blending. Additional 1:10 dilutions in 0.1% peptone can be prepared as needed. Since mold spores sediment quickly, samples should be plated within 1 min after blending (Beuchat, 1992). Spread or surface plating of duplicate 0.1 ml diluted sample is preferred over pour plating because molds develop slowly when trapped beneath the agar (Pitt and Hocking, 1997). Plates are incubated upright (prevents spores from collecting on lid) at 25°C for 5 days. After incubation, the number of plates with between 15 and 150 colonies are counted and the results are recorded as cfu/g or ml of sample (Beuchat and Cousin, 2001).

Media used for the enumeration of molds have undergone many changes over the years. Pitt (1986) suggested that the ideal medium for enumeration of molds should: (1) be nutritious enough to support fastidious molds, (2) suppress bacteria, (3) slow mold colony growth but not prevent spreading molds from growing, and (4) promote growth of the spoilage molds but suppress the growth of molds that are not involved in the spoilage of the food being evaluated. Several media are used for enumeration of molds in foods because there is no agreement worldwide on one medium. Acidified potato dextrose agar (PDA) had been used for years to enumerate molds and still is used by some food processing industries; however, it is not very nutritious, has too much glucose to adequately recover molds, and does not allow recovery of all molds because of the acids used (Koburger, 1979; Pitt and Hocking, 1997). A general purpose medium such as plate count agar (PCA) supplemented with 100 mg/l of both chloramphenicol and chlorotetracycline, followed by incubation at 20 to 25°C for 5 days was recommended over acidified PDA (Koburger, 1979). Other media that have been used included Czapek agar with 50 mg/l chloramphenicol and 50 mg/l chlorotetracycline (CZCC), dichloran rose bengal chloramphenicol agar (DRBC), dichloran 18% glycerol agar (DG18), glucose yeast extract agar with 100 mg/l chloramphenicol (GYC), malt extract agar (MEA), malt salt agar (MSA), oxytetracycline glucose yeast extract agar (OGYE), Sabouraud dextrose agar (SDA) plus 100 mg/l chloramphenicol and 100 mg/l chlorotetracycline (SABCC) and similar combinations of media and antibiotics (Hocking and Pitt, 1992; King, 1992). In the late 1970s through the 1980s, there was a global effort to standardize the methods used in mold enumeration by a group of food microbiologists and mycologists.

Based on several collaborative studies worldwide, DRBC (water activity \(a_w\) 0.99) and DG18 (\(a_w\) 0.955) have been recommended as general-purpose media for the enumeration of molds in foods using a surface or spread plating method with a 5 day incubation at 25°C for temperate regions of the world and at 30°C for tropical areas (Pitt et al., 1992). The use of these two
general-purpose media are recommended by the ICFM and information about their use and instructions for preparation can be found in Pitt and Hocking (1997) and Samson et al. (2002a). Both DRBC and DG18 are commercially available. In addition, these media and methods have been adopted in Australia (Pitt and Hocking, 1997), Canada (Douey and Wilson, 2004), several countries in the European Union (Samson et al., 2002a), and the USA (Beuchat and Cousin, 2001; Tournas et al., 2001). Several countries in Asia and Central/South America plus New Zealand use either the Compendium of Methods for the Microbiological Examination of Foods (Beuchat and Cousin, 2001) or the Bacteriological Analytical Manual (Tournas et al., 2001) for microbiological methods to enumerate molds, which recommend the use of DRBC or DG18 at 25°C for 5 days. The International Organization for Standardization (ISO) through the ISO 9000 quality series published ISO 7954 for enumeration of yeasts and molds by a colony count at 25°C in 1987 when the following media were recommended: chloramphenicol glucose yeast extract agar, PDA, or SDA (http://www.iso.org; Tortorello, 2003). The proposed new ISO method is to use DRBC for high aw foods (ISO/CD 21527.2) and DG18 for low aw foods (ISO/CD 21527.1). Based on the uses of DRBC and DG18, these media are becoming the ones of choice for the mycological examination of foods.

One limitation of the methods for enumeration of molds is that no one medium is useful for the isolation and enumeration of all molds from all foods. Hence, different media have been recommended based on the types of mold expected to be in the foods at the time of sampling. Selective media that have been recommended for the enumeration of different groups of molds from foods are presented in Table 3.2 (Beuchat and Cousin, 2001; Beuchat and Pitt, 2001; Pitt and Hocking, 1997; Pitt et al., 1992; Samson et al., 2002b). Although Beuchat and Hocking (1990) in their review of concerns for enumerating xerophiles in foods noted that either salt or sugar is needed to reduce the aw and better methods are needed because of inadequate information on stress or injury of these molds in food environments, new methods have not been developed. Since most foods do not contain only one type of mold, several media and incubation conditions may be needed for each food being analyzed in a laboratory if more than total counts are desired. Another limitation is that there is no agreement on the antibiotic to use for bacterial inhibition. Although 100 ppm of heat-stable chloramphenicol is used to inhibit bacterial growth in DRBC and DG18, Samson et al. (2002b) recommend using 50 ppm chloramphenicol and 50 ppm of fresh filter sterilized chlortetracycline (heat-labile and unstable unless refrigerated) because chloramphenicol does not inhibit bacteria from all foods.

A spiral plate count method that dispenses a known volume of diluent over an agar plate that is rotating in an Archimedes circle has been used for bacterial enumeration (Morton, 2001) but infrequently for mold enumeration (Pitt and Hocking, 1997). In this method, the amount of sample is decreased as the stylus tip moves across the plate from the center to the edge (Morton,
<table>
<thead>
<tr>
<th>Type of molds</th>
<th>Selective media</th>
<th>Incubation conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidophilic Aflatoxin producers</td>
<td>Acetic dichloran yeast extract sucrose agar (ADYS) and Aspergillus flavus and parasiticus agar (AFPA)</td>
<td>25°C for 5–7 days; 30°C for 2–3 days</td>
<td>Beuchat and Cousin (2001); Pitt and Hocking (1997); Beuchat and Cousin (2001)</td>
</tr>
<tr>
<td>Fusarium species</td>
<td>Czapek iprodione dichloran agar (CZID)</td>
<td>25°C for 5 days</td>
<td>Beuchat and Cousin (2001); Pitt and Hocking (1997); Beuchat and Cousin (2001); Samson et al. (2002b)</td>
</tr>
<tr>
<td>Heat-resistant</td>
<td>Malt extract agar (MEA) or potato dextrose agar (PDA) with 50 ppm chloramphenicol and 50 ppm chlorotetracycline</td>
<td>28–30°C for 30 days</td>
<td>Pitt and Hocking (1997); Beuchat and Pitt (2001); Samson et al. (2002b)</td>
</tr>
<tr>
<td>Ochratoxin A producers</td>
<td>Dichloran rose bengal yeast extract sucrose agar (DRYES) and dichloran yeast extract sucrose glycerol agar (DYSG)</td>
<td>20°C for 7–8 days</td>
<td>Pitt and Hocking (1997); Samson et al. (2002b)</td>
</tr>
<tr>
<td>Penicillium species</td>
<td>Dichloran rose bengal yeast extract sucrose agar (DRYES)</td>
<td>20°C for 5 days</td>
<td>Beuchat and Cousin (2001); Samson et al. (2002b)</td>
</tr>
<tr>
<td>Preservative resistant Proteinophilic</td>
<td>Acetic dichloran yeast extract sucrose agar (ADYS) and Dichloran creatine sucrose bromocresole agar (CREAD)</td>
<td>25°C for 5–7 days; 25°C for 7 days</td>
<td>Samson et al. (2002b); Frisvad et al. (1992); Frisvad (1993); Samson et al. (2002b)</td>
</tr>
<tr>
<td>Xerophilic</td>
<td>Dichloran 18% glycerol agar (DG18)</td>
<td>25°C for 7 days</td>
<td>Pitt and Hocking (1997); Beuchat and Cousin (2001); Samson et al. (2002b)</td>
</tr>
<tr>
<td>Xerophilic – extreme</td>
<td>Malt yeast 50% glucose agar (MY50G)</td>
<td>25°C for 7–21 days</td>
<td>Pitt and Hocking (1997); Beuchat and Cousin (2001); Samson et al. (2002b)</td>
</tr>
</tbody>
</table>

*Heat 100 g of product to 75°C and hold for 30 min before plating onto the agar.*
The colony count is determined by using a special counting grid that correlates the area on the plate with the volume of sample plated (Morton, 2001). Directions of the manufacturer should be followed when using this method. Zipkes et al. (1981) reported that the spiral plate count recovered 2–3.7 times more molds and yeasts than did the pour plate method using PDA with chlorotetracycline for whole oats, green beans and cheese; however, the spread plate method was also 2–3 times higher than the pour plate method. Manninen et al. (1991) reported that there were no significant differences for the pour plate method and the spiral plate count method for pure cultures of Aspergillus flavus and Penicillium camemberti; however, the laser reader had a problem counting large (10–15 mm) colonies of Rhizopus oligosporus and generally recorded them twice. When molds and yeasts from goat’s milk cheese were analyzed by the pour plate using oxytetracycline glucose yeast extract agar and the spiral plate count method, there were no statistical differences between the counts (Alonso-Calleja et al., 2002). The spiral plate count method uses only one plate and can save time and materials in the enumeration of mold in foods. Major drawbacks for this spiral method are the limited studies done with the current recommended methods for mold enumeration using spread plates with DRBC or DG18 agars and the cost of the instrument.

A dry, rehydratable film method has gained acceptance by the Association of Official Analytical Chemists (AOAC) International (Anonymous, 2002b). The principle of this method is that the dry nutrients, antibiotics and a dye indicator are on a cardboard template that has a glassine film overlay that contains the gelling agent. When 1ml of the diluent is pipetted onto the dry surface, the film overlay replaced, and the sample distributed over 30cm² using a plastic spreader, then the film becomes like a Petri dish with agar. Dry rehydratable films are incubated at 20–25°C for 5 days. Beuchat et al. (1991) found that there was interference of food particles on Petrifilm™ that made some counts of some foods higher or lower than counts on microbiological media but others were equivalent. Spangenberg and Ingham (2000) found that enumeration on Petrifilm™, DRBC and PDA were equivalent for mozzarella cheese. Taniwaki et al. (2001a) reported that the Petrifilm™ versus DRBC for enumeration of total yeasts and molds for 14 foods gave a correlation coefficient of 0.9299; however, in some cases, the counts were either over- or underestimated by Petrifilm™ compared with DRBC. Excessive colony growth of Mucor and Rhizopus were a problem with Petrifilm™ (Beuchat et al., 1990; Taniwaki et al., 2001a); however, these molds were not included in the collaborative study done to recommend acceptance as an official method of the AOAC International (Knight et al., 1997). A limitation of this method is that the small size of the film means that molds may spread, which can interfere with getting a good mold and yeast count. Also, this method does not allow the analyst to see the morphology of the mold as can be done with an agar plating method.
A method has been developed to detect total molds and yeasts in foods by using a circular plate that contains 84 wells for addition of food homogenate and medium. The principle of the method is that when yeasts and molds grow in this system, they become biochemically active and produce a colour change that is monitored in the individual wells (Feldsine et al., 2003). The final plate count of yeasts and molds is determined by a binary reaction based on the number of positive wells that give a colour change, which is correlated to a count in a conversion table (Feldsine et al., 2003). This is called a SimPlate® yeast and mold-colour indicator method. When this SimPlate® was evaluated against plating with DRBC for high aw foods (frozen fruits, fresh cheese and frozen corn dogs (hot dogs on a stick that are dipped in corn batter and deep fried)) and DG18 for low aw foods (nuts, cereal and cake mixes) in a collaborative study, all foods except nuts showed similar enumeration by plating and SimPlate® (Feldsine et al., 2003). In contrast, Spangenberg and Ingham (2000) found that the SimPlate® results did not correlate with those obtained by plating with DRBC, PDA, Petrifilm™ and hydrophobic grid membrane filter (HGMF), all of which produced similar data for Mozzarella cheese. Also, Taniwaki et al. (2001a) reported that the SimPlate® results did not correlate with those obtained by plating with DRBC, DG18, PDA and Petrifilm™ for several cereal, nut, cheese and fruit products. The presence of endogenous enzymes in raw, unprocessed cereals, fruits and vegetables may affect the SimPlate® results; therefore, it may have limited use as a replacement for total yeast and mold enumeration (Taniwaki et al., 2001a). More research has to be done with this method before it can be used to replace other plating methods for mold enumeration.

3.2.2 Filtration

The hydrophobic grid membrane filter (HGMF) method also has gained acceptance by the AOAC International (Anonymous, 2002a). The principle of the method is that a liquid sample or dilution of a solid sample, which may need to be hydrolyzed with an enzyme (different enzymes are recommended by the company depending on the food being analyzed) to break up the food, is passed through a filtration unit that has a 5 µm prefilter to remove food particles before the remaining liquid passes through a 0.45 µm membrane that has a grid pattern with hydrophobic lines that make separate compartments to trap individual cells. The filter is placed onto YM-11 agar (contains a dye indicator and bacterial inhibitor) in a Petri plate that is incubated at 25 ± 1°C for 50 ± 2 h; the squares with colonies are counted and calculated as a most probable number (MPN) value.

A collaborative study using six foods with natural contamination was done to show that the HGMF method was comparable to the 5 day pour plate using PDA supplemented with antibiotic (Entis, 1996). When 25 different foods were analyzed by the HGMF method and a 5 day pour plate using PDA supplemented with antibiotics, the HGMF method had counts that were
similar to the plating method for 22 foods but significantly lower for 2 dry foods and significantly higher for 1 dry food than those on the pour plate with PDA (Entis and Lerner, 1996). Although six genera of molds were used in this study comparing HGMF with the 5 day pour plate using PDA supplemented with antibiotics, molds such as *Mucor* and *Rhizopus*, which spread on plating media, were not used (Entis and Lerner, 1996). The results of the HGMF method highly correlated with the results from pour plating with PDA with chlorotetracycline, spread plating with DRBC and plating on Petrifilm™ for enumeration of fungi in Mozzarella cheese (Spangenberg and Ingham, 2000).

There are several limitations of this method that may be encountered, depending on the foods and molds isolated from the foods. When used with solid foods, the prefilter may become clogged with food particles that hinder the flow of the liquid with the molds onto the membrane filter. Colonies may spread over the hydrophobic grid area and make counting difficult. As with the Petrifilm™, the morphology of the molds cannot be determined by this method.

### 3.2.3 Methods used to count mold filaments

The Howard mold count was developed to determine the amount of mold fragments in tomato products; however, it has been adapted to determine the mold fragments in several other foods (Anonymous, 2000). This count is based on a standardized method that calculates the percentage of microscopic fields with mold filaments greater than one-sixth of the diameter of the field (Bier *et al.*, 2001). The Defect Action Levels (DAL) of the Food and Drug Administration (FDA) for unavoidable mold in foods, which can be found in Title 21 of the Code of Federal Regulations Part 110.110 (http://www.gpoaccess.gov/cfr/), uses the Howard mold count to set some of these action levels (FDA, 1998). Before using this method, the analyst must be able to distinguish between the cellular tissue of mold-free product, structures of contaminating animal or plant material, and the structure of common molds. The mold filaments include tubular parallel walls, cross-walls or septation that divide filaments into sections, granular appearance of cellular material, branching of hyphae, blunt ends of hyphae, and weak to no refraction of light; however, all of these characteristics will not be seen for each sample (Anonymous, 2000).

The food sample should be prepared for microscopic analysis according to the directions for that food, which are in the pages that follow the Howard mold counting method in the AOAC Official Methods of Analysis (Anonymous, 2000). The Howard mold count chamber and cover glass are cleaned to allow Newton’s rings (coloured rainbow with broken arcs of circles) to be seen when the slide is held at an angle, which allows light to be reflected from the cover glass. The cover glass is removed with a scalpel, a well-mixed sample is placed in the center of the disk and spread evenly over this
area, and the cover glass is replaced to distribute the sample over the area but not into the moat (Anonymous, 2000; Bier et al., 2001). Slides need to be remade if the distribution is not even, the sample is in the moat or the shoulder of slide or the Newton’s rings are not seen (Anonymous, 2000). Slides are examined microscopically at 90–125× magnification where each field is 1.5 mm² (Anonymous, 2000). Then 25 representative fields from two slide preparations are counted and recorded as positive when the length of ≤ three filaments (individual filament, total length of filament plus branches, total length of ≤ three filaments, total length of filaments in a clump of mold) covers more than one-sixth of the field diameter (Anonymous, 2000; Bier et al., 2001). Each field is recorded as positive or negative for mold filaments and the final calculation is reported as the percentage of positive fields (Anonymous, 2000; Bier et al., 2001).

In an attempt to develop a method to replace the Howard mold count, concentrations of ergosterol and chitin and the detection of mold using antibodies developed to different mold or extracellular fractions have been evaluated; however, they have not been commercialized. Information on these methods can be found in the following sections on methods for research and new techniques. A fluorescent-labeled lectin was used to analyze four molds (Alternaria alternata, Cladosporium herbarum, Fusarium oxysporum and Stemphylium botryosum) associated with tomatoes and was compared with the Howard mold count where \( r^2 = 0.76 \) (Potts et al., 2000). When this assay was modified and taken to commercial tomato processors for evaluation of 100 naturally contaminated raw tomato juice samples, it was over four times more precise than the Howard mold count (Potts et al., 2001a). The responses to the lectin assay differed for ten molds that were grown in either tomato juice or potato dextrose broths and added to tomato juice (Potts et al., 2001b). A United States patent (US Patent 6,833,250) was issued in December 2004 to researchers at the University of California, Davis, CA, and to a researcher at VICAM of Watertown, MA, for this method to detect and remove chitin from biological material (Potts et al., 2004). In 2002, VICAM has developed a test based on this research, which is known as the MoldQuant™-T; however, the method is still under development because there is concern whether chitin is the appropriate measure for all molds (personal communication with Barb Cohen of VICAM). Based on this research, patent and company development, rapid methods to replace the Howard mold count may be commercially available in the future.

### 3.2.4 Methods used mainly for research

Several methods have been researched over the past 40 years in an effort to detect and enumerate molds from food in a way that is better than the traditional plating methods. Indirect methods that have been used to determine mold contamination of grains and foods include the measurement of cell wall components (chitin and ergosterol), the detection of metabolites and volatile
compounds, the use of conductance, and related methods. Chitin (β(1 →4)-
linked-2-acetamido-2-deoxy-D-glucose or N-acetyl-D-glucosamine) is a
polysaccharide that is present in the cell wall of most fungi but is not found
in most plant or animal tissues; therefore, foods are hydrolyzed by acid,
alkali or enzymes to produce chitosan, glucosamine or N-acetylglucosamine,
which then are analyzed by chromatography (gas chromatography (GC) or
high-performance liquid chromatography (HPLC) and amino acid analysis),
colorimetry, microscopy (fluorescent or nonfluorescent dyes, immunofluorescent
dyes), near infrared spectroscopy and titration (Cousin, 1996). A 1 h acid hydrolysis
of chitin was done to determine mold contamination of peanuts and koji; however, the amount of glucosamine depended on the
mold present (Chen and Chiou, 1999). Chitin analysis lacked sensitivity and
reproducibility; therefore, it had limitations for continued development for
mold detection (Cousin, 1996; Gourama and Bullerman, 1995a).

Ergosterol is the major sterol found in most fungi (molds and yeasts) but
not in plants and this sterol absorbs at the 240 to 300 nm UV wavelengths;
therefore, it has been used to estimate the total fungal biomass in plants, soil
and cereal grains (Gourama and Bullerman, 1995a). Although the measurement
of ergosterol has been used to estimate the fungal contamination of plants
and cereal grains, this method has been used to a limited extent in research
on the detection of molds in foods. When ergosterol was compared to an
indirect enzyme-linked immunosorbent assay (ELISA) for detection of
Fusarium exoantigens in wheat, the linear correlations ranged from 0.66 to
0.81, depending on the sample (Abramson et al., 1998). Gourama and
Bullerman (1995b) reported that ergosterol could detect fungal growth before
spores could be detected on plate counts and that ergosterol correlated with
aflatoxin B₁ production on rice. Also, Saxena et al. (2001) reported similar
results for ergosterol, plate counts and ochratoxin A production in rice. Yong
and Cousin (2001) found that an ELISA and plate counts detected the presence
of aflatoxin-producing molds in maize stored at 21 °C after 3 days compared
with after 4 days for the ergosterol assay. The relationship between ergosterol
and colony diameters was acceptable for measuring growth of Aspergillus
flavus, Byssoschlamys fulva, Fusarium oxysporum and Penicillium commune;
however, these measurements were less consistent for Byssoschlamys nivea,
Eurotium chevalieri, Mucor plumbeus, and Penicillium roqueforti (Taniwaki
et al., 2001b). Since the assay for ergosterol does not always correlate with
mold growth, especially early growth, measurement of ergosterol is unlikely
to replace the plate count method as a rapid mold enumeration assay.

Enzymes, volatile and non-volatile metabolites, and other chemicals that
are produced by molds during their growth have been used to detect the
presence of molds in foods. Adamek et al. (1990) studied the production of
volatiles by molds inoculated onto wheat or laboratory media and found that
the volatiles produced depended on the mold and the conditions of incubation;
however, methylbutanol, methylfuran and methylpropanol were produced by
several molds. Fischer et al. (1999) reported that 3-methyl-1-butanol and
1-octen-3-ol were produced by several airborne fungi. The detection of volatiles as a measure of mold growth has been used in the development of electronic noses (Jonsson et al., 1997; Keshri et al., 1998, 2002; Magan and Evans, 2000; Olsson et al., 2000). Chiou (1997) found that the decrease in free glutamic acid in peanuts correlated with the increase in mold growth. When Mátrai et al. (2000) worked with enzymes in an effort to find a rapid test to detect the presence of molds in cereal grains, they found that an increase in invertase production in cereals and feeds within 20 h at 37 °C correlated with the growth of Aspergillus species in the A. flavus and A. fumigatus groups but not with other slower-growing Aspergillus, Fusarium or Penicillium species. To detect early mold growth in tomatoes, Polevaya et al. (2002) followed the production of acetaldehyde, carbon dioxide, ethanol and ethylene in fruit inoculated with Botrytis cinerea and found the ethylene may be the best indicator. Although many of these compounds have been used to detect specific molds or groups of molds, there may not be one compound that is useful for detecting general mold contamination of foods. The development of these methods to detect groups of molds or specific species of molds seemed to show some promise and may continue, especially in the area of electronic nose technology (see Section 3.4 for further information).

Impedance or conductance has been used infrequently to detect the presence of molds in foods because molds produce electrolytes slowly and also absorb them. Watson-Craik et al. (1989) stressed that new media for detecting molds by decreasing conductance were needed. Nielsen (1990) found that capacitance was a better measure of heat-resistant molds in apple juice than were either conductance or impedance. Sawai and Yoshikawa (2003) used an indirect assay for conductance measurement of fungi where conductivity of the NaOH solution was detected when CO₂ was absorbed. This indirect method overcame some of the concerns with both the growth medium and the absorption of electrolytes by fungi; however, it was done for only two rapidly growing molds, Aspergillus niger and Rhizopus stolonifer (Sawai and Yoshikawa, 2003). Since conduction has limitations for mold detection, this method has less potential to be used in the near future for mold enumeration.

The rapid enumeration and detection of molds in foods lag behind those of pathogenic bacterial detection because there are fewer researchers working on these methods. Additional methods that have been used experimentally to enumerate or detect molds in foods can be found in Gourama and Bullerman (1995a), Pitt and Hocking (1997), Samson et al. (2002a) and Williams (1989). More research is needed on these and other rapid methods before they can be used for routine detection and enumeration of molds in foods.

### 3.3 Identification of molds

Molds that are growing on the surface of foods can be identified to the genus level by experienced mycologists through visual observation and microscopy.
Slide wet mounts (Fig. 3.1) should be made in water; however, if drying is a concern, then a mounting medium such as 85% lactic acid with/without 1 g aniline blue/liter (Samson et al., 2002a) or 0.1% acid fuchsin (Pitt and Hocking, 1997) can be used. A sterile inoculating needle is used to remove some spores and mycelium from the colony by rubbing the needle across the colony and also going a little below the surface to get some mycelial fragments. This can be put into the water on the slide. If the mold does not release from the needle, then it can be aided with a drop of 70% ethanol and removal from the needle with the edge of a cover slip. A cover slip is placed over the preparation and it is examined under the microscope at 40× magnification. If a higher magnification is needed to see fine or detailed structures, then a drop of immersion oil is placed on the cover slip and it is viewed at 100× magnification.

Identification of molds isolated from foods by either direct or dilution plating is done by plating on the microbiological medium that was used to develop the identification key, following with microscopic techniques as described in fungal taxonomic reference books for foodborne molds (Pitt and Hocking, 1997; Samson et al., 2002a). In addition, Samson et al. (1986) compiled a list of taxonomic references that can be used to identify molds isolated from foods. Some common taxonomic resources for *Aspergillus* (Pitt, 2000), *Fusarium* (Burgess et al., 1994; Nelson et al., 1983) and *Penicillium* (Klich, 2002; Klich and Pitt, 1988) are also useful for identification of molds isolated from foods. The major mold genera associated with foods are listed in Table 3.3 along with their classification and some distinguishing characteristics. Classically, molds are identified by using taxonomic keys that describe macroscopic (Fig. 3.2) and microscopic (Table 3.4) characteristics after the molds have grown on selected media for a specific time at defined temperatures (Fig. 3.3). Currently, identification of molds needs to be done by experienced mycologists to insure that an accurate identification is done; however, few laboratories have this type of expertise because many universities have stopped teaching fungal taxonomy (Flannigan, 1997).

Seiler et al. (1994) used a 96-well microtiter plate containing different carbon, nitrogen and other chemicals to identify molds important to the dairy industry; therefore, they suggested that a physiological identification scheme could be developed to identify molds. In addition, Dobranic and Zak (1999) modified the Biolog™ microtiter plate for detecting bacteria to differentiate fungi based on functional characteristics in the presence of different tetrazolium dyes. Talbot et al. (1996) used the Biolog GN™ to determine the physiological diversity of *Fusarium compactum* strains that were isolated from different environments and suggested that this could be used only as a prescreen because the correlation from this carbon use was not as good as that noted for genetic relatedness by random amplified polymorphic DNA (RAPD). Lee and Magan (1999) also used the Biolog GN™ to determine the carbon use by *Aspergillus* and *Eurotium* spoilage molds associated with maize. These concepts were used by Biolog, Inc. with assistance from the
### Table 3.3 Characteristics of common molds isolated from foods

<table>
<thead>
<tr>
<th>Class of fungi</th>
<th>Characteristics</th>
<th>Major mold genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycetes</td>
<td>Septated mycelia, sexual spores (ascospores) in asci in the ascomata on the hyphal mass</td>
<td>Byssochlamys, Eurotium, Emericella, Eupenicillium, Monascus, Neosartorya, Talaromyces, Xeromyces</td>
</tr>
<tr>
<td>Hyphomycetes (Deuteromycetes or fungi imperfecti)</td>
<td>Septated mycelia, asexual spores (conidia), fertile hyphae or conidiophores (stipe, branch or vesicle)</td>
<td>Acremonium, Alternaria, Aspergillus, Aureobasidium, Botrytis, Cladosporium, Epicoccum, Fusarium, Geotrichum, Moniliella, Penicillium, Phoma, Trichoderma, Trichothecium, Trichoderma, Wallemia</td>
</tr>
<tr>
<td>Zygomycetes</td>
<td>Non-septated mycelia, asexual spores, sporangia with nuclei, sporangiospores, zygospores</td>
<td>Absidia, Mucor, Rhizopus, Syncephalastrum, Thamnidium</td>
</tr>
</tbody>
</table>

*Burgess et al. (1994); Nelson et al. (1983); Pitt and Hocking (1997); Samson et al. (2002a).*

**Fig. 3.2** The macroscopic characteristics of mold colonies after growth for 7 days on several selected media as shown in Fig. 3.3.

Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands and Agriculture Canada to develop an automatic identification of molds or filamentous fungi. This new system is based on the reduction of tetrazolium...
Fig. 3.3  Point inoculation method used to identify mold isolates after they have been picked from the enumeration medium by a needle, placed into 0.2% semi-solid agar with 0.05% Tween 80, and a loopful placed at three to four equi-distant points on the agar at A and B as shown above. All plates are incubated for 7 days at temperatures of 5, 25 and 37 °C before being used in taxonomic keys to identify the molds to genus and species level. ‘A’ and ‘B’ in the diagram represent two separate isolated mold colonies. CYA = Czapek yeast extract agar; MEA = malt extract agar; G25N = 25% glycerol nitrate agar.

in response to metabolism of 96 different chemicals, which include sugars, acids, amino acids and related compounds (http://www.biolog.com).

In addition to this metabolic profile, there are photographs of the molds as they grow on agar media and as they appear on microscopic slides; however, these are generally after 7 days at the given temperature. The Biolog FF Microplate™ is incubated at 26 °C for 24–96 h and includes a database of over 500 mold species in 120 genera; but Samson (2003) reported that most molds need at least 3 days to give a reaction. Samson (2003) suggested that users of this automated instrumentation obtain training on the microscopic examination of molds before using the photographic software supplied for the molds to prevent misidentification. Kubicek et al. (2003) used the Biolog FF MicroPlate™ method with indonitrotetrazolium violet (INT) as the redox dye and measured the mycelial production at 750 nm as well as the oxidation of INT to formazan for identification of Trichoderma species. They suggested that this method could be used in combination with other methods to identify mold species. Not all fungi reduce tetrazolium violet dye as do bacteria; therefore, the use of INT as the redox dye overcame limitations of the use of metabolic analysis for mold identification (Kubicek et al., 2003). The Biolog™ method for yeast identification showed a 68% correct identification for food and wine yeasts (Praphailong et al., 1997); however, no similar information is available for molds isolated from foods. This Biolog™ system needs to be evaluated by independent research groups to determine its usefulness for routine identification of molds by quality control personnel in food processing plants.
Detection, enumeration and identification methods for molds

3.4 New techniques and applications for mold enumeration and identification

In an effort to decrease the time for enumerating and identifying molds in foods, new methods are being researched. Four major areas where research is continuing on the detection and identification of molds include development of immunoassays, detection of volatile compounds, molecular techniques based on nucleic acids, and use of infrared spectroscopy and other spectral imaging systems.

Imunoassays have been developed for several different mold genera, such as *Alternaria* (Lin and Cousin, 1987; Lin et al., 1986), *Aspergillus* (Tsai 1986), *Penicillium* (Lin et al., 1987), and *Mucor* (Lin et al., 1987).

<table>
<thead>
<tr>
<th>Table 3.4</th>
<th>Microscopic characteristics used to identify molds isolated from foods(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>Observations</td>
</tr>
<tr>
<td>Mycelia</td>
<td>Septate or nonseptate</td>
</tr>
<tr>
<td></td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Branched or unbranched</td>
</tr>
<tr>
<td></td>
<td>Structures that bear conidia</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td>Conidia</td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Micro- and/or macro-conidia</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td></td>
<td>Smooth or rough</td>
</tr>
<tr>
<td></td>
<td>Borne singly, chains, clumps, etc.</td>
</tr>
<tr>
<td></td>
<td>How formed</td>
</tr>
<tr>
<td></td>
<td>Location on hyphal structures</td>
</tr>
<tr>
<td>Ascoma and asci</td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td></td>
<td>Borne singly, in chains or in clumps</td>
</tr>
<tr>
<td></td>
<td>Smooth or rough</td>
</tr>
<tr>
<td>Ascospores</td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td></td>
<td>Smooth or rough</td>
</tr>
<tr>
<td></td>
<td>Structure (flanges, ridges, etc.)</td>
</tr>
<tr>
<td></td>
<td>How formed</td>
</tr>
<tr>
<td>Cleistothecia</td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td></td>
<td>Location in relation to hyphae</td>
</tr>
<tr>
<td>Sclerotia</td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td>Chlamydospores</td>
<td>Shape and size</td>
</tr>
<tr>
<td></td>
<td>Colour and shading</td>
</tr>
<tr>
<td></td>
<td>Smooth or rough</td>
</tr>
<tr>
<td></td>
<td>Borne singly or in chains</td>
</tr>
</tbody>
</table>

\(^a\)Burgess et al. (1994); Nelson et al. (1983); Pitt and Hocking (1997); Samson et al. (2002a).

Although most of these assays were developed to detect certain genera of molds, Yong and Cousin (1995) developed a nonspecific ELISA that detected the six genera of molds in the mixture (*Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Mucor* and *Penicillium*) as well as ten other genera (*Byssochlamys*, *Eurotium*, *Leptosphaerulina*, *Monascus*, *Neosartorya*, *Phoma*, *Talaromyces*, *Trichoderma*, *Trichotecium* and *Verticillium*). In addition, immunoassays have been developed to detect mycotoxin-producing molds such as aflatoxin-producers (Tsai and Yu, 1997; Yong and Cousin, 2001) and trichotecencene- and fumonisins-producers (Iyer and Cousin, 2003). There were a few attempts to produce antibodies to ergosterol (fungal sterol) by conjugating it to a carrier protein (hemisuccinate coupled to bovine serum albumin) as a way to detect general mold contamination (Karlsson and Häggblom, 1992; Pallavi *et al.*, 1997); however, no additional research has been done with this assay because the production of ergosterol by molds is affected by the substrate and environmental conditions (Pallavi *et al.*, 1997). In Europe, four different commercial immunological latex agglutination assays became available in the early 1990s for the detection of mainly *Aspergillus* and *Penicillium* species in foods (Braendlin and Cox, 1992; de Ruiter *et al.*, 1993; Karman and Samson, 1992; Notermans and Kamphuis, 1992; Stynen *et al.*, 1992; Van der Horst *et al.*, 1992). Some of the major molds associated with foods, such as species of *Alternaria*, *Geotrichum*, *Fusarium*, *Mucor* and *Rhizopus*, could not be detected with these kits. Generally, detection of molds took from 15 to 30 min with these kits, depending on the time needed for the sample preparation. Since some of these assays were developed for the medical field, they were not readily adaptable to detect molds in foods; therefore, they are no longer commercially available. More research is needed to produce the proper antibody or antibodies that will be most useful in detecting general mold contamination of foods; thereby, allowing similar kits to become available in the marketplace.
There has been interest in detecting volatile compounds produced by molds as a way to indicate fungal growth in agricultural commodities such as grains. Börjesson et al. (1989) used gas chromatography and mass spectrometry (GC-MS) to conclude that 2-methylfuran, 2-methyl-1-propanol and 3-methyl-1-butanol were the major volatiles produced when Aspergillus amstelodami, Aspergillus flavus, Fusarium culmorum and Penicillium cyclopium were grown on wheat. In a further study, Börjesson et al. (1992) inoculated oats and wheat with three species each of Aspergillus and Penicillium, measured the volatiles produced by GC-MS, and found that 3-methylfuran was a common metabolite produced by all molds on both grains. This work with detection of volatiles produced by molds has led to the development of electronic nose biosensors for the detection of mold growth in cereal grains (Jonsson et al., 1997; Keshri et al., 1998; Magan and Evans, 2000; Olsson et al., 2000) and bread (Keshri et al., 2002). These electronic noses are a series of sensors that electrically detect volatile compounds, produce signal patterns that are fed to a computer, and analyze the patterns by artificial neural networks (ANN) or multivariate analysis (Magan and Evans, 2000; Schnürer et al., 1999). Jonsson et al. (1997) found that the signals from 15 gas sensors gave good ANN predictions of ergosterol and mold colony forming units (cfu) for wheat samples. Olsson et al. (2000) found that both the electronic nose and GC-MS were able to detect mold growth in barley. Spoilage molds in bread were detected before growth could be seen or enzymes produced (N-acetyl-β-D-glucosamine, α-D-galactosidase and β-D-glucosidase) by an electronic nose (Keshri et al., 2002). Although the use of GC-MS and electronic noses have been proposed for detection of general mold growth, some volatile organic compounds may allow detection of individual species of molds as noted by Fischer et al. (1999) for airborne fungi.

In another application of biosensor technology, Söderström et al. (2003) experimented with measuring mold growth in malt extract medium by using an electronic tongue, which is similar to an electronic nose in that it contains several metal sensors that detect nonvolatile compounds in liquid. Vlasov et al. (2002) reviewed electronic tongue technology and potential applications for analysis of liquids. More research is needed to determine if electronic noses and tongues can be used routinely for rapid detection, enumeration and identification of molds in foods.

Polymerase chain reaction (PCR) of amplified DNA using conserved sequences of mitochondrial ribosomal genes and internal transcribed spacer regions (ITS) has been used in fungal taxonomy and phylogenetic studies for several years; however, now these molecular techniques are being researched to detect specific molds, especially mycotoxin producers in grains and foods. Shapira et al. (1996) developed three primers to two enzymatic genes (ver-1 encodes versicolorin A dehydrogenase and omt-1 encodes sterigmatocystin-o-methyltransferase) and a regulatory gene (apa-2 regulates aflatoxin biosynthesis) to detect A. flavus and A. parasiticus in inoculated maize, but
the ver-1 probe was the most sensitive in this study because it detected $10^2$
spores of *A. parasiticus* after a 24h enrichment. Based on this research, Shapira *et al.* (1997) produced two chimeric proteins for genes *ver-1* and *apa-2* in *Escherichia coli* and used these to produce polyclonal antibodies for the detection of aflatoxin-producing molds; however, these polyclonal antibodies cross-reacted with all *Aspergillus* species and to a lesser extent with all *Penicillium* species.

A PCR reaction based on three genes for enzymes involved in aflatoxin biosynthesis, namely, *nor-1* (encodes norsolorinic acid reductase), *omt-1* and *ver-1* was used by Färber *et al.* (1997) to detect *A. flavus* in figs where the DNA was detected, but the sensitivity was low. These three genes were used to develop a multiplex PCR that detected the presence of these genes in *A. flavus*, *A. parasiticus* and *A. versicolor* (produces sterrigmatocystin but not aflatoxin), but only *omt-A* and *ver-1* were detected in the nonaflatoxicigenic fermentation strains of *A. oryzae* and *A. sojae* (Geisen, 1996). Mayer *et al.* (2003) developed real-time PCR against genes involved in aflatoxin biosynthesis and it detected *A. flavus*, *A. parasiticus* and *A. oryzae*, which contained the *nor-1* gene but not other molds that did not contain the genes. This research contradicts that published earlier (Geisen, 1996) for detection of *A. oryzae* with the *nor-1* gene (Mayer *et al.*, 2003). When this assay was used to detect *A. flavus* inoculated into pepper, paprika and maize, the number of gene copies of *nor-1* correlated with the number of colony forming units; however, the copy numbers were higher than the mold count (Mayer *et al.*, 2003).

Several PCR-based assays have been developed recently to detect *Fusarium* species that produce trichothecenes and fumonisins. Niessen and Vogel (1998) developed a PCR assay to the *tri-5* gene that encodes trichodiene synthase to detect *Fusarium* species that produce trichothecenes. When wheat was analyzed for DNA, deoxynivalenol and numbers of *Fusarium graminearum*, the intensity of the DNA signal increased as the amount of mycotoxin and mold increased (Niessen and Vogel, 1998). This research group further developed the method into real-time detection by using a LightCycler™-PCR combined with SYBR®Green I fluorescent dye (Schnerr *et al.*., 2001). This method detected *Fusarium* species in 29 of 30 wheat samples with 2–78% infected kernels based on microbiological analysis but did not detect *Fusarium* species in noninfected samples (Schnerr *et al.*, 2001). In an analysis of 300 wheat samples, there was a good correlation ($r = 0.96$) between detection of *Fusarium* DNA and deoxynivalenol (Schnerr *et al.*, 2002). The internal transcribed spacer (ITS) regions of several *Fusarium* species were examined to identify regions that were specific to species that produced fumonisins (Grimm and Geisen, 1998). For this research, two primers were identified and used to make a PCR assay, which was combined with a commercial ELISA kit to detect species of *Fusarium* that produced fumonisins (Grimm and Geisen, 1998). This method detected only pure cultures of molds that produced fumonisins; however, it was not used with grain or food samples (Grimm and
A multiplex PCR was developed to detect species of *Fusarium* and those that produce trichothecenes and fumonisins by making primers to conserved ITS1 and ITS2 regions of *Fusarium* rDNA and to genes involved in mycotoxin production *TRI6* (trichothecenes), and *FUM5* (fumonisins), respectively (Bluhm *et al.*, 2002). All of these primers were highly specific for their intended detections and were able to detect $8 \times 10^4$ *F. graminearum* and $4 \times 10^4$ *F. verticillioides* cfu/g of cornmeal after 24 h (Bluhm *et al.*, 2002). This was further developed into multiplex real-time PCR where three products (*Fusarium* species, fumonisin-producing *Fusarium* species and trichothecene-producing *Fusarium* species) were detected in a single reaction (Bluhm *et al.*, 2004). A method that uses a 44 h enrichment followed by PCR was introduced in 2005 to detect 50 cfu/g or more yeasts and molds by DuPont Qualicon, which continues their BAX® System (http://www.dupont.com) of detection of microorganisms in foods. The rDNA sequencing kits that were developed by Applied Biosystems (http://www.appliedbiosystems.com) for use in clinical medicine can be applied to identifying molds and yeasts in foods if the molds exist in the database.

Research is continuing on the rapid detection of mycotoxin-producing molds in raw agricultural commodities and in foods by PCR, multiplex PCR and related methods. Since these methods have been used to detect mycotoxin-producing molds, they may be able to be developed for general mold detection.

Spectrophotometric technologies, especially infrared, have been studied for their potential in detecting mold in agricultural commodities and foods. Hirano *et al.* (1998) adapted the near infrared (NIR) spectra to detect mold inside peanuts and developed a sorter to eject nuts that contained either internal or external molds. Roberts *et al.* (1991) found that near infrared reflectance spectroscopy (NIRS) could be used to measure glucosamine, which is a hydrolytic product of chitin, a major mold cell wall polymer. Delwiche (2003) found that wheat kernels could be sorted into scab-damaged, mold-damaged and sound kernels by NIRS and linear discriminant analysis software with accuracies up to 95%. Similar accuracies were reported by Wang *et al.* (2004) for sorting mold-damaged soybeans by NIRS combined with partial least squares (PLS) and neural network data analysis. Since different spectra are produced by molds and by corn, Fourier transform infrared (FTIR) photoacoustic spectroscopy (PAS), which probes the surface of solids, was used to detect *Aspergillus flavus* in corn and an artificial neural network was developed to discriminate between contaminated and uncontaminated corn (Gordon *et al.*, 1997, 1998). Hahn (2002) also used FTIR to detect and differentiate *Fusarium oxysporum* and *Rhizopus stolonifer* in tomatoes. Reflectance spectroscopy as found in NIR was combined with a hyperspectral instrument to create a new imaging technique to detect defects, including mold rots, on the surface of apples (Mehl *et al.*, 2004); however, more research is needed before it can be used for mold detection on produce. Since all of these NIR, FTIR and imaging techniques have shown success in
some applications, it is expected that further research will be needed before a definite method can be proposed for detecting molds in foods.

3.5 Future trends

Although research on the enumeration, detection and identification of molds lags behind that of bacteria, newer methods based on biosensors, immunology, metabolic activity and molecular biology could be developed and refined in the future. Some methods used for detection of bacterial metabolism also are being adapted to enumeration of molds and yeasts. Examples are the BacT/Alert Microbial Detection System of Biomerieux, which detects carbon dioxide produced during microbial growth in special developed media for specific groups of microorganisms (http://industry.biomerieux.usa.com) and Soleris (formerly BioSys) of Centrus, which detects microbial growth based on coloured compounds produced in agar columns (http://www.centrusinternational.com). Although both websites list the ability to monitor growth of molds and yeasts with their systems, there are no independently published research papers on the use of these methods for molds. Based on the research reported in the previous section and the adaptation of rapid methods developed for bacterial detection to mold detection, it would be expected that some of these methods could be refined for potential development into techniques that could be used for mold enumeration, detection and identification.

In addition to the methods just discussed, some methods are being adapted from bacteriology, such as flow cytometry, in which individual cells are measured in a liquid as they flow past a sensor. Bradner and Nevalainen (2003) studied the metabolic activity of molds in potato dextrose broth by flow cytometry, which used two fluorochromic stains (dihydroethidium, which detects aerobic metabolism by measuring reactive oxygen species, and hexidium iodide, which reacted with the cytosol and nuclei). Since enzymatic activity could be detected in actively metabolizing molds, the covalent linkage of either oligonucleotides or antibodies to fluorescein isothionate (FITC) or green fluorescent protein (GFP) could be used to study specific cellular or genetic properties in molds (Bradner and Nevalainen, 2003). Prigione et al. (2004) used flow cytometry to detect molds in air by using multiple parameters: particle size, side scatter (SSC) for granularity, red fluorescence at >620 nm after staining with propidium iodide, and microwave treating at 440 W and a frequency of 2450 MHz for 30 s to allow the mold cell walls to take up the dye. A French company has developed a flow cytometric system (D-count by Chemunex) using antibody-conjugated fluorescent dyes to detect contamination in foods (Anand and Griffiths, 2003); however, this system has only begun to be used for detection of yeasts in dairy and fruit products. A solid phase cytometric system (Fluorassure™) also has been developed where microorganisms are captured on a membrane filter that is stained with
a fluorescent dye and then scanned in an instrument using a laser (Anand and Griffiths, 2003). Although these two cytometric methods are not currently used to enumerate or detect molds in foods, they could be adapted in the future based on the fluorochrome chosen.

Biosensors based on DNA or RNA, mass spectroscopy, bioluminescence and similar systems are being developed to detect bacteria in foods (Anand and Griffiths, 2003); however, little has been done in the area of mold detection. A commercially available kit to detect molds in clinical samples, which is based on repetitive sequence-based PCR (rep-PCR), has been developed by Bacterial Barcodes and marketed through DeversiLab System (http://www.barcodes.com accessed 11 February 2005) for generating DNA ‘fingerprint patterns’ to identify strains. Healy et al. (2004) used this system to identify Aspergillus species from clinical sources and reported a 98% correlation with identification of these species by morphological and cultural methods. On the DeversiLab website (http://www.barcodes.com, accessed 11 February 2005), a general fingerprinting kit for molds is listed; however, no other information is available on its use. On the AOAC International (2003) website, there is a listing for this kit for identification of fungi in foods by DNA fingerprinting; however, there is no current activity reported on its recognition. With more research, a method based on detecting DNA may become commercially available in the future for mold detection in foods. Baronian (2004) recently reviewed the use of yeasts and molds for whole cell biosensors for different applications; therefore, in the future, some of these ideas may be adapted to the detection of molds in foods.

As more research is done, the analytical and biotechnological companies will be interested in expanding their markets to the detection, enumeration and identification of molds. Also, it is expected that new developments in bacteriological detection will eventually find adaptation to enumerate, detect and identify molds. If these methods based on DNA or RNA are developed to a degree that the analyst does not have to understand classical mold identification, then the shortage of classically trained food mycologists may not be an issue in the future. However, if the analyst needs to be able to tentatively identify the type of mold before the methods can be used, then the need for food mycologists will still exist.

New method development for the detection, enumeration and identification of molds in foods will not be based on a single method because there are many diverse molds that grow in many different foods. In the final analysis, the purpose for doing mold analysis, the rapidity with which an answer is needed, the accuracy and sensitivity of the method, the ease of doing the technique, the cost/sample, the degree of automation, and the personnel and training needed to do the technique will be the driving forces for new method adoption. The intended use of the results will be critical in future method development because different methods can be used to assess viable versus nonviable cells; to enumerate actual cell numbers; to determine presence or absence of mold cells, metabolites, mycotoxins, cellular components or genetic
materials; and to identify the molds to the genus or species or strain level. Regardless of the methods developed, there will need to be data banks established with information that is generated by research, collaborative studies, field trials and related endeavors. Also, these methods will need to be analyzed with many different types of foods from around the world. Twiddy and Phillips (1995) emphasized that mycological methods for developing countries must be inexpensive, easily done in laboratories with limited resources and personnel, and adaptable to different environmental conditions. New methods that are proposed in the future must be standardized for use and adopted worldwide by regulatory agencies, food processing companies, educational and research institutions, and trading partners.

3.6 Sources of further information and advice

Although rapid methods for mold detection, enumeration and identification still are developing, there are some resources that can be followed to find out the new or proposed changes in the future. Some web resources include the following:

http://www.foodmycology.org
http://www.cbs.knaw.nl
http://www.foodscience.afisc.au/fcc
http://www.fda.gov
http://www.atcc.org
http://www.aoac.org/testkits/kits-microbiology.html

For print material, the *Compendium of Methods for the Microbiological Examination of Foods* (Downs and Ito, 2001), *Fungi and Food Spoilage* (Pitt and Hocking, 1997), *Introduction to Food- and Airborne Fungi* (Samson et al., 2002a), Official Methods of Analysis of AOAC International and related fungal references should be consulted. Also, revisions of these print materials should be sought as they are published in the future. In addition, research on methods to detect molds will continue to appear in research journals, such as: *Applied and Environmental Microbiology*, *Biosensors and Bioelectronics*, *International Journal of Food Microbiology*, *Journal of AOAC International*, *Journal of Food Protection*, *Journal of Microbiological Methods*, *Journal of Rapid Methods and Automation in Microbiology*, *Letters in Applied Microbiology*, and related journals.

3.7 Note on registered, trademark and commercial methods

The mention of commercial methods that have a trademark or registration mark and of websites for manufacturers of equipment or test kits is neither
an endorsement of the methods nor an endorsement of one manufacturer over another. Also, the mention of these methods does not mean that they are all acceptable for mold detection, enumeration and identification or that they have been properly evaluated by independent laboratories. These methods were included in this chapter simply to let readers know what is currently commercially available for mold detection, enumeration and identification.

3.8 References


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Detection, enumeration and identification methods for molds


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84 Food spoilage microorganisms


Detection, enumeration and identification methods for molds


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4

Modelling microbial food spoilage

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4.1 Introduction

Spoilage can be defined as any change that renders a food product unacceptable for human consumption (Hayes, 1985). It occurs as a result of microbial, physical and bio(chemical) reactions and may also be caused by activities of parasites. The underlying mechanisms are very complex and may be difficult to identify; they are therefore still poorly understood. However, the loss of desirable characteristics and substantial product deviations are mainly due to microbial growth of spoilage organisms and to the effects of their synthesised extracellular enzymes such as proteases, lipases and carbohydrases. Food may become contaminated with a wide range of microorganisms at all stages of production (harvesting, processing, storage) but only a relatively small fraction of these will develop in food commodities and cause serious degradation. Microbial spoilage may lead to formation of slime, visible colonies of bacteria, yeasts or moulds, gas formation, turbidity of liquids, discoloration and changes in characteristic flavour and odour. Rejection of spoiled products is based mainly on sensory expectations and/or detection of metabolites associated with spoilage such as acetone, methylethylacetone and dimethylsulphide in beef (Stutz et al., 1991), ethanol for quality changes in thermally treated products including canned goods (AIFST, 2001) and in marinated chicken breast and rainbow trout (Randell et al., 1995) or trimethylamine in decaying fish (Dainty, 1996). It is estimated that one-third of the food world production is lost by microbial activity, which is an economically significant problem for manufacturers, retailers and consumers (Lund et al., 2000).

Development of predictive models for specific food spoilage flora is a new approach to ensure safe and stable products. Predictive models are
based on the premise that responses of microbial populations to environmental factors are reproducible and predictable. In predictive microbiology, a reductionist approach is generally adopted and microbial responses are measured under defined and controlled conditions. The results are summarised in mathematical equations which, by interpolation, can predict probable behaviour in novel conditions, i.e. those not actually tested (Ross and McMeekin, 1994). Predictive models provide an alternative to time-consuming storage trials or ad hoc microbiological investigations to define the shelf-life or onset of spoilage. Significant progress has been made in recent years, based on active international collaboration (e.g. FAIR CT98-4083 project: Predictive modelling of food spoilage), to develop computerised predictive food spoilage data bases and models for use by the food industry and regulatory authorities. Recent examples of food spoilage models for the growth of specific spoilage organisms or their enzymatic effects are summarised in Table 4.1.

### 4.2 Approaches to spoilage modelling

#### 4.2.1 History of predictive modelling

The term ‘predictive microbiology’ was first used in the 1980s when food microbiologists began to develop mathematical models to predict growth of microorganisms in foods. Predictive models have subsequently been constructed mainly for pathogens and several software packages are available, such as Food MicroModel formerly available from the UK Food Standards Agency.

<table>
<thead>
<tr>
<th>Model concerning</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Ready to drink beverages</td>
<td>Battey and Schaffner (2001)</td>
</tr>
<tr>
<td>Seafood spoilage predictor</td>
<td>Dalgaard et al. (2002)</td>
</tr>
<tr>
<td><em>Bacillus</em> spp.</td>
<td>Braun and Sutherland (2004a)</td>
</tr>
<tr>
<td><em>Brochothrix thermosphacta</em></td>
<td>Braun and Sutherland (2004c)</td>
</tr>
<tr>
<td><em>Lactobacillus curvatus</em></td>
<td>Wijtzes et al. (2001), Messens et al. (2003)</td>
</tr>
<tr>
<td><em>Lb. plantarum</em></td>
<td>Garcia-Gimeno et al. (2002)</td>
</tr>
<tr>
<td>Enterobacteriaceae (including <em>Escherichia coli</em> and <em>Aeromonas hydrophila</em>)</td>
<td>Ross et al. (2003), Fujikawa et al. (2004, for <em>E. coli</em>), Pin et al. (2004 for <em>A. hydrophila</em>), Braun and Sutherland (2005)</td>
</tr>
<tr>
<td><em>Pseudomonas/Shewanella/Acinetobacter</em> Yeasts</td>
<td>Braun and Sutherland (2003)</td>
</tr>
<tr>
<td>Lipases and proteases of food spoilage organisms</td>
<td>Jenkins et al. (2000), Braun and Sutherland (2004b)</td>
</tr>
</tbody>
</table>
Food spoilage microorganisms

(the data are now included in ComBase) or the USDA public domain Pathogen Modelling Programme. Food MicroModel also includes growth models for some spoilage organisms, e.g. *Brochothrix thermosphacta*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. There are also many individual publications concerning predictive models for foodborne pathogens (e.g. Gibson *et al.*, 1988; Buchanan and Phillips, 1990; Wijtzes *et al.*, 1993; McClure *et al.*, 1997; Miles *et al.*, 1997; Sutherland *et al.*, 1995, 1997; Pin *et al.*, 2000). Application software with kinetic models for growth of food spoilage organisms has also been developed (Zwietering *et al.*, 1992; Schellekens *et al.*, 1994; Avery *et al.*, 1996; Wijtzes *et al.*, 1998). Preparing predictive models describing growth of food spoilage organisms has expanded greatly in the last decade, although they have their origin in the 1960s when first kinetic models were constructed describing influence of temperature on fish decay (Spencer and Baines, 1964; Nixon, 1971; Olley and Ratkowsky, 1973a,b). Furthermore, Olley and Ratkowsky (1973a,b) recognised the elemental similarity of response to temperature of many spoilage processes and suggested a ‘universal spoilage curve’, i.e. the rate of response to temperature was similar regardless of the type of spoilage criterion (bacterial numbers, chemical or sensory assessment). The $T_{\text{min}}$ value of 263 K was found to be acceptable for the prediction of spoilage processes.

4.2.2 Difficulties and challenges in developing predictive food spoilage models

Although they supply retrospective information, traditional microbiological methods such as viable counting are most commonly used in data generation for predictive microbiology. Modern biochemical methods, e.g. ATP determination, impedimetric methods or bioluminescence measurement to forecast the extent of food deterioration, are of limited value since they do not give a response until large numbers of cells ($10^6$ to $10^7$ cfu/g or ml) are present, at which stage deterioration is generally imminent.

Development of an equation to predict microbial growth is not difficult, but developing models that are industrially robust presents particular challenges (Betts and Walker, 2004). Because of the complexity of food spoilage and the variety and number of spoilage organisms, modelling of spoilage is less straightforward than creating pathogen models, and their application is much more limited (Pin and Baranyi, 1998). To predict food spoilage accurately it is necessary to consider the whole microbial ecology of the food system (McMeekin and Ross, 1996). Parameters that influence the lag phase and the growth rate of microorganisms were classified by Mossel (1971):

- ‘intrinsic factors’ (e.g. pH value, water activity, redox potential, presence of antimicrobial substances as well as other physical, chemical and structural composition of the food);
- ‘extrinsic factors’ (temperature, relative humidity, etc.);
• ‘implicit factors’ (composition of food and behaviour of the microorganisms); and
• processing factors (smoke, salts, organic acids, preservatives and other additives).

However, the principal determinants affecting microbial growth are temperature, pH and water activity. Occasionally, nitrite oxygen, organic acids or other preservative substances are also considered in models. Temperature abuse is a major factor contributing to food spoilage and although these days, monitoring temperature history during food processing, distribution and storage is straightforward, the variability and uncertainty of some retail and consumer refrigerators can still present a problem.

Interpretation of temperature profiles by computer programs based on predictive microbiology allows informed decision on shelf-life and safety of foods (McMeekin et al., 1997). Early models used to interpret the effect of environmental history on microbial development employed the concept of specified spoilage levels, e.g. in any microbiological model application, the start and end-points were based on accumulated knowledge of the microbiology of processing procedure or a product in storage (McMeekin and Ross, 1996).

4.2.3 Basic approaches and concepts of modelling
The following sections will consider categorisation, approaches and construction of models for pathogens and spoilage organisms because the procedures for both are similar. There have been several recent reviews of basic modelling methods. Davey (1989) and McMeekin et al. (1993) proposed models that predict the combined effect of, for example, pH, water activity, temperature on microorganisms in linear/nonlinear Arrhenius, square root or polynomial model forms. The following scheme, proposed by Whiting and Buchanan (1993), is widely used and classifies models into three types: primary, secondary and tertiary. Primary models describe changes in microbial populations with time, secondary models describe the relationship between the primary model parameters and various environmental conditions, and tertiary models are computer tools that integrate the primary and secondary models into user-friendly software.

Different types of secondary models exist, discussed by Betts and Walker (2004), which describe different aspects of microbial response to the environment:

• **Kinetic growth models**: predict growth parameters, e.g. lag time, growth rate or time taken to achieve specific increase in numbers. These are useful for shelf-life predictions, where growth of spoilage organisms is unavoidable, but the growth rate is crucial, as it can shorten or extend the shelf-life of a product.
• **Time-to-growth models**: based on time for a specific microorganism to initiate growth, which is useful for predicting time needed by an organism for toxin production or enzyme synthesis.

• **Growth/no-growth (probabilistic) models**: define the boundary of environmental conditions allowing microbial growth, or determine probability of growth occurring under any set of conditions within a given time period.

• **Inactivation/death-models**: describe the death or inactivation kinetics of pathogenic or spoilage microorganisms as affected by heat or other modern preservation techniques such as irradiation, ultraviolet light, high pressure and are therefore applicable for analogously treated products.

• **Survival models**: predict ability of microorganisms to survive in an environment unfavourable to growth. These are particularly useful for foodborne pathogens with low infective doses such as *Listeria monocytogenes*, *E. coli* O157 or *Campylobacter jejuni*.

Mathematical models are also frequently classified as **mechanistic** and **empirical** models. **Empirical** models are pragmatic in nature and describe a dataset in a useful mathematical relationship, without considering the intrinsic mechanism by which these data are generated. A **mechanistic** model describes rather the process, either directly observable or unobservable, that generates those data and provides interpretation of the response observed of the underlying mechanisms. Among the existing predictive models, none is purely mechanistic, rather a mixture of the two is applied (Ross *et al.*, 2000), i.e. empirical models can help to define certain qualitative features, and a more mechanistic model may be developed.

Furthermore, the question of whether to develop **generic** or **specific** models has been a matter for debate among predictive microbiologists. **Generic models** are those that have been produced in microbiological media adjusted as required to represent a range of environmental conditions. Using this approach it is necessary to accept that the growth rate of inoculated microorganisms will almost invariably be more rapid than in any food of similar environmental conditions, since there will be no constraints by competition from indigenous organisms or the structure of the food itself. Consequently, it may result in predictions from the model which can be extremely conservative or ‘fail-safe’. This contrasts with the potential for ‘fail-hazardous’ predictions derived from survival or inactivation models attributable to protective (as opposed to inhibitory) effects of unmodelled factors in the food. It is therefore advisable to verify and validate the applicability of generic models in foods of particular interest.

**Specific models** are those in which a particular food itself has been modified to different specifications of pH, water activity and/or preservative concentration and incubated at one or more temperatures. This can result in a plethora of models of relatively and, in some cases, extremely limited applicability. Furthermore, it is highly labour-intensive to prepare a range of models using this procedure. Nevertheless, a number of models based on
direct estimates of growth of microorganisms inoculated into foods have been published (Nicolai et al., 1993; Abdullah et al., 1994). The dynamic nature of the spoilage process in particular indicates that empirical product-orientated models rather than kinetic models may be the most useful for predicting the effect of different storage conditions on shelf-life, or the onset of food spoilage. An iterative approach is preferred to a general predictive modelling approach. Factors influencing spoilage can be identified by step-wise comparison of results from model systems and food inoculation experiments (Dalgaard, 1995). Therefore Dalgaard (1995) suggested the following two-step procedure for evaluation and prediction of fish spoilage. Firstly, product-based investigations to identify (a) the reactions for spoilage (SR), e.g. production of metabolites, (b) the specific spoilage organisms (SSO) as a part of the total micro-flora responsible for SR reactions; and (c) the spoilage domain (SD), i.e. the range of environmental conditions over which a particular SSO is responsible for spoilage. Secondly, growth responses of the SSO are modelled within the range of its SD. Figure 4.1 shows an example of the SSO-hypothesis.

It is important in this context not to assume that because an organism is present in high numbers, it will inevitably be the primary cause of spoilage of the particular food. For example, spoilage of chilled vacuum- or modified atmosphere-packed cod had always been attributed to large numbers of Shewanella putrefaciens. However, Dalgaard (1995) could identify Photobacterium phosphoreum as the actual spoilage organism, since despite being present in lower numbers, it produced more of the main spoilage metabolite trimethylamine than Sh. putrefaciens.

Nevertheless, according to Gram et al. (2002), neither the total viable count (TVC) nor the number of specific spoilage organisms present is a reliable direct predictor of the sensory quality of a food, e.g. Braun and
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Sutherland (2003) detected proteolytic activity and synthesis of lipases at temperatures of 2–6 °C when pseudomonads achieved numbers of only $10^4$–$10^5$ cfu/ml. Nevertheless, Ellis et al. (2002) noted a correlation between spoilage of chicken meat at 20 °C and a TVC of $10^7$ cfu/g 

Pseudomonas spp.

4.3 Developing spoilage models

4.3.1 Planning

The nature of the organism of concern and the problem it causes will partly determine the type of model required (McMeekin et al., 1993). Different types of models were outlined in the previous section. Factors that need to be considered when designing experiments to generate data upon which the model is based include quantity of data, inoculum size, experimental design and construction of the models and will be described below.

4.3.2 Data generation and analysis

Experimental design

The experimental design can also have an important effect on the predictive capability of the models ultimately produced and should consider the environmental conditions relevant to the food of interest. Several models have been based on the central composite design, which assumes constant variability of data above and below a central or optimum point (Palumbo et al., 1992; Abdullah et al., 1994; Guerzoni et al., 1996; Cheroutre-Vialette and Lebert, 2000). However, this may not necessarily be the case with biological systems, including microbial growth in media or foods, since, as growth conditions deviate further from the optimum, the growth rate will become progressively slower, with greater potential variability. An alternative and more realistic approach is an empirical experimental or ‘edge’ design, founded on the basis that the further the data produced are from optimum conditions, the greater will be the variability of the data. To minimise variability of the model, experiments should be designed in such a way that less data are generated in conditions where growth would be rapid and to concentrate data generation in conditions where growth is likely to be slower and more erratic, i.e. towards the growth/no-growth interface at each temperature. This will help to ensure that the model will provide reliable and realistic predictions in the sub-optimal conditions that generally prevail in refrigerated foods. Figure 4.2 shows an example of the FAIR CT98-4083 project for the combinations of environmental conditions where growth of the 

Pseudomonas cocktail occurred.

Selection of strains and size of inoculum

The strains of microorganisms used to develop food spoilage models are of
considerable importance. Since it is not feasible to test large numbers of strains against numerous combinations of environmental conditions which are strain-specific and may not represent a worse-case scenario of bacterial growth or within a food, often mixtures or cocktails of strains have been used for growth models. To increase user confidence in the ability of those models to predict with accuracy throughout the range of environmental scenarios, it is advisable to include at least one strain in the cocktail that is particularly resistant to each environmental condition under investigation. However, it is likely that interactions (synergisms/antagonisms) may occur between the different strains of microorganisms in the cocktail, resulting in considerable advantages for selection or growth of siderophore-producing pseudomonads (Gram et al., 2002) or, alternatively, one strain from the cocktail may become predominant.

The choice of growth parameters to model will also depend on the intended use of the model. In relation to the onset of spoilage or to the determination of shelf-life of a product, the time for a specific increase in numbers may be an important consideration. However, the range of conditions over which the model is to be used should be defined carefully as empirical models should not be applied beyond the range of factors used in their construction. Experimental work can be reduced significantly, by the use of suitable screening experiments (McClure et al., 1994).

Furthermore, many of the techniques rely on statistical measures of performance; however, an important aspect which should not be overlooked is whether the model makes biological sense. Poorly designed or over-parameterised models may give a good fit to the data but the predicted response may be erroneous. A biological and mathematical interpretation should be given to the results (Betts and Walker, 2004).
As an example, within the EU project FAIR CT98-4083, which focused on the development of generic spoilage models for perishable foods, the main food spoilage organisms were categorised into six groups of broadly similar organisms (pseudomonads, yeasts, Enterobacteriaceae, *Br. thermosphacta*, lactic acid bacteria and bacilli). The multiple strain cocktails were inoculated into liquid media with pH and water activities pre-adjusted to conditions representative of foods and stored for up to 6 weeks at temperatures between 2 and 20°C. Since the inoculum levels for growth conditions should be between $10^2$ and $10^3$ cfu/ml (McClure *et al.*, 1994) an initial concentration of $10^3$ cfu/ml was taken. At intervals during growth microbial numbers were estimated, and additionally enzyme synthesis (lipases) and activity (proteases) and organic metabolic compounds related to increases in the number of microorganisms were measured.

**Measurement of relevant components**

Growth of food spoilage or pathogenic organisms
The most commonly employed technique for data generation is the estimation of TVC of spoilage or foodborne pathogenic organisms during growth in specific environmental conditions. Detailed description of the procedures has been extensively documented, e.g. Gibson *et al.* (1988), McClure *et al.* (1993), Pin and Baranyi (1998) and Braun and Sutherland (2003, 2004a–c). Alternative methods for estimating viable counts include:

- Optical density (OD) measurements, initially difficult to model because turbidity is not measurable below numbers of $10^5$–$10^6$ cfu/ml. However, modifications of experimental procedures and mathematical developments in handling data derived from OD methods mean that the technique has become more useful (Hudson, 1993; Baranyi and Pin, 1999; Rasch *et al.*, 2002). A modification of this technique by Neumeyer *et al.* (1997a) used turbidimetric measurements calibrated against viable counts to determine generation times and construct a model for *Pseudomonas* spp.
- Impedance or conductance measurements were used by Deák and Beuchat (1994) to prepare a prediction model for food spoilage yeasts.
- Luminescence techniques constitute a very rapid but cost-intensive method and are not widely used to generate data for predictive microbiology.

Extracellular microbial enzymes
In particular with food of animal origin substantial product deteriorations are attributed to the effect of extracellular microbial proteases and lipases (Kraus, 1961; Fehlhaber, 1992; Baltes, 2000; Belitz *et al.*, 2001; Braun, 2003) and should be considered in the development of food spoilage models.

Enzymatic activity can be investigated either by determination of the decrease in concentration of the raw substrate or by measurement of increase in concentration of degradation products during the reaction catalysed by the enzyme (Kalisz, 1988). In order to make sensitive and specific measurements
of the often small enzyme quantities, the catalytic activity of the enzyme is used. The majority of existing methods, as, for example, photometric measurements, the Hull test, the Prescott and Wills measurement, the Azocasein method for proteases, or the pH-stat method (titration), the Wilhelmy plate and the Stead method for lipases, supplies very precise results but photometric extinction measurements are usually accomplished without exception in liquid media, most of which require special equipment.

Because of its relatively simple execution and the reliability, the agar diffusion method in diverse modifications is widely applied (Griffiths et al., 1981; Marshall and Marstiller, 1981; Christen and Marshall, 1984; Kouker and Jäger, 1987; Stepaniak et al., 1987) and was also favoured for the FAIR CT98-4083 project to generate enzymatic data to model spoilage. Casein agar (adjusted to the defined pH values and water activities) was used to detect proteolytic enzyme activity. Aliquots of 0.4 ml of enzyme-containing, bacteria-free filtrate were placed into rings positioned on the agar and incubated at temperatures corresponding with the growth temperatures (Braun and Sutherland, 2003). The diameter of the digested agar region expressed as a measurement of area (cm²) is proportional to the enzyme activity, which was confirmed by different authors (Griffiths et al., 1981; Kouker and Jäger, 1987). To measure synthesised lipases, the Reflektoquant Lipasetest (Merck) was used (Braun et al., 2002), which is based on the conversion of Br₂Cl⁻indoxylcaprylate by lipases to form a blue dye, the concentration of which is determined reflectometrically with a measuring range of 10–400 µg/l.

It was found that the amount produced or activity of the enzyme did not relate to the TVC of the microorganisms under investigation at low temperatures (2–6°C) in particular, confirming that there may not be a direct relationship between microbial number and enzyme activity or amount of enzyme produced. Most enzyme synthesis took place during the late exponential and early stationary phase of growth. Since the curve of Baranyi et al. (1993) was developed to fit the exponential phase of microbial growth, difficulties were encountered in fitting it to the enzymatic data; consequently it was not possible to produce a satisfactory model for enzyme production or activity. Other ways of developing models from these data are being considered.

Nevertheless, in further investigations (funded by Deutsche Forschungsgemeinschaft) to describe the enzymatic activity, proteases and lipases of 55 spoilage-causing bacterial strains of 13 different species were produced under optimal conditions after an incubation of 72h in fat- and protein-enriched nutrient broth and measured by the agar-diffusions test. Tween, tributyrin, gelatine and casein were used as substrates. To consider hydrolytic effects in heated products, the bacteria-free enzyme-containing filtrates were also tested after using different heating programmes (60°C/60 min, 65°C/30 min, 71°C/15 min, 75°C/5 min), resulting in two linear mathematical models (‘predictive enzymology’), which have been verified and validated in milk. The ‘short time’ model provides predictions for both untreated and heated extracellular lipolytic and proteolytic enzymes of
Ps. aeruginosa, Ps. fluorescens, A. caviae, A. hydrophila, Proteus mirabilis, Pr. vulgaris, B. cereus, B. subtilis, Staphylococcus aureus, St. epidermidis, Micrococcus luteus, Clostridium perfringens and Serratia marcescens over 4 days (Braun and Fehlhaber, 2002) within the three-dimensional matrix of environmental conditions, including variation in temperature from 2 to 37°C, pH values from 4.0 to 7.3 and water activities from 0.8 to 0.98. The ‘long-time’ predictive model focuses on extracellular lipolytic enzymes synthesised by Ps. aeruginosa, Ps. fluorescens, A. hydrophila, St. aureus and Serratia marcescens within a temperature range from –2 to +7°C, pH values from 4.0 to 6.3 and water activities from 0.95 to 0.995, and delivers predictions over 38 days (Braun and Fehlhaber, 2003).

Volatile compounds
In principle, it may be anticipated that the quantities of metabolites resulting in off-odours and/or off-flavours (i.e. spoilage) produced by the activities of microorganisms during growth, should be related directly to the number of organisms in the food or broth. However, metabolic pathways of spoilage organisms may vary under the different environmental conditions and this should be clarified by preliminary experiments.

Chromatographic methods and Fourier transform infrared (FTIR) spectrometry are useful techniques to identify and estimate volatile compounds. Gas chromatographic techniques were used by Guerzoni et al. (1990) to measure volumes of carbon dioxide (CO₂) evolved during growth of S. cerevisiae to predict shelf-life of fruit-based products and within the FAIR CT98-4083 project for quantifying growth of Br. thermosphacta, Pseudomonas and related genera and Enterobacteriaceae stored at temperatures above 10°C. Measurements of CO₂ evolved during growth of bacteria could be subjected to growth-curve fitting in the same way as viable count data. However, CO₂ evolution curves are not analogous to viable counts, since CO₂ production did not show consistent correlation with environmental factors. Consequently, ‘lag’ was derived from the individual curve fittings as a primary parameter and can be interpreted as the time taken for the bacteria to reach a high enough number for CO₂ to become detectable. It should therefore be noted that the model for evolution of CO₂ should not be considered to be a model for rate of evolution of CO₂; rather, it should be considered as a model for time to initiation of evolution of CO₂, representing the beginning of overt spoilage of a food.

The technique was not feasible for the cocktail of yeast strains because of differing rates of evolution of CO₂ by the different constituents of the cocktail. Also in this project, the accumulation of 3-methyl-butanol (3-MB) has been investigated as a measure of growth by Br. thermosphacta to model growth and spoilage by this organism (Prajapat, 2004). Figure 4.3 shows predicted values for TVC and time to initiation of evolution of CO₂ at a temperature of 9°C, pH 5.98 and NaCl concentration of 4.5% (w/v). The time taken to detect CO₂ is about 50 h, corresponding to a TVC for Br. thermosphacta
of about $10^5$ cfu/g and this is likely to be the point at which spoilage is incipient.

While volatile metabolic compounds produced by microorganisms can be extracted from the headspace above the medium or food, non-volatile metabolic compounds such as D- and L-lactic acid, acetic acid and ethanol produced by lactic acid bacteria, for example, need to be extracted from the substrate itself before quantification. FTIR spectroscopy is a technique that can be used to carry out rapid, multi-component analyses of systems (Vannini et al., 1996) and to record changes with time of functional groups on the basis of absorbances of the spectrum of specific wavebands. It was used by Ellis et al. (2002) to determine the progress of spoiling chicken meat at 20°C.

### 4.3.3 Construction of models

According to Whiting and Buchanan (1993) the predictive microbiology classification scheme consists of three stages (primary, secondary, tertiary models) but in this section only the two-stage procedure will be addressed. The primary stage of construction of a model includes, firstly, determination of growth responses (growth, survival, inactivation) of the microorganisms to the selected environmental conditions as a function of time. Secondly, estimation is needed of parameters such as the growth rate/specific growth rate, final cell concentration, time to achieve a given quantity of cells or suitable metabolites and death rate as autonomous and history-independent variables. Lag time is a more difficult parameter from the modelling point of view, because of the dependence of both the prior history of the inoculated cells and actual environment.

Generally, construction of predictive growth models requires fitting of a curve to the data generated. The sigmoid Gompertz curve, or some modification
of it (e.g. Zwietering et al., 1990), and the curve of Baranyi et al. (1993), which fits a straight line to the exponential phase and is therefore often considered more appropriate for microbial growth, are frequently used. Data for inactivation and survival models can be handled in a similar way to those of growth models. The main difference between growth and inactivation modelling becomes obvious when comparing stationary phase of growth curves and ‘tailing’ of death curves, following the exponential growth or death phase, respectively. The maximum population level is an autonomous parameter of the growth models, but this is not the case with the tailing of inactivation curves (Baranyi, 2004). Survival data are difficult to model successfully because the survival/death phase is not necessarily exponential in nature.

The secondary stage of modelling characterises parameters derived from primary modelling approaches as a function of the environmental factors such as temperature, pH value and water activity. Different modelling approaches are possible:

- **Arrhenius-type models**, where the natural logarithm of the growth rate is inversely proportional to absolute temperature.
- **Bělehrádek or square root models**, which are based on the linear relationship between the square root of the growth rate and the difference between the growth temperature and the theoretical minimum growth temperature, to which in both cases (Arrhenius-type models and Bělehrádek models) terms may be added for water activity or pH value or other factors.
- **Polynomial equations** based on response surface methodology (RSM) where no assumptions about relationships between growth controlling factors and growth rate are made, and the effects of several variables on growth rate are determined simultaneously.
- **A more recent approach** has been the application of Artificial Neural Networks (ANN) described by Najjar et al. (1997), Hajmeer et al. (1997) and Geeraerd et al. (1998). This is a highly interconnected network including many processing elements capable of performing a parallel computation for data processing, inspired by the elementary principles of the nervous system. The technique was used by Garcia-Gimeno et al. (2002) to construct a predictive model for the growth of *Lb. plantarum*. Advantages include lower standard errors of prediction compared with RSMs, ability to ‘prune’ the ANN, i.e. to remove unnecessary parameters (weights) during training of the network without losing capability and an ability to handle situations where parameters are not uniformly distributed. Cheroutre-Vialette and Lebert (2000, 2002) confirmed the possible use of this alternative approach by construction of dynamic models for *L. monocytogenes* based on a recurrent neural network. Neural networks can ‘learn’ from previous examples by iteration, without the need for first hand knowledge of the relationships between variables.

However, careful consideration of the experimental design (see Section 4.3.2) is advisable to improve reliability of all of these models.
4.3.4 Validation, verification and maintenance of predictive models

Validation is a term that has been widely applied to any assessment of the success or ‘goodness-of-fit’ of a model (Betts and Walker, 2004). Neumeyer et al. (1997a) explained validation as the process of comparing response times predicted by the model to the response time observed in food products, which is broadly accepted. An assessment of predictive models against data used for their development should always be done because it is then possible to detect, at an early stage, underlying problems with the data or with the model being used. One of the simplest measures of the goodness of fit of a model is to visually compare predicted and observed data (Bratchell et al., 1990; Baranyi et al., 1999).

An example from the FAIR CT98-4083 project is shown in Fig. 4.4, which compares the predicted curve for *Pseudomonas* and related genera with the actual curve derived from measured data. With two-stage modelling techniques, both the primary and secondary fits to the data can be examined (Betts and Walker, 2004).

For the FAIR CT98-4083 project, the following procedural definitions for verification and validation were used:

- **Verification**: inoculation of the same cocktail of microorganisms that was used to construct the model into sterile foods, or foods with low numbers of indigenous organisms followed by incubation at a given temperature with regular enumeration of inoculated organisms and measurement of enzyme production or activity and increases with time of metabolites. This resulted in a growth curve which could be compared with the curve for the same environmental conditions produced in the medium, if available, or with a curve predicted by the model (Sutherland, 2003). This provides an indication of the intermediate error, attributable to the food itself, distinct from errors introduced by the presence of indigenous microorganisms (Pin et al., 1999).

![Figure 4.4](image-url)  
**Fig 4.4** Comparison of predicted growth (broken line) with measured growth (solid line) of *Pseudomonas* and related genera at 2 °C, pH 6.0, NaCl 2.5% (w/v).
• Validation: foods were allowed to spoil ‘naturally’ (i.e. without inoculation) at given temperatures and estimates were made of both TVC and numbers of specific groups of spoilage microorganisms, using selective media. Consequential increases in organic metabolites and enzyme synthesis or activity were also recorded. This discloses the overall error of the model, including the effects of the presence of other organisms and the food itself (Pin et al., 1999). However, success in validation using this procedure can be limited if the microorganism in question is not present or not detected in the food.

It is important to assess the entire range of conditions over what region the model has been tested. Each model will have an interpolation region or a minimum convex polyhedron (MCP; Baranyi et al., 1996) which means the region within the boundaries of the experimental matrix under which data have been generated. Figure 4.5 shows the MCP for Br. thermosphacta generated within the FAIR CT98-4083 project. Predictions outside this region (i.e. extrapolated) are less or not reliable.

Another important aspect of model evaluation is the use of correct data transformation. Many measures of microbial response are not normally distributed and are therefore transformed. For time-based data (lag time, time to reach a target) the log\(_{10}\) or natural (ln) transformation (Buchanan et al., 1993) is used; for growth rate, the reciprocal of the value, i.e. 1/time (Ross et al., 2003) or the square root of time (Garcia-Gimeno et al., 2003) is often used.

In general, models for pathogens should be fail-safe, whereas if models for spoilage organisms are too fail-safe, they may be of limited benefit. A spoilage model with a tendency to fail-dangerous may still be useful if its accuracy is good. A commercial decision would need to be made on the value of that model for a given food product. Challenge test studies in the

![Fig. 4.5 Strict interpolation region or MCP for the model of Br. thermosphacta.](image)
products of concern could be done to verify the model for that particular formulation (Betts and Walker, 2004).

Useful possible verification options are summarised by Betts and Walker (2004):

- where food studies can be done in the same laboratory with the same microorganisms with a high level of control;
- to achieve improved validation with data obtained from naturally contaminated foods; or
- against independent existing literature data that do not use the microorganisms or exactly reproduce conditions used in the construction of the model.

However, many disadvantages are associated with the use of public domain data (Sutherland and Bayliss, 1994) and Neumeyer et al. (1997b) recommended use of validation data that have been derived in controlled conditions, where full details of the growth conditions are known.

Finally, the performance of models can be judged using several statistical analysis techniques. The most commonly used criteria are summarised below; for more detailed information, see Betts and Walker (2004).

- Multiple correlation ($R^2$) as an overall measure of the accuracy of prediction.
- Root mean square error (RMSE) describes the variability remaining in the fitted model.
- Standard deviation of the residuals ($S_{yx}$) or standard error represents the amount of deviation that may be expected for any predicted value from the model.
- Bias factor ($B_f$) and accuracy factor ($A_f$) are two different indices to measure the overall agreement between predicted and observed values (Ross, 1996); $B_f$ is commonly used as a measure of model performance and provides a common criterion for comparison of different models; $A_f$ was refined by Baranyi et al. (1999) by percentage discrepancy ($%D$) term to allow comparison with other models.

Once a predictive model has been validated and instituted, in accessible software, i.e. it becomes a tertiary model, it is necessary to set up a procedure for maintaining the model and to recognise when it is becoming inappropriate or outdated. Draper and Smith (1981) recommended that the model should be periodically checked by statistical methods and standard quality control charts to discern more subtle forms of deviations.

### 4.4 Applications of spoilage models

#### 4.4.1 Practical use and benefit of spoilage models

Food microbiology has historically relied on extensive empirical studies to
assess the microbiological safety, quality and spoilage potential of foods. These were often of limited value because only a few variables could be tested at one time (Deák and Beuchat, 1994). The purpose of modelling is to reduce biological, chemical and/or physical phenomena to systems of mathematical equations with the advantage that a larger number of diverse factors can be considered, and therefore predictive models reflect to a greater extent realistic conditions where simultaneous interactions take place. A predictive model must describe the facts of interest with sufficient accuracy to facilitate design, operational or management decisions without requiring actual testing, which is either too expensive, too time consuming, unsafe, or all of the above. Additionally, and as a general benefit, it can be seen that the modelling approach to food microbiology and food spoilage provides a broad basis for comparison of data from diverse sources on the growth of microorganisms in foods.

The proportion of the food industry regularly using predictive models is increasing and will continue to do so, with further progress towards making predictive models and microbiological response data such as ComBase more freely available. Predictive models can be used in a proactive way, to try to prevent problems before they arise, or reactively to allow a rapid response in the solution of a particular problem (Jones et al., 1994). A successful use of predictive models by the industry for specific applications depends on the availability of appropriated application technologies; for instance the Food Spoilage Predictor is commercial software that can read and evaluate temperature profiles collected by electronic temperature loggers (McMeekin and Ross, 2002) and has numerous applications in the assessment and management of seafood safety.

Successful applications of predictive models are discussed by Sumner and Krist (2002) for the Australian meat industry and many models have been published for a number of organisms including spoilage organisms of importance to the dairy industry such as *A. hydrophila* (Palumbo et al., 1991, 1992; Hudson, 1992; Pin et al., 2004), *St. aureus* (Broughall et al., 1983; Broughall and Brown, 1984), or *B. cereus* (Baker and Griffiths, 1993).

However, it is advisable that potential users of predictive models develop their own in-house verification data.

In general, predictive models are powerful tools that can be applied in establishing food specification and processing requirements, shelf-life evaluation, in assessing food safety and quality and during developing hazard analysis critical control point (HACCP) systems, and as an educational tool. These will be discussed more fully below.

### 4.4.2 Product development and modification

Predictive models provide a better understanding of the impact of each variable and therefore can help to quantify the effects of several formulation factors (Whiting, 1995). This allows product developers to select a 'preservative
system’ (Kabara, 1981) that provides a hostile environment to spoilage organisms or pathogens.

Leistner and Gorris (1995) were instrumental in the development of the hurdle technology concept and in recent years have obtained much practical experience in the global application of this successful approach in the food industry. The so-called hurdle effect is also transferable to the ‘enzymatic stability’ of a product, i.e. corresponding combinations of pH, temperature and water activity, available from ‘predictive enzymology’ models (Braun and Fehlhaber, 2002, 2003) or from databases prepared by the FAIR CT98-4083 project, theoretically allow a reduction or a complete inhibition of enzymatic effects in particular. For example *Ps. fluorescens*-lipases are not synthesised at 6°C in combination with pH 7.5 and water activity of 0.98, or if previously synthesised, will be inhibited in their activity at temperatures below 7°C combined with pH 5.0 or water activity of 0.85. However, from the point of view of product quality, it is not generally possible in practice to change environmental conditions by, for example, increasing acidification of a product.

During development of a new food product, or during reformulation of an existing one, there are considerable advantages in being able to predict the consequences, in terms of growth and enzymatic effects of specific microorganisms, that cause deterioration of food and/or foodborne diseases. Predictive models, accessible through user-friendly software that permits the operator to simulate changes to the environmental factors controlling the growth of relevant microorganisms (e.g. temperature, pH, water activity, type and concentration of preservative), allow determination of the likely spoilage status of different formulations of a particular food. Nevertheless, models do not completely replace microbial testing or the judgement of a trained and experienced food microbiologist.

### 4.4.3 Shelf-life determination

Historically established methods such as the $Q_{10}$ Approach, the Accelerated Shelf Life Testing (ASLT) and the Weibull Hazard method can be applied. The $Q_{10}$ Approach is the relationship of the reaction rate constants at temperatures that differ by 10°C and is frequently used by the foodstuffs industry to express temperature dependence. It is also the basic principle of the ASLT, used mainly at the pilot plant phase of product development (Fu and Labuza, 1994). The latter procedure involves storage of the product at higher temperatures than normal, with periodical testing up to the end of shelf-life. This provides data rapidly, but has obvious inherent hazards. The Weibull Hazard method, which indicates the proportional probability of deterioration of food on the basis of cumulative evaluation of measured or observed values as a function of the time (yes/no evaluation using the Weibull function) provides reliable shelf-life data for chilled (Gacula and Kubala, 1975) and frozen food (Tomasicchio *et al.*, 1989), ice-cream (Wittinger and
Predictive models in product development provide an alternative approach to shelf-life predictions, which is different from product studies. Accurate predictions of likely shelf-life are very important to ensure product quality, and may provide commercial advantages, e.g. evaluation of the possibility of marketing a food on an international basis.

Examples of shelf-life assessment using predictive models include the approach of Zwietering et al. (1996) who used three different models and two literature sources to determine numbers of *B. cereus* in pasteurised milk at the point of consumption and concluded that the models could give appropriate prediction. The process risk model of Rasmussen et al. (2002) determined the shelf-life of Atlantic salmon fillets based on the *Pseudomonas* growth model of Neumeyer et al. (1997b). Further application of a shelf-life decision system (SLDS) predicted the quality of marine fish (Koutsoumanis et al., 2002). Another EU-funded project focused on the development of a Safety Monitoring and Assurance System (SMAS) for meat products, which integrates kinetic models for dominant meat pathogens and spoilage bacteria, risk assessment techniques and the capacity to monitor single product temperature history with time temperature integrators (TTIs), into an effective chill chain decision and management tool. In the ‘Real World Shelf Life Technology’ TTIs appearing as self-adhesive labels on perishable products (Fu and Labuza, 1994; Taoukis and Labuza, 1998) have also became accepted. They are based on mechanical, chemical, enzymatic or microbiological systems, which change irreversibly as a function of the ambient temperature and are usually expressed as a change in colour.

### 4.4.4 Implementation of spoilage models in quality assurance programmes

Generally, the food industry conducts a safety assessment when designing new products, utilising new production processes or changing manufacturing specifications. Scientific knowledge and practical experience are applied to assess raw material suppliers, hygienic layout of premises and hygienic design of manufacturing equipment. The scientific concepts and tools that have been developed in support of product development, such as predictive modelling of microbial behaviour (growth, inactivation, survival), are increasingly becoming incorporated into the framework of quantitative microbial risk assessment, e.g. summarised by Hope et al. (2002) for *Salmonella* Enteritidis Risk Assessment (SERA) for shell eggs and egg products. The objectives of this risk assessment were to establish the baseline risk of foodborne illness from *Salmonella* Enteritidis, to identify and evaluate potential risk mitigation strategies, and to further identify data gaps related to future research efforts.
When new products are developed, informed and qualified judgement that considers potential microbiological hazards and control measures as part of the HACCP concept is applied, and predictive models can assist in establishing limits for critical control points or in the context of process control and determination of optimum processing conditions for a food product. Predictions of thermal death rates for relevant spoilage and pathogenic microorganisms during heat processing of a food can allow adjustment of the process to ensure destruction or depression of microorganisms. Furthermore, use of models that predict growth rates at chill temperatures for given organisms allows optimisation of the cooling regime to prevent or limit the growth of organisms that may survive processing procedures. If there is in practice a temporary failure of temperature control over a batch of chilled products, the batch may need to be destroyed where there is a danger of pathogen growth. Application of a predictive model constructed to predict ‘worst possible scenarios’ in terms of spoilage may mean that damage limitation measures could be implemented, e.g. a reduced shelf-life. Additionally, predictive microbiology delivers a rational basis for drafting of guidelines by regulatory agencies.

Finally, predictive models also have an important role in education in that they allow simple demonstration of microbial behaviour and risks without the need for expensive laboratory exercises. Their use in the training of non-experts in the food industry remains to be explored but some university courses are starting to cover this topic and to provide students with opportunity to use and learn from the models.

4.5 Limitations of models

The reasons for the limitations of models or predictions therefrom are diverse and will be outlined in this section.

4.5.1 Nature of models or predictions

Mathematical models have been established as an important tool for the industry and have been integrated into modern quality assurance systems (McMeekin and Ross, 2002). However, predictions provide imperfect representations of the consequences of the numerous processes that affect microbial growth in food, and should be considered at best as a simplified impression of reality. Baranyi (2004) stated that ‘mathematical modelling is the art of omitting the unnecessary’. However, ‘the unnecessary’ depends on the aim of the modelling, which is becoming increasingly refined.

Nevertheless, differences between predictions and observations do not necessarily imply that the model is defective; rather that our knowledge of some food ecosystems is incomplete and factors other than those used in model development have an effect on microbial behaviour (McMeekin et al.,
Further discussions of problems with predictive models are outlined by Knockel and Gould (1995), McMeekin et al. (1997) and ter Steeg and Ueckert (2002).

4.5.2 Complexity of food spoilage

Compared with modelling growth kinetics of pathogens, modelling the process of spoilage is much more complicated and interactive, because reactions and specific spoilage organisms may change as a function of product characteristics and storage conditions (Dalgaard et al., 2002). Mathematical models generally contain simplifications and omit elements of the system that would make the model impractically complex. The differences that could occur between laboratory-based investigations and ‘real’ conditions may be due to the infinite complexity of foods, and considering individual environmental factors in isolation. Moreover, studies of the effect of the prior history of the microbial cells are relatively limited and the effects of strain variations and differences between individual cells have been insufficiently investigated. These limitations will be discussed in detail below.

Food structure

Predictions based on data obtained from broth experiments are often applicable to growth of organisms in structured foods, but there are also many cases where the food structure results in a different microbial behaviour, invariably slower growth, i.e. the model gives fail-safe predictions (Wilson et al., 2002). Some foods are uniformly liquid in structure, with possibly some suspended material, e.g. fruit or vegetable juice, but most are structured as gels, oil-in-water or water-in-oil emulsions, gelled emulsions (Wilson et al., 2002) that support microbial growth, often in micro-environments within the major structure. A perceived limitation of models is, if prepared in homogeneous systems, they may be considered not representative of conditions within the structure of a food. Attempts to explore micro-environments and the activities of microorganisms within them, in order to improve modelling capability, were reported by Wilson et al. (2002).

Influence of environmental factors within the food chain

In realistic conditions of food distribution, retailing and up to storage by the consumer, temperature may vary and there is concern that predictive models obtained from data produced under consistent growth conditions may not provide realistic responses. Furthermore, pH value and/or water activity of a product can change during manufacture and storage, e.g. in fermented sausage pH decreases owing to metabolic activities of the starter organisms and water activity declines as the sausage dries. Moreover, the fermentation and drying processes are carried out at different temperatures. Products of this complexity present a considerable challenge in predictive microbiology.

Some attempts have been made to model the effects of temperature
fluctuations on growth rates of microorganisms, e.g. Koutsoumanis (2001) in raw fish, Jones et al. (2004) for *E. coli*, and Baranyi et al. (1995) for *Br. thermosphacta*. Baranyi et al. (1995) introduced the factor ($\alpha_0$) which is a measure of the physiological condition or ‘readiness to grow’ of a cell (Baranyi and Roberts, 1994). Specifically, the lower the $\alpha_0$ value, the longer the cells take to adjust to a new environment. Predictions of growth rate were well represented by observed data when temperature was decreased stepwise from 25 to 5°C, but not when the decrease was from 25 to 3°C (Baranyi et al. 1995) and it was hypothesised that at the lower temperature the physiological state of the cells was altered, possibly introducing additional lag. A new approach was also introduced by Lebert et al. (2003) to predict microbial growth in dynamic water activity conditions.

**Interactions of microorganisms**

The distribution of microorganisms in food is not necessarily uniform and, moreover, in real situations, perishable foods generally contain an indigenous or adventitious mixed microflora, which can affect the growth rate of the organism(s) of interest and consequently cause differences from predicted growth rates.

The mechanisms dictating which microorganisms become dominant in the microflora are documented by Gram et al. (2002). Changing environmental factors may lead to selective advantages for a particular fraction of microorganisms, e.g. production of siderophores by pseudomonads to utilise the limited amount of iron in the medium. Another mechanism is described as metabiosis: a form of inter-dependency, meaning the reliance by one organism on another to produce a favourable environment, e.g. Cousin and Marth (1977) observed increased growth of lactic acid bacteria in milk pre-inoculated with Gram-negative psychrotrophic bacteria. Furthermore, some Gram-negative bacteria such as pseudomonads, and Enterobacteriaceae demonstrate the ability to coordinate expression of certain phenotypic traits (‘quorum sensing’) through bacterial communication, using $N$-acyl homoserine lactones (Gram et al., 2002). Several authors have made attempts to characterise the interactions of bacteria and some examples are described in the next section.

**4.6 Future trends**

As a consequence of improved computational capability, predictive models and databases of microbial growth/inactivation/survival responses will become more accessible and widespread, less cost-intensive and will allow more accurate description of microbial responses to defined environmental factors, facilitating more realistic and accurate predictions. Growth, survival or inactivation models for pathogens, or kinetic models for the growth of food spoilage microorganisms including software available at a minimal price or
free have demonstrated the potential of predictive microbiology to wide
groups of users in industry, academia and government regulatory agencies.
Also, substantial collaboration should be encouraged at national and
international levels to avoid duplication.

Developments of predictive models in the next generation will address
limitations mentioned in the previous section and should include the following.

First, modelling of bacterial lag phases, since information on the distribution
of lag times of individual cells is important for risk assessors. If this phase
can be extended indefinitely, conventional spoilage will not take place. It is
recognised that growth models generally do not predict the lag phase duration
of microorganisms as efficiently as the growth rate. The lag phase is affected
by a number of factors, including the physiological state of the inoculum,
and inoculum size (Baranyi and Roberts, 1994). The previous history of the
microbial population can be influenced by many conditions (McMeekin
et al., 1993), and can introduce considerable variation into the length of the
lag phase (Robinson et al., 1998, 2001; Augustin and Carlier, 2000). However,
the growth rate of a microorganism is an intrinsic feature of that organism,
I.e. it depends only on the current environmental conditions. Techniques
such as flow cytometry will allow single cell investigations and will be
valuable for elucidation of the underlying mechanisms controlling growth of
microbial populations, including injury repair and adaptation mechanisms
(ter Steeg and Ueckert, 2002). The theory of Baranyi and Pin (2001) to
evaluate the behaviour of the population as a whole from observing many
individual cells was recently validated experimentally by Elfwing et al.
(2004) using a flow chamber and an automated image analyser to derive
statistical distributions, following observation of divisions of thousands of
single cells.

A new approach for lag phase predictions was introduced by Baranyi
(2002) and recent trends in predictive modelling of microbial lag phenomena
are summarised by Swinnen et al. (2004) and Kutalik et al. (2005). Successful
lag models were applied by Smelt et al. (2002) for the effect of heat stress
on the lag times of Lb. plantarum, by Whiting and Bagi (2002) for the lag
phase of L. monocytogenes, and by Francois et al. (2003) for Lactococcus
lactis strains.

Second, quantifying on a larger scale the interactions between the
microorganisms present in foods since microbial growth can be modulated
by the activities of other microorganisms present. Pin and Baranyi (1998)
attempted to characterise interactions between spoilage organisms by
inoculating liquid medium with either one organism group alone or four
groups of organisms together including pseudomonads, Br. thermosphacta,
lactic acid bacteria and Enterobacteriaceae. Pseudomonas were the organisms
least affected by other groups and Enterobacteriaceae the most affected.
Amanatidou et al. (1999) quantified the coexistence of spoilage organisms
in a food system by means of mathematical models.

Gimenez and Dalgaard (2004) used a novel approach to model and predict
simultaneous growth of *L. monocytogenes* and spoilage organisms in cold-smoked salmon. These developments contribute greatly to the ability to predict growth of microorganisms in food, in which there is often an initial mixed microflora before one strain or group becomes dominant.

Third, developing models to describe the functionality of beneficial microorganisms in food. Lactic acid bacteria are traditional starter cultures for production of fermented products to achieve beneficial changes in the food and inhibit growth of pathogenic or spoilage organisms. The control of undesirable organisms is due to the production of metabolites such as bacteriocins, and to exopolysaccharides, which can be used to improve the texture of foods such as fermented milks (De Vuyst and Degeest, 1999). Growth models for lactic acid bacteria have been developed by Wijtzes et al. (2001). Vereecken and Van Impe (2002) created a model that combined growth and metabolite (lactic acid) production, and Rasch et al. (2002) constructed a model to predict the behaviour of the antibacterial compound reuterin produced by *Lb. reuteri*. Leroy et al. (2002) developed models to predict exopolysaccharide yield by *Streptococcus thermophilus* and bacteriocin production by *Lb. sakei*.

Fourth, newly developed and applied technologies in the food processing arena will enhance

- modelling of behaviour of microorganisms associated with new preservation techniques, e.g. models by Rodrigo et al. (2003) for inactivation of *Lb. plantarum* by pulsed electric fields and Marquenie et al. (2002) on effects of ultraviolet light and heat treatment on spoilage fungi on strawberry and sweet cherry; and
- modelling of packaging design such as Jacxsens et al. (2002) for equilibrium modified atmosphere packages of fresh-cut vegetables or permeation of gases through porous polymeric films used for modified atmosphere packaging (Del-Valle et al., 2003).

Fifth, prediction of microbial activity at the growth/no-growth interface. Decline in microbial numbers does not necessarily occur rapidly in conditions inimical to growth; microorganisms can survive for an extremely long time while undergoing a gradual decrease in number, suggesting that physiological modification or a degree of adaptation of the cells is taking place (McClure et al., 1996). Because of the problem of erratic growth, resulting in irregular growth curves, if indeed a growth curve can be prepared at all, existing interface models are mainly probability-based (Ratkowsky and Ross, 1995; Presser et al., 1998; Masana and Baranyi, 2000).

Sixth, modelling of thermal inactivation processes which have been developed in recent years, mainly for aerobic spore-formers, e.g. *B. subtilis* (Wuytack and Michiels, 2001; Jagannath et al., 2003), *B. stearothermophilus* (Fernandez et al., 1996; Tejedor et al., 2001), *B. cereus* (Leguerinel and Mafart, 2001) and also for *Br. thermosphacta* (Bellara et al., 1999) and for bacterial enzymes (Braun and Fehlhaber, 2002, 2003) will add to our understanding of the behaviour of food-associated microorganisms.
Seven, to improve the reliability and to reflect conditions for food during retailing and storage modelling of the microbial growth of bacteria under suboptimal conditions, e.g. the model of Bernaerts et al. (2002) for *E. coli K* 12 under suboptimal time-varying temperature conditions will be enhanced in the future.

Eight, using data of molecular microbiology for predictive microbiology, e.g. information on expressing of diverse genes to get stress adapted.

### 4.7 Sources of further information and advice

Several predictive models for spoilage microorganisms and foodborne pathogens are available through the Internet. Some useful sites include:

- The Seafood Spoilage Predictor (SSP) program, available at http://www.dfu.min.dk/micro/ssp contains predictive models for spoilage of fresh fish by *Photobacterium phosphoreum* and *Sh. putrefaciens* (Dalgaard, 1995; Dalgaard et al., 2002).
- The Food Spoilage Predictor (FSP), which includes the *Pseudomonas* model of Neumeyer et al. (1997a), can be found at http://www.hdl.com.au/html.body_fsp.htm.
- The Pathogen Modelling Program (PMP), for growth predictions for foodborne pathogens is available through a link at http://www.arserrc.gov.
- The Growth predictor (GP), provided from the UK is available at www.ifr.ac.uk/safety/growthPredictor.
- The DMfit Program, which fits growth data by linear and non-linear regression with the curve of Baranyi et al. (1993) is accessible through the website of the UK Institute of Food Research at www.ifr.bbsrc.ac.uk.
- Another free-web-based database of food microbiology data is ComBase (www.combase.cc) as a collaboration between the Food Standards Agency and the Institute of Food Research, UK, and the USDA Agricultural Research Service and its Eastern Regional Research Center, USA, containing large amounts of microbial growth and survival curves that are the basis for numerous microbial models.
- It is anticipated that a website will be constructed to allow access to the spoilage models and data on enzymatic synthesis and activity being prepared in the FAIR CT98-4083 project, which should be accessible in the future through the website of London Metropolitan University (www.londonmet.ac.uk).
- A predictive microbiology services not available in the public domain is the ‘Forecast’ bureau service (Campden and Chorleywood Food Research Association) delivering growth predictions for spoilage organisms of chilled stored foods.
- Finally, the two linear models on enzymatic activities of selected spoilage organism are available at Leipzig University (www.uni-leipzig.de/~lmh).
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5

Determining the stability and shelf-life of foods
G. Betts, Campden and Chorleywood Food Research Association, UK

5.1 Introduction

No food product is able to maintain its original and optimal quality indefinitely. During storage it is inevitable, therefore, that some deterioration will occur that will ultimately render the product unacceptable for consumption. The time during which it remains stable and retains the desired quality is called the shelf-life. During this time the product should:

- remain safe;
- comply with any label declaration of nutritional data;
- retain the desired sensory, chemical, physical and microbiological characteristics of that product (IFST, 1993).

This chapter is concerned primarily with how the microbiological issues impact on product shelf-life. However, it is also worth noting that in some products microbiological issues may play little or no role in product stability. In such foods, it is important to consider how nonmicrobiological factors impact on product shelf-life. In some products microbiological growth may be limited and these other nonmicrobiological factors become more important.

5.1.1 Changes affecting the quality of food products

There are many different chemical and biochemical changes that can occur in food products that will limit their shelf-life. In some food groups, e.g. dried products and sterilised foods, where microbial growth is prevented, it is the chemical and biochemical changes that will be of most importance in determining the shelf-life. Table 5.1 shows some examples of nonmicrobial
deterioration processes which will impact on the sensory quality (texture, appearance, odour and flavour) of products. It is important to decide during initial product concept discussions, which of the attributes are most likely to restrict the shelf-life of the product (Fig. 5.1), as this will help design the product formulation and processing specifications.

**5.1.2 Microbial factors affecting shelf-life**

Unless they have received a commercial sterility heat process, i.e. \( > F_0 3 \) (heat treatment equivalent to 3 minutes at 121°C), most foods will contain microorganisms that have the potential to grow and spoil the food. Each food type will have its own unique spoilage flora and the contribution of these microorganisms to the deterioration in product quality will be dependent on the process design, product formulation characteristics and subsequent storage conditions. For example, perishable chilled foods will be more susceptible to general microbial spoilage than will pasteurised acidic products, where the product intrinsic property of low pH, combined with a heat process, will select for a few specific spoilage organisms.

The growth of microorganisms present in any foodstuff can be controlled by careful formulation of the product and by the choice of storage conditions. The particular factors which can influence microbial growth are called ‘intrinsic’ or ‘extrinsic’ factors. Intrinsic factors are attributes of the food itself such as the level of acidity or amount of salt present. Extrinsic factors are environmental factors to which a food is exposed, for example, temperature and atmosphere. Table 5.2 gives a list of common intrinsic and extrinsic factors which can be used to control the growth of spoilage organisms.

In order to choose the levels of factors required to control microbial growth, knowledge is required on the growth characteristics of relevant organisms for each product type. Some limiting values for growth of spoilage organisms can be found in the literature (Table 5.3) and are a useful guide for assisting in product formulation in order to achieve a desired shelf-life with respect to microbial growth. It is worth noting that such information assumes only one inhibitory factor is present at once or that the other factors present

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**Table 5.1** Nonmicrobial spoilage defects

<table>
<thead>
<tr>
<th>Description</th>
<th>Causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rancidity</td>
<td>Oxidation of fats. Exacerbated by elevated temperature, light or oxygen levels.</td>
</tr>
<tr>
<td>Colour loss</td>
<td>Bleaching or fading of colour due to enzymatic action. Exacerbated by light.</td>
</tr>
<tr>
<td>Staleness</td>
<td>Associated with high fat products. Enzymatic changes, e.g. oxidation.</td>
</tr>
<tr>
<td>Separation</td>
<td>Formation of separate layer, e.g. in mayonnaises and sauces.</td>
</tr>
<tr>
<td>Dryness</td>
<td>Loss of moisture from product. Exacerbated by elevated temperature.</td>
</tr>
<tr>
<td>Limpness</td>
<td>Product has lost turgidity, e.g. cut vegetables. Enzymatic action.</td>
</tr>
</tbody>
</table>
are near optimal for growth. In foods that have a number of factors present, the minimum values may be higher.

Other limiting values for growth of these spoilage organisms may be given in other chapters of this book. The boundaries for microbial growth...
will differ between studies dependent on whether the study was done in laboratory media or food, and whether the study used a particularly resistant strain of the organism or one with more ‘typical’ characteristics.

**Intrinsic factors**

**pH**

In the acid environments found in many food products, the growth of microorganisms may be inhibited. Microorganisms may well die at reduced pH but this will be dependent on the final pH achieved, the type of acid used and the storage temperature. Death is generally more rapid in low pH foods stored at ambient temperatures where the metabolic activity of the cells is higher than at chill temperatures. The inside of a bacterial cell is at a pH close to neutral and needs to be maintained at this level for the organism to grow. Therefore growth is often slowed or prevented in acidic environments, as microorganisms use increasing amounts of energy in an attempt to counteract the effects of the acid molecules and maintain the correct pH inside the cell.

The main types of acid used in food manufacture are known as organic acids or ‘weak acids’ and include acetic, citric, malic, tartaric and lactic. A characteristic of these acids is that they exist in two forms (dissociated and undissociated) depending on the pH values. This is shown below for acetic acid.

\[
\text{Low pH, e.g. 4.5–5.5} \quad \text{High pH, e.g. 7.0 inside} \\
\begin{array}{c}
\text{found in foods} \\
\text{microbial cells}
\end{array} \quad \begin{array}{c}
\text{CH}_3\text{COOH} \\
\text{CH}_3\text{COO}^- + \text{H}^+
\end{array} \\
\text{Undissociated molecule} \quad \text{Dissociated molecule}
\]

This is important for food preservation because only the undissociated form of the acid is able to enter the bacterial cell. Charged molecules such as the dissociated form are not able to cross the bacterial membranes. All organic acids have a particular pH (the pKₐ) value at which half of their molecules...
are undissociated. These are shown in Table 5.4 for some acids. The acids work most effectively as the pH approaches or falls below their pK\textsubscript{a} value, as more acid is in the undissociated state and is thus able to enter the microbial cell.

With organic acids, it is not just the final pH that is effective but the acid ions themselves have an antimicrobial effect. Generally, the acids are most effective in the order, acetic > lactic > citric > tartaric. Not all acids used in food manufacture are organic acids, for example phosphoric acid is used in the beverages industry. The main effects of non-organic acids are due to the general reduction in pH rather than any additional effects due to the acid ions themselves.

**Water activity**

Microorganisms need water to survive. Any reduction in the amount of water available in their environment will cause them to dehydrate and lose their turgidity, and ultimately will inhibit or stop their growth. Loss of water can occur from drying processes where water is evaporated from the foods using heat. More often, in food preservation, the reduction in water will be as a result of the food constituents binding with the water so that it is unavailable for the microorganisms to use. The term ‘water activity’ describes the amount of water that is freely available in a food system. Proteins and fats will bind the water to some extent but the main food constituents responsible for binding water are salt and sugars. Compounds capable of reducing water activity are known as humectants.

Reducing the water activity (\(a_w\)) of the environment can have a dramatic effect on microorganisms, increasing the lag phase and decreasing the growth rate. Each organism has a minimum \(a_w\) below which it cannot grow. This limiting \(a_w\) value will depend on the type of humectant used. Salts (e.g. NaCl, KC1) and sugars (e.g. glucose and sucrose) produce a similar response to each other during the growth of bacteria; glycerol acts differently. Bacteria can generally tolerate higher levels of glycerol as the glycerol is able to enter

<table>
<thead>
<tr>
<th>Acid</th>
<th>pK\textsubscript{a}\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>4.75</td>
</tr>
<tr>
<td>Benzoic</td>
<td>4.20</td>
</tr>
<tr>
<td>Citric</td>
<td>3.14 (4.77, 6.39)\textsuperscript{2}</td>
</tr>
<tr>
<td>Lactic</td>
<td>3.08</td>
</tr>
<tr>
<td>Malic</td>
<td>3.40 (5.11)\textsuperscript{2}</td>
</tr>
<tr>
<td>Proprionic</td>
<td>4.87</td>
</tr>
<tr>
<td>Sorbic</td>
<td>4.80</td>
</tr>
<tr>
<td>Tartaric</td>
<td>2.98 (4.34)\textsuperscript{2}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}pK\textsubscript{a} the pH value at which 50% of the acid is in its undissociated form.

\textsuperscript{2}These acid molecules have more than one acid group. Figures in brackets refer to dissociation value of second or third acid group.
the cell freely and does not cause the osmotic stress mechanisms seen with non-permanent solutes. For example, in *Pseudomonas fragi*, and lactic acid bacteria (LAB), it has been shown (Sperber, 1983) that the minimum $a_w$ allowing growth when NaCl was used was 0.957 for *Ps. fragi* and 0.963 for LAB. When glycerol was used, the minimum $a_w$ for growth was 0.94 and 0.928 respectively.

**Microbial interactions**

Chemical and physical parameters are often the main factors determining the growth of spoilage organisms in foods; however, interactions between microorganisms themselves may also affect their growth and spoilage activity (Gram *et al.*, 2002).

Organisms compete for the same substances or may produce inhibitory metabolites, which changes the local environment, or directly affect other organisms. The growth characteristics of the weaker organism will be affected by the stronger competitor. In many cases, the final maximum population of the weakest organism is decreased (Buchanan and Bagi, 1999; Duffy *et al.*, 1999) although this may not occur until levels of the competitor have reached high levels, e.g. $10^8\text{ g}^{-1}$ (Malakar *et al.*, 1999; Steele and Stiles 1981).

Microbial interaction is not always easy to measure but can contribute greatly to the stability of many foods. For example fermented meat products rely on the use of lactic acid bacteria as starter cultures. The rapid growth of these organisms and the acid they produce contribute to the stability and safety of these products.

**Extrinsic factors**

**Storage temperature**

For perishable products stored at chill temperatures, the choice and maintenance of temperature can have a large effect on shelf-life. All microorganisms have a minimum temperature below which they cannot grow. Even psychrotrophic organisms will have a reduced growth rate at very low (deep chill) temperatures.

**Modified atmosphere**

The use of modified atmospheres can have a large effect on the shelf-life of a product. Day (1992) gives a good review of modified atmosphere packaged (MAP) goods.

Chilled foods are often stored in a mixture of gases where oxygen is excluded in order to minimise growth of aerobic spoilage organisms such as *Pseudomonas* species. Typical gas mixtures will contain carbon dioxide (CO$_2$) at a level of 25–40% and nitrogen (N$_2$) at 60–75%. The use of such a gas mixture will change the microbial population within a food. While the growth of *Pseudomonas* will be minimised, facultative spoilage organisms such as lactic acid bacteria, Enterobacteriaceae and yeasts (all of which can grow with or without oxygen) may dominate.
There is also the potential for growth of anaerobic pathogens such as *Clostridium botulinum* in MAP foods. Owing to these risks, it is currently recommended in the UK that the shelf-life of chilled MAP or vacuum packaged foods should be restricted to 10 days or less if they do not contain sufficient controlling factors, i.e. pH of 5.0 or less, $a_w$ of 0.97 or less, aqueous salt level of 3.5% or greater; heat treatment equivalent to 90°C for 10 min (Betts, 1996). This applies to all VP/MAP foods stored between 3°C and 8°C.

For some foods, e.g. raw meat, a gas mixture containing elevated levels of oxygen is used in order to prolong the visual quality of the product. For ambient-stable snack products such as crisps and nuts, an atmosphere excluding oxygen, e.g. 100% N₂ or even argon, may be used to minimise rancidity due to fat oxidation and this prolongs the shelf-life (Day, 2001).

### 5.2 Product and process design

The shelf-life of a food product is a direct consequence of the product and process design. Generally the less processing a product receives, the shorter the shelf-life; conversely, the more processing it receives, the longer the shelf-life.

In the previous section various intrinsic and extrinsic control factors were described. In product and process design, the art is to combine these factors in such a way as to maximise product quality during the desired shelf-life. Each factor in itself is unlikely to achieve product stability but together they can act synergistically to achieve effective preservation. The use of multiple control factors is called hurdle technology and has been initially described in relation to fermented meat products (Leistner, 1995).

Factors such as heat treatment, storage conditions, salt/water activity, pH and preservatives are the building blocks of product and process design. There are a number of key ways in which they may be combined.

#### 5.2.1 Storage conditions

Food products are generally divided into three main categories, dependent on their storage conditions:

- **Frozen foods.** These are often stored at −18°C and have a shelf-life of 6 months to 2 years. Frozen foods do not support the growth of microorganisms and therefore the shelf-life will not be limited by microbial activities. Enzymatic reactions are also slowed down at frozen temperatures and the shelf-life is likely to be limited by textural changes such as ice-crystal formation and moisture loss or by biochemical changes such as rancidity.

- **Chilled foods.** These are generally stored at <8°C and typically have a
shelf-life of 1–6 weeks. Storage at chill temperatures will reduce the growth rates of microorganisms but many spoilage organisms are able to grow at refrigeration temperatures as indeed are some pathogens. In these foods the choice of heat treatment, pH and water activity will be crucial to destroy or stop the growth of any remaining microorganisms.

- **Ambient stable foods.** These will either have a suitable heat treatment, which is intended to destroy target microorganisms in the product, or the product will be sufficiently inhibitory in terms of pH, water activity or preservative level to prevent the growth of microorganisms likely to survive the heating process. These products will typically have a shelf-life of 6 months to 2 years.

### 5.2.2 Heat treatment

With the exception of raw products, most foods will have some heat process during manufacture. There are three main categories of heat treatment used to stabilise foods.

**Pasteurisation to inactivate vegetative microorganisms**

Typically a process of $70\,^\circ\mathrm{C}/2\,\text{min}$ or equivalent ($z$ value of $7.5\,^\circ\mathrm{C}$; the $D$ value is the time required to destroy 90% (or 1 log) or the bacteria present and the $z$ value is the number of Celsius degrees that result in a 10-fold change in the $D$ value) is given to chilled food products. This is primarily aimed at achieving a 6 log reduction in *Listeria monocytogenes* and other vegetative pathogens but it is also sufficient to inactivate most Enterobacteriaceae, *Pseudomonas* and yeasts that could spoil chilled foods. It would also inactivate most lactic acid bacteria.

**Pasteurisation to inactivate psychrotrophic or acid tolerant spore-formers**

A process of $90\,^\circ\mathrm{C}/10\,\text{min}$ or equivalent ($z$ value of $9\,^\circ\mathrm{C}$) is also given to chilled food products that are vacuum packed or modified atmosphere packed and have a shelf-life of greater than 10 days. This process is designed to achieve a 6 log reduction of psychrotrophic strains of *Clostridium botulinum* but will also inactivate vegetative spoilage organisms. This process could also be applied to any products in which psychrotrophic *C. botulinum* has been identified as a realistic hazard.

A process of $95\,^\circ\mathrm{C}/5\,\text{min}$ or $95\,^\circ\mathrm{C}/10\,\text{min}$ or equivalent ($z$ value of $8.3\,^\circ\mathrm{C}$) is given to acidic ambient stable products. It is designed to inactivate acid-tolerant spore-formers that could grow and spoil the product if present after heat treatment, i.e. *Clostridium butyricum*, *Bacillus polymyxa*.

**Sterilisation to achieve commercial sterility in canned goods**

A process equivalent to 3 min at $121.1\,^\circ\mathrm{C}$ ($F_o3$) based on a $z$ value of $10\,^\circ\mathrm{C}$ is used to achieve a 12 log reduction of mesophilic *C. botulinum*. It will also inactivate all vegetative microorganisms and the majority of spore-forming...
organisms capable of causing spoilage in temperate zones. In practice, a process higher than $F_{o3}$ may be required in order to inactivate any mesophilic spores of higher resistance.

In tropical zones, a process in excess of $F_{o3}$ may be required to inactivate heat resistant spores such as *Bacillus stearothermophilus*. For example, this organism can have a $D$ value of several minutes at 121°C and a process in excess of $F_{o20}$ may be needed for products where this organism is considered a risk.

### 5.2.3 Determination/validation of meat processes

There are several key stages involved in the design of an appropriate heat treatment for a food product:

1. Define the product/process parameters.
2. Identify target microorganisms.
4. Commissioning heat process equipment.
5. Process validation/verification.

Gaze (1992) gives guidance on the design of pasteurisation processes for a range of food types based on the stages described above.

During stage 1 the intrinsic properties of the food in terms of, for example, pH and salt should be established and also whether there are any restrictions on the maximum heat treatment that can be applied due to the quality of the product. Raw fish and meat may withstand heating to only 60°C or 70–90°C respectively, while product such as pickles may need a higher temperature to inactivate enzymes.

Identification of target microorganisms (stage 2) can then be done based on the information obtained in (stage 1) and data obtained on the heat resistance characteristics. This may be available from the literature or could be collected from practical studies in the product. Any data suitable from the literature on heat resistance in neutral environments may not be relevant to products that are acidic or high in salt as these factors will affect the heat resistance characteristics.

The heat resistance data can be used to determine the times and temperatures required to achieve an appropriate reduction in numbers of target microorganisms. Typical processes will be based on achieving between a 6- and 12-log reduction dependent on the target microorganism.

During the commissioning of the heat processing equipment, it is essential to ensure that the target heat treatment is achieved throughout the product. For in-pack thermal processes achieved, for example, in a static retort, there is likely to be a variation in temperature achieved on different shelves and different positions in the pallets. Temperature measurements should be done to ensure the target heat process is achieved in all parts of the equipment being used.
Once the processing equipment has been commissioned, it is useful to carry out a validation/verification test to ensure the correct lethality is being achieved. This is particularly useful for aseptic filling systems where it is more difficult to monitor the temperature throughout the process. One system for doing this is to immobilise microorganisms in a suitable matrix and to process them with the product (Gaze, 1992). The actual reduction in numbers of microorganisms achieved by the process can then be determined.

5.2.4 Product design
In products that are single-layered or homogeneous in nature, the assessment of product stability and shelf-life is relatively simple. For products that are multi-layered or have separate components, such as ready meals or chilled deserts, the assessment of product stability may need to be done separately for each component. As a minimum this should be done as a theoretical exercise during the product concept stages. The shelf-life of the total product will be limited by the component with the shortest individual shelf-life.

In addition, it may be necessary to consider what will happen at the interface between two different components or layers. If a low moisture content, which relies on low water activity for its stability, is placed next to a high-moisture component, it may well absorb moisture at the surface and become unstable.

5.3 Design and operation of challenge tests
5.3.1 Challenge tests and shelf-life testing
Challenge testing and shelf-life evaluation are both designed to determine the time period during which a product will remain safe and stable. Safety with respect to food poisoning organisms is not covered in this chapter but the principles outlined below for spoilage organisms could be applied to foodborne pathogens. It should, however, be noted that for pathogens with low infective doses (e.g. *Salmonella*, *E. coli* O157), simply preventing growth is not an adequate safeguard. Such organisms must be prevented from entering the food or completely eliminated from it using a suitable process.

During the development of products there are two different aspects that should always be considered:

- Is the product safe and stable during normal production and storage conditions, taking into account the growth of any natural flora present? This can be determined using shelf-life evaluation trials where the product is stored under normal storage conditions and evaluated for any changes in levels of naturally occurring organisms.
- Is the product likely to be safe and stable during its shelf-life if it became contaminated with undesirable microorganisms that may pose a threat to
Determining the stability and shelf-life of foods

the stability of the product, i.e. are the product formulation and storage conditions inherently safe with respect to the inoculated organisms? This can be determined using challenge testing where product is deliberately inoculated with potential spoilage organisms and evaluated for any changes in levels of the inoculated organisms during storage.

Shelf-life testing

The shelf-life of a new food should be assigned after completion of practical trials to assess the deterioration in product quality likely to occur during normal storage conditions. Betts et al. (2004) describes the shelf-life evaluation sequence for chilled food products, although the same criteria can be applied to other food groups.

Shelf-life evaluation is usually done at three key stages of product development (Fig. 5.2). It is a logical sequence of events which describes the actions taken from first product concept to on-going production, as summarised below.

Phase I: Pilot scale

- **Product concept**: during the initial stages, the identity of the product will be defined in terms of the intended market, the brand image and the product design and packaging. As part of the product concept, it is important to have an idea of the target shelf-life that would be required for the product to be commercially viable.

- **Process and product characteristics**: the product will need to be more closely defined in terms of the factors likely to affect the shelf-life, for example, pH, $a_w$ or heat treatment (see Section 5.1.2). An estimate of the likely shelf-life can be made from this information. It is also possible to gain an idea of the likely quality changes that will limit life, e.g. microbial growth for perishable foods, staling for baked goods, rancidity for high-fat products.

- **Preliminary shelf-life assessment**: product samples can be made on a small scale in the test kitchen and stored under the intended storage conditions for the desired shelf-life. Samples of the product can be evaluated for factors likely to deteriorate throughout life as defined above. It is not usual to do extensive testing at this stage as there may be changes in product formulation that could occur at the scale-up stages.

Completion of the preliminary assessment should provide sufficient information to allow a decision to be made on whether the target shelf-life is likely to be achieved.

Phase II: Factory trials

This is where the main part of the shelf-life assessment should be done. The product should be manufactured on the equipment to be used during routine production. It is an opportunity to evaluate any changes likely to occur in the product shelf-life as a result of moving production to a larger scale. It is
important before any trials are done that the product safety has been assessed by doing a Hazard Analysis Critical Control Points (HACCP) study. If the safety of the product is assured then the main part of the shelf-life assessment can be done on product samples that have been manufactured using routine equipment.

- **Choice of storage conditions:** it is important when choosing storage conditions to ensure that the samples of product will be exposed to
conditions of time and temperature that are similar to those likely to be in operation during manufacture, retail distribution and storage as well as purchase by the consumer. A protocol is given by Betts et al. (2004) for appropriate times and temperatures to represent likely conditions seen during the shelf-life of chilled foods.

- **Sampling frequency**: the frequency of sampling times will depend on the expected shelf-life of the product. For a short shelf-life (i.e. < 1 week) chilled product it may be advisable to test samples every day, whereas for a longer shelf-life (e.g. up to 2 months) then testing every week may be sufficient. At each sampling point a minimum of three samples should be evaluated and ideally these should be taken from different production runs in order to account for any variability that is likely to be seen in different batches of the product.

- **Which tests to conduct**: any factors that were identified as being likely to affect the quality of the product should be evaluated during sample testing. Such tests may be key microorganisms, chemical factors such as rancidity or sensory changes such as changes in flavour, odour or appearance.

- **Interpretation of results**: the levels of each of the factors measured during the shelf-life trial should be assessed against the acceptance criteria for levels of bacteria or for acceptable deterioration in key quality parameters. The shelf-life should be set as the last sampling date before the key levels were exceeded. It should be determined whether the proposed shelf-life based on the results from the trial meets the target shelf-life set in the initial product concept meeting. If it does not, then a decision needs to be made on whether a shorter shelf-life is appropriate with respect to the commercial viability of the product. If it is not, then some reformulation of the product may be required. The shelf-life evaluation sequence would need to be repeated on the new product formulation.

Phase III: Full-scale production

During routine production of the product it is advisable to do periodic checks on samples to show that the shelf-life set during the initial trials is still valid as minor changes to ingredients or processing conditions may affect the shelf-life. In addition, any changes to product recipe, processing parameters or ingredients supplier may affect the shelf-life and this should be evaluated in a product and process review.

In shelf-life evaluation, it is only those microorganisms naturally present in the batch of product that will be able to grow during storage. Ideally, under good manufacturing conditions and using a HACCP approach to product manufacture, there will be minimal chance of undesirable food spoilage organisms being present. The product conditions will have been chosen to control those organisms likely to be present during routine production.

However, it is possible that on occasions during routine production, the product may be contaminated with different spoilage organisms not present
in the batch used for shelf-life evaluation. If this occurred, then the manufacturer would need to know whether the product was likely to remain stable during normal storage conditions or whether it would be likely to spoil. This ‘what if’ scenario is the rationale behind the use of challenge testing trials.

Challenge testing
Microbiological challenge testing is the laboratory simulation of what could happen to a product during manufacture, distribution and subsequent storage. It is important that the challenge test scenario is realistic. The reasons for undertaking the challenge test need to be defined and some consideration must be given as to how the results will be used. For example, it may be possible to show growth of a particular spoilage organism in a product. However, if it is unlikely that such an organism would ever contaminate the product, it should be considered whether it is advisable to do the challenge test.

Challenge testing involves inoculation of the product with relevant microorganisms that have the potential to be contained in raw material or introduced into the product at some stage during production and distribution and holding of the product. It can be done under a range of controlled environmental conditions to establish product stability in the case of food spoilage organisms. There are four main stages to challenge testing: (i) an appropriate experimental design; (ii) an inoculation procedure; (iii) a test procedure; and (iv) interpretation of results (Notermans et al., 1993).

Design of challenge test
Challenge testing can be applied to new or existing products to:

• determine the safety of the product;
• determine the potential for spoilage of the product;
• assess the stability of new recipes or reformulated products.

Safety is the prime consideration when producing food for commercial sale and it must be ensured that the product represents a minimum hazard to the consumer. However, this is outside of the scope of this chapter and only spoilage issues will be considered further. The use of the HACCP approach to identify food poisoning organisms which should be used in challenge tests is described by Notermans and in’t Veld (1994).

Organisms likely to contaminate and spoil a product will depend on the formulation characteristics. In aerobically packed chilled food products, *Pseudomonas* spp. are often responsible for spoilage, while in low-pH preserved foods, e.g. mayonnaises, specific strains of yeasts may be responsible, e.g. *Zygosaccharomyces bailii*. In mildly heat processed foods the organisms of concern may be spore-formers, e.g. *Bacillus polymyxa*. Table 5.5 gives some examples of spoilage organisms, which may be useful to use in challenge tests for various food types.

Having identified which organisms are to be used, it is necessary to consider
what particular aspects of the product and storage conditions are to be challenged and where it is likely that contamination will occur. Challenge tests can be used to assess contamination of the product before heating, after heating and during filling or after opening of the final package by the consumer. It is important to define where contamination is likely to occur as this will define the preparation procedures to be used for the test organisms (Fig. 5.3).

Challenge tests can be used to assess the effect of storing the product under a variety of controlled storage conditions. These should include conditions to simulate abuse conditions, which may be encountered during distribution and consumer handling after purchase.

Another important aspect to consider during design of a challenge test is the number of samples to be evaluated at each sampling point. There should be sufficient data obtained to allow for statistical analysis if required and, where possible, triplicate samples should be evaluated as a minimum. In addition, the number of sampling times needs to be defined. Ideally there should be a minimum of five sampling times, one at the beginning of life, one at the end and three spaced throughout the total time period. The greater the number of sampling times, the more useful the data obtained as it will give information of the rate of microbial growth at various points throughout the shelf-life.

In some high acid or heavily preserved products, it is not unusual for levels of microorganisms to stay constant; they may even decrease over a period of hours, days or weeks before beginning to increase. Sufficient sampling times should be done throughout the usual shelf-life to ensure that any initial decrease in microbial levels is not taken to be the end-point of the trial and so subsequent growth following this is missed.

Each experiment needs to be adequately controlled to ensure that the results obtained are meaningful. These should include positive controls, i.e. laboratory media in which the organisms used in the challenge test are known to grow. It may be useful to include negative controls in which growth is not expected to occur. The use of such controls will ensure that the behaviour of

<table>
<thead>
<tr>
<th>Food group</th>
<th>Spoilage organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chilled</td>
<td><em>Pseudomonas</em> (if non-MAP product)</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
</tr>
<tr>
<td></td>
<td>Lactic acid bacteria (if MAP product)</td>
</tr>
<tr>
<td>Ambient stable</td>
<td>Yeasts, <em>e.g.</em> <em>Z. bailii</em></td>
</tr>
<tr>
<td>High acid</td>
<td><em>Bacillus polymyxa</em></td>
</tr>
<tr>
<td></td>
<td><em>Clostridium butyricum</em></td>
</tr>
<tr>
<td></td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>Cream-based products</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
</tr>
<tr>
<td>Ambient stable</td>
<td>Yeasts</td>
</tr>
<tr>
<td>Low $a_w$</td>
<td>Moulds</td>
</tr>
</tbody>
</table>

Table 5.5 Typical spoilage organisms for use in challenge tests
the test organism is as expected under known conditions, and this will give confidence that the results seen in the product are correct.

Inoculation procedures
The choice of organisms for use in challenge tests is very important as they must provide a realistic challenge to the product. If the organisms chosen were particularly resistant to a preservative used in the product, then they may grow, whereas other less-resistant strains would not. Conversely, if the organisms used were very sensitive to the antimicrobials used, then they would fail to grow and the product would appear to be safe or stable when in fact it may have failed the challenge test had more realistic organisms been chosen.

Fig. 5.3  Product preparation stages for a chilled recipe meal and potential stages for contamination with spoilage organisms.
The cultures chosen should ideally have been isolated from a food source similar to the product under test, or conditioned by growing them in a product sample, or laboratory media with similar characteristics (Notermans and in’t Veld, 1994). Cultures from recognised culture collections, e.g. National Collection of Industrial and Marine Bacteria, may be preferable as they allow full traceability for comparison between different challenge tests but should be checked to ensure that they behave in a similar manner to freshly isolated strains. It is preferable to use more than one strain of each microorganism in order to provide a greater challenge to the product. Organisms can be used separately or mixed together as a single inoculum containing different spoilage genera. The use of separate organisms will represent the ‘worst-case’ situation. There will be less competition for the organism and it may therefore grow quicker than if it were present in a mixed culture. This option is expensive as the test would need to be repeated for each organism.

The use of a mixed cocktail is likely to be more realistic as it will include the microbial interactions likely to be seen in food products. Individual strains may grow more slowly than they would if tested singly due to the competition. Conversely they may grow more quickly due to the presence of nutrients made available by growth of the other organisms present. This option is more cost effective as it involves only one test.

Other important considerations are the volume of inoculum used, and method of inoculation. In terms of inoculum volume, a minimal amount should be used so that the characteristics of the product, such as $a_w$, are not affected. Rose (1987) details inoculation procedures for liquid, dry and intermediate moisture products. The number of organisms per unit weight or volume must be realistic. The levels must be high enough to be easily detected, e.g. minimum level of 100 cells per gram of product; however, they should not be so high that they easily overcome the preservation capability of the product. Conducting a challenge test with an inoculum size of $10^6$ cells per gram is unrealistic and is likely to lead to product failure, unless the preservation system used is intended to achieve a high reduction in numbers of test organisms, in which case such levels may be necessary.

The organisms should ideally be conditioned to grow under the stress factors encountered in the food product. This will depend on the likely source of the contaminant, but, for example, *Pseudomonas* contaminating a chilled product may well come from the factory environment and thus be adapted to grow under chill temperatures. Similarly, an acid-tolerant yeast or lactic acid bacteria containing fruit juice, pickles or sauces may well have adapted to a high-acid environment. The ‘conditioning’ of organisms to the stress they are likely to encounter in foods can have a large effect on the results of the challenge test. Conditioned organisms may well have a shorter lag time or faster growth rate when introduced to the product and will pose more of a challenge.

If it is considered that the only point of likely contamination is prior to a heating step, then it is appropriate to expose the test organism to a similar
heat process before being used in the challenge test as this will ensure it is in the same condition it is likely to be in when present in routine production.

When inoculating the product, care must be taken to ensure that the process of inoculation does not affect the intrinsic/extrinsic parameters of the product itself, for example the $a_w$ or preservative concentration, while the inoculation of MAP or vacuum packs may affect the intra-pack gaseous environment, unless suitable precautions are taken.

Interpretation of results
Before the challenge testing begins it is important to define the criteria of acceptability of the product. It may be that no growth of the spoilage organisms is allowed. In this case, the end of lag phase and signs of growth within the target life would constitute the failure of the challenge test. Alternatively a certain growth may be allowed, e.g. up to a maximum of $10^4 \text{cfu g}^{-1}$ and the point at which this occurs will be the end of the challenge test. If the specified end-point is reached earlier than anticipated then the product should be reformulated or the processing and storage conditions adjusted accordingly.

If the product survives the challenge test, i.e. the test criteria are not reached during the desired shelf-life, then the product can be considered suitable in relation to the specific conditions tested should it happen to become contaminated with spoilage organisms with similar characteristics to those tested.

If there are any changes, however small, to the product formulation, processing, storage, distribution or retail conditions then the results of the challenge test can no longer be considered to be reliable.

5.3.2 Variation in microbial growth kinetics
The growth kinetics of any microorganism under a defined set of conditions shows a level of innate variability. Therefore, the results from a challenge test or shelf-life trial can only ever be an indication of the range of potential growth characteristics of an organism. For this reason it is important that challenge tests and shelf-life trials contain a sufficient level of replication to ensure that the range of likely growth kinetics is determined. Ideally a minimum of triplicate packs should be examined on each sampling occasion. In addition, product from different batches could be used if it is considered likely that different batches of the product will vary.

It may be advisable to shorten the working shelf-life of the product slightly until sufficient data are available on product quality throughout shelf-life on product manufactured during routine production (Betts et al., 2004). Another way to assess the reliability of the data from shelf-life trials is to compare it with predicted shelf-life based on mathematical models (see Section 5.3.3).
5.3.3 Use of predictive models in challenge tests

Chapter 4 in this book is dedicated to modelling microbial food spoilage. In this chapter the specific use of models in challenge tests is described.

Predictive microbiological models involve the use of mathematical equations to estimate the probable growth of bacteria under different conditions. They are developed using laboratory data from one set of experimental conditions and can be used to predict the likely responses under new sets of conditions not previously tested. Modifications of new or existing recipes can be evaluated on the computer using a predictive model very quickly and easily, before embarking on expensive laboratory experiments or pilot-scale production runs.

Predictive models have been developed extensively over the past 15 years, during which time a number of predictive modelling systems have become available in the public domain. Currently there are a number of systems that can be accessed by the food industry to help assess the shelf-life of products, for example, Pathogen Modelling Program (PMP; www.arserrc.gov), Growth Predictor (www.ifr.ac.uk) and the Campden and Chorleywood Food Research Association (CCFRA) FORECAST Service (www.campden.co.uk).

With respect to product safety, there are a number of models that can be used to assess the likely growth, survival or death of foodborne pathogens under a variety of environmental conditions. PMP and Growth Predictor include models for *Salmonella*, *Listeria* and *Escherichia coli* O157, *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum*.

It is important to be able to predict the growth of food spoilage organisms when considering the likely stability and shelf-life of food products. The FORECAST system contains models for specific spoilage groups, for example; *Pseudomonas* species, Enterobacteriaceae, lactic acid bacteria or for a mixture of spoilage organisms relevant to food commodities, fish products and meat products. Some of the spoilage models (Table 5.6) can be used to help establish the shelf-life of food products. The models can also be used as a valuable addition to traditional challenge test studies. The likely growth of spoilage organisms at different contamination levels can be predicted.

Predictive models can be used to obtain data on the growth rates of bacteria which can be applied to all stages of the manufacturing process, for example:

- new product development;
- changes to product recipes;
- estimating microbiological shelf-life;
- setting microbiological specifications;
- trouble shooting, for example, to determine the effect of breakdown of storage temperatures.

Before using predictive models for any of these stages, it is important that consideration is given to the correct choice of organism, just as it is important to choose the right organisms for challenge studies. It is also important to
note that predicted models should not be used as a replacement for challenge
test studies. However, if the predictions are verified against data obtained
from real foods and the model is shown to be reliable for a particular food
group, then models can be used as a reliable indication of likely growth if a
product becomes contaminated with spoilage organisms.

One of the simplest measures of the goodness-of-fit of a model is to
visually compare predicted and observed data (Bratchell et al., 1990; Baranyi
et al., 1999). Figure 5.4 shows the basic form of such a graphical representation.
Predicted values are plotted against observed values on appropriate axes and
the closer the points fall to the line, the better the agreement between predicted
and observed values.

A more quantitative assessment can be made of the fit between predicted
and observed growth data using performance measures. Ross (1996) proposed

<table>
<thead>
<tr>
<th>Model</th>
<th>Temperature (°C)</th>
<th>NaCl (% aq)</th>
<th>aw</th>
<th>pH</th>
<th>Other conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas1</td>
<td>0–15</td>
<td>0.0–4.0</td>
<td>(0.970–1.000)</td>
<td>5.5–7.0</td>
<td></td>
</tr>
<tr>
<td>Bacillus spp.2</td>
<td>5–25</td>
<td>0.5–10</td>
<td>(0.946–1.000)</td>
<td>4.0–7.0</td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae3</td>
<td>0–30</td>
<td>0.5–10</td>
<td>(0.943–1.000)</td>
<td>4.0–7.0</td>
<td></td>
</tr>
<tr>
<td>Yeasts (chilled foods)4</td>
<td>0–22</td>
<td>0.5–10</td>
<td>0.870–1.000</td>
<td>2.6–6.0</td>
<td></td>
</tr>
<tr>
<td>Yeasts (fruit/drinks)5</td>
<td>0–22</td>
<td>–</td>
<td>(0.946–1.000)</td>
<td>2.0–7.0</td>
<td>0–60% Sucrose (w/v)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0–20% Ethanol (v/v)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0–1000 Potassium sorbate</td>
</tr>
<tr>
<td>Lactic acid bacteria6</td>
<td>2–30</td>
<td>0.5–10</td>
<td>0.870–0.989</td>
<td>3.0–6.0</td>
<td></td>
</tr>
<tr>
<td>Meat spoilage7</td>
<td>2–22</td>
<td>0–6</td>
<td>4.6–7.0</td>
<td></td>
<td>0–240 KNO2 (ppm)</td>
</tr>
<tr>
<td>Fish spoilage8</td>
<td>2–22</td>
<td>0–6</td>
<td>(0.943–1.000)</td>
<td>4.5–8.0</td>
<td></td>
</tr>
</tbody>
</table>

1 Pseudomonas fluorescens, Ps. putida, Ps. fragi, Ps. aeruginosa.
2 Bacillus polymyxa, B. pumilus, B. firmus, B. coagulans.
3 Proteus mirabilis, Klebsiella pneumoniae, Citrobacter freundii, Enterobacter cloacae, Hafnia alvei.
5 Saccharomyces cerevisiae, Sacch. carlsbergensis, Pichia anomola, Pich. carsonii, Candida stellata.
6 Lactobacillus casei, Lac. sake, Leuconostoc mesenteroides, Pediococcus pentosaceus, Ped. acidilactis, Lac. plantarum, Lactococcus lactis, Carnobacterium divergens, Carn. gallinarum.
7 Leuconostic mesenteroides, Lactobacillus sake, Carn. divergens, Ps. fluorescens, Ps. putida, Brochothrix thermosphacta, Serratia liquefaciens, Serr. marcescens.
8 Ps. fluorescens, Acinetobacter calcoaceticus, Flavobacterium indologenes, Lactobacillus viridescens, Lac. plantarium, Moraxella sp., Aeromonas hydrophilia, Aer. sobria, Serratia liquefaciens, Hafnia alvei, Lactococcus lactis, Shewanella putrefaciens, Photobacterium phosphoreum.
two different indices for determination of model performance. The bias factor ($B_f$) is a measure of the overall agreement between predicted and observed values. It will indicate whether, on average, the predictions lie above or below the line of equivalence, and by how much. Equal weighting is given to over-prediction, where the predicted value is greater than the observed, and under-prediction where the predicted value is smaller than the observed. Perfect agreement between observed and predicted values would give a $B_f$ of 1. A value larger than 1 would indicate that on average, the predictions were larger than the observed values and would represent a fail-dangerous model, e.g. a $B_f$ of 1.2 would indicate that the predictions were 20% larger than observed values. A value less than 1 would indicate a fail-safe model where, on average, the predictions were shorter than observed. A fail-safe model is one where the predicted growth of the organism is faster than that likely to be observed in practice. For example, it may predict that it would take 8 days for spoilage organisms to reach the threshold level required for spoilage to be observed, when in the food matrix it took 10 days for such growth to occur. A fail-dangerous model represents the opposite situation. It may predict 10 days for critical levels to be reached when in practice it took only 6 days for sufficient growth to occur.

In calculation of the $B_f$, the over (+) and under (–) predictions can cancel each other out. They do not therefore show the absolute errors in the model. This can be achieved by the use of the accuracy factor ($A_f$). This is based on a similar equation to the bias factor but disregards whether the difference between the predicted and observed value is positive or negative. As all differences are now positive, the $A_f$ value will always be equal to (if there is perfect agreement) or greater than one. An accuracy of 1.5 means that on average, the predicted value is 50% different (either smaller or larger) from the observed value.

If a $B_f$ is calculated for verification data then the answer can be interpreted according to these guidelines where four categories of $B_f$ values are proposed (Betts and Walker, 2004):

- Good model – $B_f$ 0.9–1.05.
- Acceptable model – $B_f$ 0.7–0.9.
• Use with caution – $B_t$ 1.06–1.15.
• Unacceptable model – $B_t$ <0.7 and >1.15.

Provided verification of a predictive model falls within an acceptable or good category based on the criteria above, then it can be used to assess the likely growth of spoilage organisms in new products in the same generic food category.

5.4 Future trends

5.4.1 Reduction in food preservatives
Over recent years there has been a consumer-driven trend for manufactured foods to contain fewer artificial preservatives. More recently this has been followed by an initiative by the Food Standards Agency to reduce the level of salt in many processed foods, including meats and baked goods, by up to a half. This can have a large effect on the shelf-life of a product. Any change in product formulation should be evaluated and, if necessary, a new shelf-life trial should be done. Predictive models as described in Section 5.3.3 are useful tools to evaluate the effect of changes in level of preservatives. For example, the effect of reducing salt in a chilled product can be seen in Fig. 5.5. Predicted growth of Enterobacteriaceae is shown at 5°C for a product (pH 6.5) containing 3% salt and 1.5% salt. Based on a maximum level of $10^4$ Enterobacteriaceae per gram, the shelf-life would be approximately 5.5 days for the product with 1.5% salt and approximately 9.5 days for the product with 3% salt.

In the future, there are likely to be diverse requirements for the shelf-life of food products. On the one hand, manufacturers are striving to find ways to increase the shelf-life in order to improve manufacturing efficiency, while on the other hand there is the pressure to reduce additives and preservatives in products, which will reduce the shelf-life of foods. Alternatives to the

![Fig. 5.5 Growth of Enterobacteriaceae at 5°C in a chilled food product at pH 6.5 with 3% (w/v) or 1.5% (w/v) salt.](image)
5.4.2 Use of microbiological criteria to determine end of shelf-life
Currently one of the major determinants of the end of shelf-life is the level of general microorganisms present in the products. For a number of years the PHLS (now HPA) has given guidance on the recommended levels of aerobic colony count, which are considered to be acceptable or unsatisfactory for a range of ready-to-eat food products (latest edition PHLS, 2000). Most food manufacturers or retailers will work to meet these or similar guidelines.

However, in many cases, a high level of aerobic colonies does not necessarily equate to a product which is unsatisfactory. This will be determined by the different microbial groups within the total population. For example, chilled meat products may be able to support a population of $10^8$ lactic acid bacteria without any deterioration in product quality. However, according to the generally acceptable aerobic colony counts previously mentioned, any value exceeding $10^7$ per gram would be unacceptable for any food category.

Studies should be done to identify a more useful microbiological indicator of end of shelf-life. This could include specific spoilage organisms or specific microbial metabolite indications of incipient spoilage.

5.4.3 Effects of global trade on shelf-life
With the ever-expanding markets for processed foods, there is the potential for increased export of foods manufactured in temperate zones to those in warm or tropical zones. This global trade may necessitate re-evaluation of the shelf-life of existing products to cope with the increased ambient (and possibly refrigeration) temperatures and the potential increase in humidity. The whole product design may need to be re-evaluated as part of a HACCP review as the change in storage conditions may make the product unstable. Predictive models, which include effects of storage conditions, may be a useful tool for shelf-life prediction in the future.

5.5 Sources of further information and advice
Particularly useful publications on shelf-life evaluations and antimicrobials are:

*Evaluation of Product Shelf-life for Chilled Foods*. CCFRA Guideline No. 46 (Betts et al., 2004).

5.6 References


PUT, H.M.C. and DE JONG, J. (1980). The heat resistance of selected yeasts causing spoilage


Part II

Managing food spoilage
6

Managing microbial food spoilage: an overview
C. de W. Blackburn, Unilever Colworth, UK

6.1 Introduction

Microbial spoilage contributes to the vast amount of food that is wasted and the associated financial losses (Kantor et al., 1997). Consumer demands, trends and drivers, involving paradoxical requirements for a wider range of less heavily preserved/processed foods of improved quality and guaranteed safety, with longer shelf-lives and increasingly competitive prices, present increasing challenges in the control of microbial spoilage for all those involved in the food industry. In a consumer survey, ‘price’ was the most important purchase criterion for food (mentioned by 66% of respondents), followed by ‘freshness/not spoiled’ (37%) and ‘quality’ (33%) (Röhr et al., 2005). Faced with a global marketplace where the competition is intense, unpredictable and unruly, there is no margin for error (Miller-Smith, 2000) and the efficient and successful management of spoilage is essential.

Although there are no food spoilage management systems per se, the control of food spoilage can be linked with many of the safety and hygiene systems, processes, practices and procedures that have been developed and many of which are commonplace within the food industry. As with food safety management, the management of food spoilage needs to be applied throughout the supply chain (from ‘farm to fork’, ‘plough to plate’). It also needs to be implemented during the transition from food product development to manufacture (from ‘concept to consumer’). A quality assurance-based approach to identifying the relevant spoilage hazards and controlling them can be integrated with that of safety hazards. The use of a ‘stable by design’ approach and implementation by means of Hazard Analysis Critical Control Point (HACCP)-like principles together with all the associated prerequisite programmes (PRPs) can also be harnessed to help manage food spoilage.
Food spoilage is part of a continuum involving the identification of potential microbial, chemical and physical hazards, followed by their control to prevent a spectrum of consequences ranging from product spoilage to consumer illness, injury or even death. Consequently, food spoilage should be managed using an integrated approach, but in the context of this book the focus of this chapter will be on microbial spoilage hazards and their control.

### 6.2 Food preservation

Food preservation can be defined as the process of treating and handling food in such a way as to stop or greatly slow down spoilage and prevent foodborne illness while maintaining nutritional value, texture and flavour. To be effective, preservation must be equal to, or greater than, the microbial ‘challenge’ that the food product is presented with. The metaphor of a cliff edge (Cole et al., 1994) has been used to represent the point at which the preservation exactly matches the microbial challenge (Fig. 6.1). The cliff top represents preservation options that are sufficient to control the microbial challenge, with a reduction in preservation ultimately leading to a catastrophic failure (the rocks below) at the point when the microbial challenge exceeds the product preservation.

This metaphor is clear for cases in which the presence of a pathogen or its toxin defines the cliff edge and the subsequent drop represents food poisoning. However, in some cases (e.g. some microbial spoilage hazards) this metaphor may be better described by a less precipitous cliff slope where catastrophic failure is less applicable and ‘unacceptability’ is less easily defined (Fig. 6.1). Also, in many cases this ‘cliff edge’ cannot be approached because...
more severe preservation and processing limits are required to control spoilage microorganisms. Pressure to reduce preservation is potentially at odds with the goal of producing safe and stable food products, but there is clearly a great benefit, both for food producers and consumers, of getting as near to the ‘cliff edge’ as possible. In terms of spoilage there is perhaps more scope for compromising between the risk of spoilage and the benefits of, a more competitive product (e.g. improved quality and/or reduced cost). In these cases, an acceptable compromise between quality and spoilage might be represented by a point on a slope either prior to, or independent of, a safety cliff edge. In addition, rather than an actual point, a region may better represent the variability and/or uncertainty of the particular hazard under consideration. Knowledge is important for defining the safety and stability ‘topography’ of the product and for defining an acceptable position relative to this.

6.3 Spoilage hazards

Food spoilage is the process leading to a product becoming either undesirable or unacceptable for human consumption (with associated changes involving alterations in taste, smell, appearance or texture). Interestingly, in the definition by Singleton and Sainsbury (1993) mention is made of the food also sometimes becoming toxic.

Food spoilage may be caused by a variety of mechanisms, including microbial, chemical and physical reactions, and in many cases spoilage can be clearly defined in these distinct terms (Fig. 6.2). Microbial spoilage is often due to the growth and/or metabolism of spoilage bacteria, yeasts or moulds. Chemical spoilage may be via nonmicrobial enzymic action, oxidation or non-enzymic browning. Examples of physical spoilage include water loss; increase in moisture of dry foods; freezer burn; and recrystallisation of frozen foods. However, there are occasions when the cause and/or manifestation of spoilage is a combination of these different types of hazards, e.g. physical changes to the product or its container caused by chemical reactions or microbial growth and metabolism (Fig. 6.2).

Therefore, microbiological spoilage needs to be considered in the context that it is just one mechanism, albeit extremely important, that contributes to defining when a food is undesirable or unfit for human consumption.

6.4 Microbial safety and spoilage

Microbiological safety and spoilage of food are often separated and in many cases there is a clear reason for doing so: most foodborne pathogens will not be responsible for spoiling a food product (unsafe food may appear organoleptically unchanged) and most spoilage microorganisms are not
pathogenic. However, there are several exceptions where this separation is less distinct (Fig. 6.2). A prime example is that of the growth of certain moulds that under some conditions can lead to mycotoxin formation. The growth of some spoilage microorganisms can lead to the production of biogenic amines, such as histamine. Although naturally present in many foods such as fruit, vegetables, meat, fish, chocolate and milk, biogenic amines can also be produced in high amounts by spoilage microorganisms through the activity of amino acid decarboxylases (Paleologos et al., 2004). Biogenic amines can be found as a consequence of microbial activity in foods such as wine, fermented meat and fish products, cheeses and fermented vegetables and excessive consumption of these amines can be a human health concern (Suzzi and Gardini, 2003).
There are spoilage organisms that have been implicated in causing food poisoning but whose ‘true’ pathogenicity is uncertain, or at least under debate (e.g. some non-*cereus* Bacillus spp.). In addition there are spoilage organisms that might be considered to be opportunist pathogens (e.g. some *Aeromonas* spp.). Opportunist pathogens are generally harmless, but under certain circumstances, such as when the body’s defences are impaired, they may invade the tissues and cause disease. These are distinct from ‘true’ pathogens that are adapted to overcome the normal defences of the body and invade the tissues; with subsequent growth and/or production of toxins that damages the tissues and causes the manifestation of disease (Greenwood, 2002).

It is well recognised that non-pathogenic background flora of foods can interact with and affect the growth of pathogenic organisms. Although the growth of some non-pathogens, e.g. lactic acid bacteria, can inhibit pathogen growth (Jay, 1992), other organisms have been shown to activate growth. This so-called ‘metabirosis’, where one organism alters the environmental conditions in such a way as to allow the growth of others, is often associated with a change in pH. Studies have been documented in which the growth of some yeasts, moulds or spoilage bacteria in acid foods can increase the pH, allowing the growth and toxin production of *Clostridium botulinum* (Odlaug and Pflug, 1979; Notermans, 1993). Bacterial soft rot has been shown to lead to an increase in the prevalence and concentration of *Salmonella* in fruit and vegetables (Wells and Butterfield, 1997), although fungal rots did not have such a marked effect (Wells and Butterfield, 1999). Growth promotion of *Listeria monocytogenes* has been demonstrated on apples by growth of the fungus *Glomerella cingulata*, although another fungus, *Penicillium expansum*, inhibited growth (Conway *et al*., 2000).

The possibility of faster, or more extensive, growth of pathogens because of a reduction in the competition from spoilage microorganisms, can either be due to their removal (e.g. washing fruit; Zagory, 1999), or their growth inhibition (e.g. using modified atmosphere packaging; Francis *et al*., 1999).

The growth of microorganisms and subsequent gas production in hermetically sealed containers may also lead to physical injury. Such potential safety hazards include swelling cans causing injury when opened, pressurised glass containers exploding or cracking when being handled or opened, and pressurised plastic bottles causing explosive release of the lid when opened.

In the eyes of the consumer there is often no clear distinction between spoilage and safety. A spoiled food will often be perceived as unsafe; the fact that illness may be as a result of a psychological or organoleptic reaction rather than actual food poisoning is again a scientific distinction rather than a socially meaningful one. This lack of distinction between safety and spoilage is often mirrored in the eyes of the law. For example, in EU legislation (Anon., 2002) it is stated that ‘food shall be deemed to be unsafe if it is considered to be: (a) injurious to health; (b) unfit for human consumption’. Possible reasons for rendering a food unfit for human consumption are stated as being ‘contamination, whether by extraneous matter or otherwise…"
putrefaction, deterioration or decay’. This consumer perception and legal position have an associated impact on how the food industry itself views food spoilage, with a food spoilage incident potentially as ‘damaging’ to a food brand as a food-poisoning outbreak. This goes to emphasise the importance of managing safety and spoilage in an integrated way.

### 6.5 Management systems

There has been an inexorable move from quality control (QC) to quality assurance (QA) in the management of microbiological safety hazards in food, the focus being on preventative control measures rather than a more reactive approach centred around testing and inspection (Blackburn, 2003). Ross and McMeekin (2002) represented the potential interplay of the fundamental elements of pathogen management: the building blocks of scientific/industry knowledge; risk assessment as a decision support tool; and HACCP as the mechanism for translating quantitative, risk-based, food safety strategies into practical pathogen management systems to achieve the overall objective of ‘safe’ food.

It is proposed that, microbial spoilage management could also be transposed onto this model (Fig. 6.3). The overall objective could be extended to ‘safe

![Fig. 6.3](image_url)  
**Fig. 6.3** Fundamental elements of microbial food spoilage management.  
(Source: Ross and McMeekin, 2002).
and stable’ food and, in addition to food safety objectives (FSOs) as targets (Gorris, 2005), this could be extended to the concept of an acceptable level of spoilage (e.g. spoilage/defect/failure rate). Scientific and industry knowledge relevant to microbial spoilage hazards and their control would be critical. The combination of intrinsic and extrinsic factors will determine the dominant microflora of a particular food and hence the spoilage hazards that need to be identified and controlled (see Chapters 7–23). For example, the interaction between pH and water activity ($a_w$) can have a dramatic impact on the growth of some of the major groups of microorganisms associated with a food (Fig. 6.4; Hocking and Christian, 1995). HACCP and/or HACCP-like principles together with associated prerequisite programmes (PRPs) would be the mechanism for achieving this objective with quality control points (QCPs) in combination with critical control points (CCPs) to control the safety and spoilage hazards (see Section 6.7). In addition to the potential of risk assessment as a tool to provide a link between scientific/industry knowledge and HACCP/PRPs, challenge testing, shelf-life assessments and predictive spoilage models currently support decision making relevant to spoilage (see Section 6.6).

### 6.6 Management tools

#### 6.6.1 Microbiological risk assessment

In a food safety context, the formalised meaning of risk assessment has evolved primarily from the Codex Alimentarius Commission (CAC) definitions (FAO/WHO, 1995), where risk assessment is the primary science-based part

![Fig. 6.4 Schematic illustration of the combined influences of pH and $a_w$ on microbial growth. (Source: Hocking and Christian, 1995)](image-url)
of risk analysis, dealing specifically with condensing scientific data to an assessment of the human health risk related to the specific foodborne hazard, and risk analysis according to these definitions also comprises risk management and risk communication (Schlundt, 2000). Microbiological risk assessment approaches have been utilised primarily by regulatory bodies and researchers in order to determine the best and/or most effective risk management options (Gale, 1996; Lawrence, 1997; Gofﬁ et al., 1999; Schlundt, 2000; Kelly et al., 2003; McLauchlin et al., 2004).

However, the food and beverage industry is beginning to apply these risk assessment approaches in order to help better manage microbial pathogens (Membré et al., 2005, 2006; Syposs et al., 2005). The risk assessment outputs have enabled decisions to be made about heat process optimisation (Membré et al., 2006) and shelf-life determination (Membré et al., 2005). Extension of this concept to risk assessment of spoilage hazards is possible. However, risk assessment is not without disadvantages in that it is time consuming and requires a great deal of detailed knowledge and considerable skill to implement.

6.6.2 Challenge testing and shelf-life assessment
Challenge testing and shelf-life assessments are often key to determining the safety and/or stability of a food product. Challenge testing is more frequently associated with artificial inoculation of a food product with relevant pathogens to determine their fate (growth, survival or death) under a defined set of conditions. Shelf-life assessment is more frequently associated with the use of ‘naturally’ contaminated food samples in order to determine the extent to which shelf-life is limited by the growth of spoilage microorganisms. These techniques are covered in more detail elsewhere (Chapter 5) as are the methods for enumerating microorganisms (Chapters 1–3) and/or metabolic end-points (Chapter 1).

Although from a practical perspective the assessment of safety and stability/shelf-life can be, and may have to be, determined separately, it is important that the results are brought together so that decisions regarding the product formulation, processing and storage conditions are made with all the relevant information.

6.6.3 Predictive models
Much of the focus of predictive microbiology has been on the modelling of infectious or toxigenic pathogens. However, in many cases it is the growth of spoilage microorganisms that will determine shelf-life, or the product design will be inherently safe (e.g. low pH, low \( a_w \)) such that only spoilage microorganisms will be capable of growth. Predictive models are increasingly being developed for spoilage microorganisms, or their metabolites, and this subject is covered in more detail elsewhere (Chapter 4).
In the same way that predictive models can help to eliminate, reduce or optimise challenge testing, spoilage models have the potential application to aid shelf-life determination as well as distribution and storage condition assessment, product formulation and reformulation, process design, risk assessment, PRPs and HACCP (Vestergaard, 2001; Blackburn, 2003; Chapters 4 and 5).

6.6.4 Microbiological testing and criteria

The philosophy of utilising preventative quality assurance-based approaches to food safety is to eliminate the reliance on finished product testing and reduce such testing to a level appropriate for HACCP verification. In reality, poorly validated and/or implemented HACCP plans have often not allowed this benefit to be realised and finished product testing is still performed extensively. However, even with a scientifically valid and effectively implemented HACCP plan, the requirements for microbial testing have been redistributed to, and focused on, HACCP validation, verification and PRP activities (Blackburn, 2003). Pathogen testing is beyond the scope of this book, but is covered in detail elsewhere (ICMSF, 2002; McMeekin, 2003). Within this context, spoilage microorganisms and indicator microorganisms are widely used for microbiological criteria.

A microbiological criterion for food defines the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot (CAC, 1997; ICMSF, 2002). Microbiological criteria are essentially of three types: standards, guidelines and specifications (IFST, 1999). A standard is a microbiological criterion contained in a law or regulation where compliance is mandatory and is usually focused on safety. Standards have been withdrawn by some countries (e.g. Australia and New Zealand) as they were not considered to be justified as supporting a public health objective, being more relevant to quality and spoilage issues (Hassell and Salter, 2003). A specification is a microbiological criterion applied to raw materials, ingredients or the end-product, which is used in a purchase agreement.

A guideline is a microbiological criterion applied at any stage of food processing and retailing which indicates the microbiological condition of the sample and aids in identifying situations requiring attention for food safety or quality reasons. Guidelines arise from many sources including the food industry, enforcement agencies and national and international committees and are especially applied to indicator organisms. An example of microbiological criteria guidelines that have been published and revised several times are those for some ready-to-eat foods sampled at the point of sale (PHLS, 2000). Microbiological guideline criteria have an important role to play as part of verification of compliance within HACCP and PRPs to demonstrate acceptability of a process step, procedure or the finished product (see Section 6.7) and their relevance extends to small and/or less developed businesses (Friedhoff et al., 2005).
6.7 Management mechanisms

6.7.1 Product design
A food product design can be defined as the process and formulation factors intended to give the product its characteristics and allow it to meet customer expectation. Microbial contamination can then be defined as the presence of types or numbers of microorganisms not envisaged in the product design.

In food manufacture, the overriding microbiological concern is that of safety. Safety assurance is best obtained by focusing on ‘safety by design’ with a combination of formulation and processing conditions that will ensure that pathogenic microorganisms are controlled (in the design). Provided that the necessary PRPs are in place (e.g. good manufacturing process, GMP, good hygienic practice, GHP) then HACCP is used to ensure that the safe design is implemented and that ‘operational safety’ is maintained. However, from a business perspective the control of spoilage is important and this is particularly true in the setting and implementing of thermal processes based on process criteria (Gaze, 2005). From a safety perspective any thermal process must target the most heat-resistant pathogen identified as a hazard that needs to be controlled by inactivation. However, this heat treatment may not be sufficient to control spoilage microorganisms and although the product might then be safe it may not meet the required degree of microbial stability. For example, an $F_0$ of 3 is the minimum process criterion for a 12-log reduction of proteolytic *Clostridium botulinum*, but most sterilisation processes are higher to target more heat-resistant spore-forming spoilage organisms such as bacilli (e.g. *Geobacillus stearothermophilus*) and other clostridia (e.g. *Clostridium thermosaccharolyticum*). There are often conflicting pressures to minimise the heat treatment to improve the organoleptic quality of a product against increasing the heat treatment to target the most heat-resistant organism and/or strain thereof that could be present.

The use of a ‘stable by design’ approach and implementation by means of HACCP-like principles together with all the associated prerequisite programmes can also be harnessed to help manage food spoilage.

6.7.2 HACCP
HACCP is a food safety management system that uses the approach of identifying and evaluating hazards and controlling them at critical control points (CCPs) in the supply chain. HACCP was originally designed for manufacturing environments, but because of the difficulties with its introduction in food service operations adaptations of the HACCP approach have been developed (Griffith, 2002). The widespread introduction of HACCP has promoted a shift in emphasis from end-product inspection and testing to the preventative control of hazards during production, especially at the CCPs. HACCP is targeted primarily at safety, but the same principle can be applied to the control of spoilage.
In Australia in the 1990s, the recognition that customers expect safe food but discriminate on quality when making their buying decision, led to the development of two key voluntary, third party certified standards focused on using HACCP principles for both quality and safety (Peters, 1998). Both standards were developed in 1995 after significant research into customer expectations, and small-to-medium size business development capabilities. Customer-defined product specifications became the key to developing these HACCP-based QA standards. The HACCP principles are used to identify quality control points (QCPs) and quality points (QPs) in the process.

It was envisaged that HACCP would provide a panacea for safety, but failure to do this has been attributed to a number of factors. One of the biggest problems concerns CCPs, both in the confusion surrounding the relationship between PRPs and HACCP, and the tendency to include quality as well as safety issues (Mortimore and Mayes, 2001). Killen (2001) described experience at Heinz where HACCP studies were initially focused on safety and spoilage issues were left to one side. However, it was recognised that spoilage problems highlighted potential hazard issues and the team decided that spoilage problems should be logged as they were identified during the HACCP study for each production line, and revisited once the HACCP systems for all the production lines were operating successfully, when they could be dealt with separately and managed by non-CCPs. Killen (2001) went on to state that in retrospect, although it produced some lively debate among the HACCP teams about the potential safety implications of spoilage problems, this decision proved very sensible. It allowed the teams to focus more effectively on the key hazards and CCPs, and to produce a practical HACCP system for each production line.

The potential difficulty that arises in HACCP studies that incorporate both safety and quality control points, and the necessity for inexperienced companies to try to separate the two in order to deal with the non-safety points through PRPs and the safety-critical points through CCPs, has been highlighted (Suwanrangsi, 2001). The same author mentions how inexperienced customer auditors sometimes require a manufacturer to include additional, unnecessary, non-critical CCPs.

Regardless of whether spoilage hazards are considered during the HACCP study their control may need to be incorporated prior to HACCP implementation. Indeed, microbiological control based on safety alone could well lead to the proliferation of spoilage microorganisms. In many cases the critical limits of CCPs may need to be more severe to control microbial spoilage hazards as compared with safety hazards alone. A good example is in the setting of heat processes, where a minimum treatment is likely to be defined by the most heat-tolerant pathogen that needs to be controlled, but relevant spoilage organisms often have greater heat resistance, thus requiring the setting of longer times and/or higher temperatures.
6.7.3 Prerequisite programmes (PRPs)

It is generally agreed that the most successful implementation of HACCP is done within an environment of well-managed PRPs (Mortimore and Mayes, 2002). Although definitions vary, the concept of PRPs does not differ significantly from GMPs. GMP is concerned with the general (i.e. non-product specific) policies, practices, procedures, processes and other precautions that are required to consistently yield safe, suitable foods of uniform quality. With regard to catering/food service businesses, good catering practice (GCP) is analogous to GMP (Griffith, 2002). GHP is the part of GMP that is concerned with the precautions needed to ensure appropriate hygiene and as such tends to focus on the prerequisites required for HACCP.

Operational control of microbial spoilage is inextricably linked with safety and therefore HACCP. In the same way, the PRPs necessary for an effective HACCP are equally important to control spoilage hazards. The goal is about reducing the level of microbial contamination entering the product from all the potential sources and limiting opportunities for microbial growth and controlling microbial inactivation steps. Although GMP cannot substitute for a CCP, collectively it can minimise the potential for hazards to occur, thus eliminating the need for a CCP. The implementation of effective GMP will control ‘general’ or ‘establishment’ hazards, many of which would include potential spoilage microorganisms that would otherwise have to be controlled by a CCP. Failure to have GMP in place will inevitably lead to a large number of CCPs in the HACCP plan covering both ‘general/establishment’ hazards and product specific ones.

Generally, GMP/GHP requirements include a range of different procedures, practices, etc. (Table 6.1). There are a number of these requirements that can be particularly important for the management of spoilage microorganisms and deserve special attention here.

Cleaning and disinfection procedures for plant and equipment are important in order to prevent or reduce the risk of contamination in the manufacturing environment. Here cleaning and disinfection procedures require careful optimisation and implementation. In particular, management is required to prevent selective pressure leading to the proliferation of populations of specialised or even preservative-resistant strains.

The microbial quality of raw materials is of particular importance with respect to controlling microbial spoilage, particularly for those raw materials that receive very little, if any, further processing. Reducing the levels of microorganisms will often lead to an increased shelf-life of both the raw material and the finished product. In this respect good agricultural practices (GAP) and good animal husbandry practices (GAHP) play an increasingly important role. Microbiological specifications for raw materials and ingredients may need to be reviewed and modified to meet the product design targets and negotiation with suppliers and/or the application of supplier approval (e.g. an approved supplier list) may need to take place. In addition, microbiological testing and on-going trend analysis to monitor suppliers may be helpful.
Depending upon the product and preservation system the level/incidence of target microorganisms will define the required GHPs. To ensure the hygienic operation of each process step, the prevention of recontamination is important. This may incorporate knowledge of the physiological condition of potential contaminating spoilage organisms and how this might impact on their subsequent recovery, lag phase (germination and outgrowth for spores) and growth rate.

With regard to pest control, carriage of spoilage microorganisms can be of particular relevance. For example, there is some evidence to suggest that spoilage yeasts may be introduced into food and beverage processing facilities via insect vectors such as bees and wasps (see Chapter 10). Air management has become an essential feature of mould control in food businesses where they pose a significant hazard (see Chapter 18).

Product rework (an important economic consideration) is often a weak point in GMP as in addition to the possible production of heat-stable toxins, high levels of microorganisms and/or enzymes can potentially be reintroduced into the product if not carefully controlled.

### Table 6.1 Examples of good hygienic practices (GHPs)

<table>
<thead>
<tr>
<th>Area, practice or procedure</th>
<th>Scope</th>
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</thead>
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<tr>
<td>Food manufacturing premises</td>
<td>Hygienic design and construction</td>
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<tr>
<td>Machinery</td>
<td>Hygienic design and construction</td>
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<td>Cleaning and disinfection procedures</td>
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<tr>
<td>General hygienic and safety practices</td>
<td>Microbial quality of raw materials</td>
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<td></td>
<td>Supplier quality assurance</td>
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<td>Hygienic operation of each process step</td>
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<td>Hygiene of personnel</td>
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<td>Labelling and traceability systems</td>
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<td>Transportation</td>
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</table>

Sources: Brown (2002); Mortimore and Mayes (2002).

### 6.7.4 Spoilage incident management

Quality assurance processes should prevent microbiological contamination (levels and types of microorganisms not envisaged in the product design), but these systems are never perfect all of the time, and sometimes there is a
failure of control (or a failure to recognise hazards and implement the necessary control) at a stage in the supply chain. Therefore, companies must consider the possibility of unsafe or spoiled product reaching the market and post-launch management systems need to be in place to deal with these situations. This could include monitoring systems (e.g. customer complaints), traceability and recall procedures (Venugopal et al., 1996; Anon., 2002), and an in-house capability or knowledge of third parties capable of investigating cases of potential microbial contamination.

There are a number of ways that microbial contamination in finished product may be discovered. If this information comes from an in-house, or third-party, test or observation (e.g. microbial numbers out of specification in a final product sample) it is more likely that the product is still under the manufacturer’s control in a warehouse or cold store, depending on the product’s shelf-life and the length of time between sampling and getting results. However, depending on the shelf-life of the product, the supply chain logistics and the rapidity of the test used and the frequency of sampling, the product may be in the distribution system or with the consumer.

The information may also come directly from the consumer, via a complaint, and then the product is obviously out of manufacturer/retailer control. Although this may indicate microbiological contamination, it may be caused by consumer misuse or abuse. Consideration to how much consumer abuse the product design should be able to withstand is an important but difficult consideration that needs to be made during the HACCP study.

Regardless of the source of the information, one of the first considerations is whether the contamination is microbiological. The simple answer is ‘not always’ as has been discussed previously (Fig. 6.2). For example if the result is an observation of container swelling this may not necessarily mean gas production (e.g. overfilling) and, if it does, gas production does not necessarily mean microbial growth (e.g. chemical reactions). In addition, there may be a potential safety risk associated with a spoilage incident that needs to be considered.

Assuming that a non-microbial problem can be ruled out, another consideration is whether the result is genuine. There is always the possibility of a ‘false-positive’ result from laboratory contamination or cross-contamination from controls, or contamination from packaging (e.g. yeast/mould in aseptically packed beverage). In addition, the use of an unvalidated method; insufficient controls; difference in methodology; and identification errors and difficulties (e.g. product residues/particles appearing similar to microbial colonies) may give rise to erroneous results.

There are a number of important aspects in managing a potential microbiological incident or non-conformance (Kilsby, personal communication). The first step is to obtain information and define the problem. This involves determining what actions, if any, have been taken and determining whether the affected product is under the manufacturer’s control (i.e. can it be blocked) and to define an interim action for the ‘at-risk’ material. The
The next step is to assemble existing data (e.g. QA/QC results, consumer complaints, CCP data, CCP critical limits, line performance records) and to analyse them so that a hypothesis for contamination can be defined and the ‘at-risk’ period defined. Here, methods that are used to indicate changes in performance, such as cumulative sum (or cusum) and exponentially weighted moving average control charts (Montgomery, 2005), can be useful. If a hypothesis cannot be formed then either the relevant expertise is lacking or more time is required to understand the problem before any further analysis is done. If not already done, and if appropriate, a sampling protocol should be defined and carried out. The analyses should be relevant and targeted to test the hypothesis that has been defined. Finally, there should be output in the form of decision making for immediate action (regarding affected product and production) and learnings to implement relevant improvements to preventative measures.

6.8 Application of spoilage management systems

6.8.1 Product design and development

The development of a new food product can be viewed as a series of broad stages from product concept through to product launch and on-going manufacture (Fig. 6.5). The product concept stage provides an opportunity to understand the marketing requirements, which must then be translated into a product design. At this stage a consideration of the hazards (including microbial hazards) that affect both the safety and quality of the product needs to be addressed. The product design can be defined as the process and formulation factors intended to give the product its characteristics and allow it to meet customer expectation.

It is essential to ensure that products are safe by design and meet the required degree of microbial stability and/or shelf-life. In addition to ensuring safety, improving and/or optimising the product design can also improve shelf-life and/or quality. Alternatively, it may also highlight a need to redesign the product.

The product design will affect the extent to which preventative measures are required to control microbial contamination. The robustness of the preservation system determines the level of challenge that can be withstood and therefore the level of factory hygiene design and operation required. Product designs range from heavily preserved products intended to be ambient stable and multi-use, to single use, minimally preserved chilled products with short shelf-lives.

Translating the product design into the HACCP framework will ensure that the appropriate hazards are managed. The relative importance of spoilage microorganisms should be identified during the hazard analysis stage of the design HACCP and may range from a general consideration of quality in frozen products to the limiting factor in the shelf-life of a chilled product.
At this stage design control points (DCPs) can be identified. However, as the manufacturing line and factory may not have been agreed or identified, a detailed discussion on PRPs may not be possible (and would probably not be appropriate), although the scope of the required PRPs that would need to be in place should be defined.

Validation of the design HACCP plan may involve a number of activities (Scott, 2005). The production of kitchen-scale samples will provide the opportunity to start any shelf-life assessments and challenge tests that are appropriate. Depending on the product design the shelf-life or stability of the product may ultimately be defined by the necessity to control spoilage microorganisms rather than just pathogens. The results of these validation activities may confirm the safety and stability of the product design, but they may also require the modification to DCPs or a modification to the product design, which may then require further validation activities prior to further scale up of production.
As the product design evolves and stabilises then the design HACCP can be used as a basis for the operational HACCP and the DCPs re-evaluated and translated as appropriate into CCPs as and when the design is transferred to the factory line. At this stage quality control points (QCPs) can be identified, although it is important that the inclusion of microbial spoilage hazards in the HACCP plan and product design does not detract from sufficient resource being given to safety. The required PRPs can also be assessed in detail to ensure that they are in place and adequate to control all the hazards that are not covered by specific CCPs or QCPs.

In parallel, the production of pilot-scale samples will give the opportunity to evaluate whether scale-up of production is likely to give rise to microbiological problems not envisaged in the product design and further detailed information required to further define CCPs, QCPs and PRPs.

Once production starts and, depending on the product, the distribution system is filled in readiness for product launch, there is the opportunity to ensure that the HACCP plan is fully implemented. Verification activities are likely to be at their most intense at the early stages and a high level of monitoring (e.g. finished product testing) may be appropriate. After product launch, monitoring of consumer complaints plays a key role to verify that the product design is meeting consumer expectation.

The HACCP plan and PRPs need to be kept up to date. The HACCP plan should be reviewed when any part of the product design or production process changes, in the case of a previously unrecognised hazard being identified, or after regular periods of time (e.g. annually), whichever happens first.

### 6.8.2 Supply chain

Microbial spoilage must be managed throughout the food product supply chain where there are many sources of contamination and routes of transmission (Fig. 6.6). Raw materials and ingredients will have an associated microflora, which may contain potential spoilage microorganisms that have to be controlled in the subsequent product design. Even if apparently absent by testing, the risk of key spoilage organisms being present will need to be considered in the hazard analysis. In addition, the level of spoilage microorganisms in raw materials will have an impact on the subsequent product design (processing and preservation) and on subsequent shelf-life or product stability. Prevention of contamination over and above the maximum level acceptable within the product design will need to be controlled by appropriate CCPs or PRPs (e.g. supplier assurance, microbiological specifications, storage conditions).

During manufacture there are other sources of contamination, including equipment surfaces, factory environment, unprocessed product, people, air and water. There may also be the opportunity for organisms in the raw materials or intermediate products to grow, or a failure to inactivate them sufficiently at one or more of the processing steps. The finished product itself may actively select for the growth of specific types of spoilage
Fig. 6.6 Managing microbial spoilage through the product supply chain.
microorganisms. In particular, product residue on equipment surfaces or in the environment may provide the perfect environment and opportunity to select for high levels of spoilage microorganisms.

After manufacture there is the potential for contamination during distribution. With chilled products there is the opportunity for surviving microorganisms to grow, the rate being related to the temperatures within the cold stores and chilled transportation. It should be borne in mind that temperature abuse will increase the rate of microbial growth and potentially decrease the shelf-life depending on assumptions made during the shelf-life assessment studies. In addition, severe temperature abuse may allow the growth of organisms not considered to be significant hazards and therefore not controlled in the product design. An example here might be ambient-stable products distributed to tropical countries where ambient temperatures might allow the growth of thermophilic microorganisms. For all finished products there is also the potential for recontamination, usually via damage to primary packaging or faulty packaging equipment. During distribution there is the continuing potential for recontamination by damage to packaging. Within retail there are similar opportunities for recontamination and microbial growth, although the very high temperatures that could be experienced during shipping are unlikely.

Finally there is the consumer, certainly the most unpredictable and yet the most important part of the supply chain. From purchase onwards there is the potential for abuse of the product with regard to storage temperature (e.g. poor refrigeration, failure to refrigerate after opening), storage time (exceeding ‘use-by’ or ‘best-before’ dates) and product use (e.g. undercooking). For multi-use products the potential for recontamination must be considered in any open shelf-life that is part of the product design. In addition there are an increasing number of consumers who may be more susceptible to foodborne disease, which must be considered in the product design and which may affect the hazard analysis (e.g. the inclusion of opportunist pathogens).

### 6.9 Future trends

In addition to playing a pivotal role in improving the nutritional quality of food, ensuring its safety and preventing foodborne disease, food technologies reduce losses due to spoilage or contamination and are thus vital in the prevention of malnutrition and starvation (WHO, 1995). However, the issue of adequate nutrition in developing countries is increasingly being joined by the apparently paradoxical problem of obesity (Caballero, 2005). The expansion of the global marketplace is likely to continue driven by the pressure to reduce supply chain costs together with increasing consumer demand for choice and for fair trade. The drive to reduce food processing and preservation shows no signs of diminishing and this will put increasing pressure on the food industry to manage microbial spoilage, which will require a range of strategies.
There are many so-called new, emerging and alternative processes and preservatives (López-Rubio et al., 2004; Guinane et al., 2005; Mañas and Pagán, 2005; Patterson, 2005) that have the potential to be commercialised, or be implemented more widely, to control microbial spoilage depending on the target microorganisms and the product concerned (see Chapters 7–23). However, there is plenty of scope to better understand and optimise existing processing and preservation options. This could be achieved by utilising newly available tools, such as microbiological risk assessment (MRA) approaches (Membré et al., 2005, 2006) and genomics technology (Brul et al., 2002; van der Vossen et al., 2005).

MRA approaches could help to define and communicate spoilage risk and hence give opportunity for more informed spoilage management decisions. These techniques also have potential to facilitate further the use of combinations of processing and preservation options (both conventional and new), so-called ‘hurdle’ technology, and to help identify and integrate the most effective controls to ultimately give better management of microbial hazards. Genomics technology may provide a more mechanistic (rather than deterministic) understanding of microbial inactivation, resistance, injury, resuscitation, spore germination and growth, which could lead to more targeted and efficient preservation.

In the same way that from a safety perspective ‘emerging’ pathogens need to be considered (see Motarjemi and Adams, 2006) the possible occurrence of emerging spoilage organisms should be assessed (e.g. Alicyclobacillus and psychrotrophic clostridia, see Chapter 21). This should be contrasted with re-emerging spoilage organisms due to the reduction in preservation (e.g. certain moulds in bakery products, see Chapter 18). With the potential for the transfer of genetic material (e.g. plasmids) the possibility of spoilage or non-pathogens acquiring pathogenicity needs to be considered. In addition, increasing life expectancy and improvements in medicine will mean that the potentially ‘at-risk’ proportion of consumer populations will increase, further blurring the boundary between spoilage and safety.

There is some concern that the indiscriminate use of disinfectants has the potential to lead to microbial resistance in the future. For example, Triclosan (Irgasan) is a broad-spectrum bisphenol biocide, which is increasingly being incorporated into plastics and fabrics in addition to such products as handsoaps, lotions and toothpastes. There is some evidence that certain genetic mutations could confer an increased bacterial resistance to Triclosan and because the biocide has the same target (a fatty acid synthesis enzyme: enoyl reductase) as some current therapeutic agents (e.g. the anti-tuberculosis drug isoniazid) this could also lead to antibiotic resistance (McBain and Gilbert, 2001). However, the counter-argument is that there are reports that refute these widely publicised, yet unsupported, hypotheses that the use of antibacterial products facilitates the development of antibiotic resistance in bacteria (Cole et al., 2003).

Overall it seems clear that a multidisciplinary approach is needed in the
management of microbial spoilage. Although the new ‘omics’ techniques have a role to play, ‘traditional’ microbiology skills are still essential. A worrying trend that has been voiced (see Chapter 18) is the potential for decisions made by management personnel who often do not have technical knowledge. Bioinformatics, the science of managing and analysing biological data using advanced computing techniques, has also much to offer (e.g. in risk assessment approaches and microbial characterisation). Physics and engineering are critical, particularly translating design into operational control as well as chemistry and biochemistry to understand the impact of food chemistry and preservatives on microorganisms. There are huge challenges ahead and the correct balance of all these skills will be critical.

6.10 Sources of further information and advice

6.10.1 Key books

6.10.2 Web sites
Foods Standards Agency: http://www.food.gov.uk
Food and Agriculture Organization of the United Nations: http://www.fao.org
World Health Organization: http://www.who.int/en/

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Managing microbial food spoilage: an overview


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7

Managing microbial spoilage in the dairy industry

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7.1 Introduction

Milk is an excellent medium for bacterial growth. Uncontrolled growth of undesirable microbes negatively impacts dairy product yield, shelf-life and sensory characteristics, which can lead to significant economic losses for the dairy industry. Identifying sources of contamination and controlling growth of microorganisms in raw milk and in processed products has been an ongoing challenge for the dairy industry. Enforcement of regulatory standards for milk quality and application of improved sanitation practices and processing technologies throughout milk collection, processing and distribution has led to increased product safety, quality and shelf-life.

In this chapter, the various types of microorganism associated with milk and dairy product spoilage are discussed. Sources and prevalences of these microorganisms and implications of microbial contamination on the quality of raw and processed milk products are described. Finally, current and emerging techniques for reducing and controlling bacterial numbers in raw and pasteurized milk products with the primary goals of improving product quality and extending shelf-lives are presented.

7.2 The range of spoilage microorganisms – a historic perspective in the USA

7.2.1 Raw milk

Bacterial counts in raw milk serve as indicators of herd health, the hygienic conditions under which the milk was harvested and stored, and the milk’s
spoilage potential. Raw milk quality, as reflected by somatic cell and bacterial numbers, also directly influences the yield and quality of products derived from it (Klei et al., 1998; Ma et al., 2000). Bacterial numbers in raw milk can vary dramatically, ranging from less than 1000 cfu/ml in milk collected aseptically from healthy cows to greater than $10^7$ cfu/ml (Boor and Murphy, 2002; Chambers, 2002). Principal sources of microbial contamination in raw milk include the interior of the udder, the external surface of the teat and udder, and the dairy equipment (Chambers, 2002). In the USA, the Food and Drug Administration’s Pasteurized Milk Ordinance (FDA, 2001) describes regulatory requirements and quality standards for raw milk and for Grade ‘A’ processed milk products intended for interstate commerce (FDA, 2001). Current (2006) microbial quality standards for raw milk require less than 100 000 cfu/ml Standard Plate Count (SPC) for individual farm milk and less than 300 000 cfu/ml for commingled milk prior to pasteurization (FDA, 2001). Although raw milk microbial standards vary by country, regulations generally specify maximal allowed bacterial counts in order to ensure the safety and quality of processed milk products (Table 7.1).

Production, collection and handling practices influence the initial microflora present in raw milk (Lafarge et al., 2004). The predominant microflora in raw milk typically include lactic acid bacteria, Streptococcus spp., Pseudomonas spp., Staphylococcus spp., Micrococcus spp. and yeasts (Lafarge et al., 2004). Microorganisms found in raw milk can deteriorate finished product quality through various mechanisms. Raw milk storage temperature and duration will greatly influence the predominant microflora and rate of deterioration (Lafarge et al., 2004). Raw milk microflora can be grouped into categories by optimal growth temperature ranges (i.e. mesophiles, psychrotrophs and thermodurics). A mesophile is an organism that grows well between 20 and 45 ºC, and has an optimal growth temperature near 37 ºC. Psychrotrophs are defined as organisms that can grow at 7 ºC or less and have an optimum growth temperature between 20 and 30 ºC (Collins, 1981). Thermoduric organisms are capable of surviving pasteurization or other relatively high heat treatment (Jay, 2000). Mesophilic organisms, typically lactic acid bacteria,

| Table 7.1 Bacterial and somatic cell count (SCC) limits for raw milk intended for pasteurized milk products |
|---------------------------------|---------------------------------|---------------------------------|
| Country                        | Producer raw milk               | Plant raw milk                  |
| USA$^a$                        | 100 000 cfu/ml                  | 300 000 cfu/ml                  |
|                                | 75 000 000 SCC                  |                                 |
| Canada$^b$                     | 50 000 cfu/ml                   | 50 000 cfu/ml                   |
|                                | 50 000 000 SCC                  |                                 |
| European Union$^c$             | 100 000 cfu/ml                  | 300 000 cfu/ml                  |
|                                | 40 000 000 SCC                  |                                 |

$^a$FDA (2001).  
$^b$CFIS (2002).  
$^c$EEC (1994).
can proliferate in raw milk exposed to inadequate cooling conditions for extended periods of time (Boor and Murphy, 2002). The presence of mesophilic lactic acid bacteria in raw milk at levels greater than 10^6 cfu/ml causes defects related to lactic acid production, such as the ‘malty’ flavor produced by strains of *Lactococcus lactis*.

Fermentation of lactose by lactic acid bacteria may also result in a sour taste and curdling of casein proteins when the milk is heated (Harding, 1995). Raw milk spoilage by lactic acid bacteria has become uncommon in regions of the world in which the use of efficient refrigeration systems is widespread. To illustrate, storage of raw milk at 4 °C for as little as 24 h has been demonstrated to shift bacterial populations away from mesophilic populations such as lactic acid bacteria and toward those that are better able to reproduce under refrigerated conditions (e.g. *Pseudomonas* spp.) (Lafarge *et al.*, 2004). Hence, when milk is rapidly cooled and maintained at appropriate refrigeration temperatures (defined by the US Pasteurized Milk Ordinance (2001) as cooled to 45 °F (7 °C) or less within 2 h after milking), control of psychrotrophic organisms is of primary concern in protecting raw milk quality. Poor sanitation at the farm and inadequately cleaned processing equipment have been identified as the main sources of psychrotrophic contamination in raw milk (Cousin, 1982). A broad range of psychrotrophic bacteria have been isolated from milk. Gram-negative psychrotrophs include *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Acinetobacter*, *Achromobacter*, *Aeromonas* and *Alcaligenes*. Genera of Gram-positive psychrotrophs isolated from raw milk include *Corynebacterium*, *Microbacterium*, *Micrococcus* and spore-forming *Bacillus* and *Clostridium* (Cousin, 1982; Hayes and Boor, 2001; Chambers, 2002).

*Pseudomonas* spp. are among the most prevalent psychrotrophic spoilage organisms isolated from milk. *Pseudomonas* species frequently found in raw milk include *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas fragi* and *Pseudomonas aeruginosa* (Champagne *et al.*, 1994; Ralyea *et al.*, 1998; Boor and Murphy, 2002). As some of these microbes produce heat-stable lipolytic and proteolytic enzymes that can endure pasteurization, the presence of psychrotrophs such as *Pseudomonas fluorescens* in raw milk can contribute to quality deterioration throughout processed product shelf-life, even after the original bacteria have been destroyed by pasteurization (Cousin, 1982). Bacterial lipases can produce rancid, unclean or fruity off-flavors. Proteases degrade milk proteins to release bitter peptides and may cause curdling or gelation of the milk (Patel and Blankenagel, 1972; Baker, 1983; Hayes and Boor, 2001).

Thermoduric species found in raw milk are also commonly isolated from processed milk. Thermoduric spore-forming organisms that survive pasteurization and that are able to reproduce in processed products under refrigerated conditions can decrease product quality and shelf-life (Boor *et al.*, 1998). These organisms may enter the raw milk supply through environmental sources, such as vegetation or bedding, and through milking
equipment (Griffiths and Phillips, 1990). Heat resistant organisms isolated from raw milk include *Microbacterium*, *Micrococcus*, *Bacillus*, *Alcaligenes*, *Corynebacterium* and *Clostridium* (Hayes and Boor, 2001). *Bacillus* spp. and *Paenibacillus* spp. are of particular concern because of their ability to grow under refrigerated storage conditions and to reduce product quality (Muir, 1996; Fromm and Boor, 2004).

A cow infected with mastitis generally sheds microorganisms into her milk, thereby increasing the total bacterial count. The most common organisms identified in bulk milk from a mastitis-infected herd are *Streptococcus agalactiae*, *Streptococcus uberis* and *Staphylococcus aureus* (Bramley et al., 1984; Murphy and Boor, 2000). Although most frequently associated with mastitis infections, contamination of bulk tank milk by organisms such as *Streptococcus uberis* can also result from inadequately cleaned equipment (Bramley et al., 1984; Murphy and Boor, 2000). Microorganisms that cause udder infections, such as *Streptococcus* spp., can be present in very high numbers in raw milk. Infected animals may contribute more than $1 \times 10^7$ bacteria/ml (Chambers, 2002).

In addition to increasing total bacterial numbers in raw milk, mastitic cows shed somatic cells which have the potential to deteriorate milk quality. The current US regulatory limit requires that the somatic cell count (SCC) not exceed 750,000 per ml for Grade ‘A’ milk (FDA, 2001). SCC is useful as a measure of milk quality. High SCC suggests the presence of mastitis infection in the herd. In a study in which cows were intentionally infected with *Streptococcus agalactiae*, the resulting high SCC (849,000 cells/ml) was associated with increased lipolysis and proteolysis in the pasteurized fluid milk products (Ma et al., 2000). Sensory defects in these products included rancidity and bitterness.

SPCs less than 10,000 may be achieved by excluding milk obtained from mastitic cows from the bulk tank, by maintaining a clean milking environment and by adequate cleaning of the milking equipment (Murphy and Boor, 2000). Owing to its impact on processed product quality, controlling contamination of raw milk throughout collection, handling and storage is a critical step in ensuring product safety and keeping quality.

**Microbial enumeration and identification**

Methods for evaluating milk quality include bacteriological tests such as SPC, Preliminary Incubation Count (PIC), Lab Pasteurized Count (LPC) and Coliform Count. The SPC estimates total aerobic bacteria numbers in the milk. The PIC consists of incubating the milk at 12.8°C for 18 h prior to performing a SPC and is considered to be useful for detecting the presence of bacteria that entered milk from soiled equipment or other exogenous sources (Marshall, 1992). The underlying theory behind the PIC is that microbial flora typically associated with cows (e.g. *Streptococcus* spp.) will not grow significantly when held at the PIC temperature/time combination, whereas microorganisms that are present in raw milk due to poor hygienic practices,
typically Gram-negative psychrotrophs, will increase in number under these conditions. The SPC obtained following the PIC incubation is compared with the SPC of an unincubated sample to determine if bacterial numbers have increased significantly during the holding period. A PIC that is more than 3–4-fold higher than the original SPC is considered to reflect inadequate protection of the milk from exogenous contamination (Murphy and Boor, 2000). The LPC consists of pasteurizing the raw milk by heating to 63 °C for 30 min, after which the milk is immediately cooled to 10 °C and plated (Marshall, 1992). The LPC is used to enumerate thermoduric bacteria, which typically enter raw milk through contamination from sources such as unclean equipment and soiled cows (Murphy and Boor, 2000). Coliform count is used as a measure of fecal or environmental contamination.

### 7.2.2 Processed fluid milk

Microbial quality standards for Grade ‘A’ pasteurized milk and milk products in the USA include a bacterial limit of 20 000 cfu/ml whenever the product is offered for sale (FDA, 2001). As with raw milk microbiological standards, acceptable limits for bacterial counts in pasteurized milk vary by country (Table 7.2). Following pasteurization, bacterial numbers in milk are generally less than 1000 cfu/ml (Boor and Murphy, 2002). While microflora present in freshly pasteurized milk generally represent thermoduric organisms present in the raw milk, microorganisms also can be introduced into pasteurized milk during processing, as post-pasteurization contaminants.

**Table 7.2** Microbial standards for pasteurized milk products

<table>
<thead>
<tr>
<th>Country</th>
<th>Total bacteria&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Coliform bacteria&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 000</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>( m = 10 000 )</td>
<td>( m = 1 )</td>
</tr>
<tr>
<td></td>
<td>( M = 25 000 )</td>
<td>( M = 10 )</td>
</tr>
<tr>
<td></td>
<td>( n = 5 )</td>
<td>( n = 5 )</td>
</tr>
<tr>
<td></td>
<td>( c = 2 )</td>
<td>( c = 2 )</td>
</tr>
<tr>
<td>Canada&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European Union&lt;sup&gt;d&lt;/sup&gt;</td>
<td>After 5 days at 6°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( m = 50 000 )</td>
<td>( m = 0 )</td>
</tr>
<tr>
<td></td>
<td>( M = 500 000 )</td>
<td>( M = 5 )</td>
</tr>
<tr>
<td></td>
<td>( n = 5 )</td>
<td>( n = 5 )</td>
</tr>
<tr>
<td></td>
<td>( c = 1 )</td>
<td>( c = 1 )</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total bacteria and coliform bacteria counts given as the upper limit of cfu/ml for the USA. For Canada and the EU, two tiered limits are given, with allowable results based on number of samples: \( n \) = number of sample units (sub-samples) to be examined per lot; \( m \) = maximum number of bacteria per g or ml of product that is of no concern (acceptable level of contamination); \( M \) = maximum number of bacteria per g or ml of product, that if exceeded by any one sample unit (sub-samples) renders the lot in violation of the regulations; \( c \) = maximum number of sample units (sub-samples) per lot that may have a bacterial concentration higher than the value for \( m \) but less than the value for \( M \) without violating the regulations.

<sup>b</sup>FDA (2001).

<sup>c</sup>CFIS (2002).

<sup>d</sup>EEC (1994).
Post-pasteurization contamination in the dairy processing environment is the main source of spoilage microorganisms found in conventionally pasteurized (high-temperature short-time) fluid milk. Post-pasteurization contamination generally occurs when microorganisms are introduced into the pasteurized product through contact with contaminated processing equipment, airborne contaminants or dairy plant personnel. Gram-negative organisms, predominantly the genus *Pseudomonas*, have been identified as the most common post-pasteurization contaminants responsible for fluid milk spoilage (Cousin, 1982; Kozlowski *et al*., 1993; Ternström *et al*., 1993). The gram-negative *Enterobacter*, *Klebsiella*, *Alcaligenes*, *Acinetobacter* and *Flavobacterium* also have been isolated from pasteurized milk (Cousin, 1982). As Gram-negative psychrotrophic organisms typically do not survive pasteurization temperatures, their presence in pasteurized milk is generally considered to indicate post-pasteurization bacterial entry into processed products. Post-pasteurization contamination sources include inadequately sanitized pasteurizers and filling machines, airborne contaminants and contact surfaces (Ternström *et al*., 1993; Gruetzmacher and Bradley, 1999).

When sources of Gram-negative post-pasteurization contaminants are eliminated from the dairy plant environment, fluid product shelf-lives are extended. In dairy plant environments in which Gram-negative post-pasteurization contamination has been reduced, the presence and growth of psychrotrophic, spore-forming, heat-resistant microorganisms such as *Bacillus* spp. and *Microbacterium* spp. has been recognized as the next hurdle in extending the shelf-lives of conventionally pasteurized fluid milk (Shehata and Collins, 1971). *Bacillus* species frequently isolated from pasteurized milk include *B. cereus*, *B. circulans*, *B. mycoides* and *B. licheniformis* (Meer *et al*., 1991; Crielly *et al*., 1994). In addition to *Bacillus*, species of the genus *Paenibacillus* have been identified as predominant microflora in pasteurized milk in which the presence and growth of heat-sensitive psychrotrophs (e.g. *Pseudomonas* spp.) has been controlled (Fromm and Boor, 2004). The combined ability to survive pasteurization and to grow at refrigeration temperatures gives these organisms the potential to cause spoilage (Olson, 1963; Cromie, 1991).

Psychrotrophic spore-formers such as *Bacillus* and *Paenibacillus* can enter the milk supply from various sources. Contamination of milk by these organisms appears to occur at the farm level as well as in the dairy plant. On the farm, these organisms may be introduced through inadequately cleaned milking equipment and bulk tank surfaces (Phillips and Griffiths, 1986; Griffiths and Phillips, 1990). Spores of the bacteria introduced into raw milk can survive pasteurization and germinate and grow in the processed product (Griffiths *et al*., 1986; Griffiths and Phillips, 1990). Thermudoric psychrotrophs can also enter products through post-pasteurization contamination (Davies, 1975; Coghill, 1982; Lin *et al*., 1998). To illustrate, Lin *et al*., 1998 found a larger diversity of strains among *B. cereus* isolates in pasteurized milk products than in the raw milk, suggesting that contamination takes place in the dairy plant.
Thermoduric psychrotrophs deteriorate product quality through production of heat-resistant microbial enzymes, which cause sensory defects that decrease product shelf-life (Meer et al., 1991). The proteolytic and lipolytic activities of these enzymes produce off-flavors such as bitter, unclean, fruity and rancid (Cousin, 1982). Milk defects associated with Bacillus spp. can include coagulation of milk proteins and off-flavor development such as sour, yeasty, unclean and rancid (Boor and Murphy, 2002). B. cereus and B. mycoides have been associated with sweet-curdling of milk and bitty cream defects (Meer et al., 1991). Non-spore-forming psychrotrophic bacteria, mostly strains of Pseudomonas spp., also secrete heat-stable extracellular lipases and proteases (Cousin, 1982; Champagne et al., 1994; Sorhaug and Stepaniak, 1997). These enzymes can degrade milk components and contribute to milk spoilage even after the organisms are destroyed by thermal processing (Mayerhofer et al., 1973; Garcia et al., 1989; Sorhaug and Stepaniak, 1997). Hydrolytic activity of lipases secreted by Pseudomonas spp. can cause milk to taste rancid, bitter or unclean. Casein degradation by proteases causes milk clotting and gelation in addition to giving a bitter flavor (Dogan and Boor, 2003).

Although pasteurized milk spoilage is predominantly due to bacterial contamination, yeasts and molds may also be present as contaminants. Yeasts and molds generally enter the raw milk supply from environmental sources (Fleet, 1990). Adequate pasteurization of milk will destroy yeasts and molds present in raw milk prior to heat treatment (Viljoen, 1998). Therefore, isolation of yeasts and molds from pasteurized milk generally indicates post-pasteurization contamination. The presence and growth of yeasts and molds contributes to musty or fruity flavors in milk (Hayes and Boor, 2001).

### 7.2.3 Processed dairy products

**Cheese**

Microbial growth in cheese is influenced by factors such as moisture content, water activity, redox potential, aerobic or anaerobic conditions, pH, acidity, and salt level (Johnson, 2001). Bacterial contamination of cheese products may occur during processing and post-processing handling where good sanitation practices are not in place. Spoilage microorganisms in cheese also may originate from low-quality raw milk. For example, thermoduric species of Bacillus, Clostridium, Lactobacillus, Microbacterium, Micrococcus and Streptococcus can survive milk heat treatment and grow in some cheese products (Johnson et al., 1990; Peterson and Marshall, 1990; Hull et al., 1992).

Predominant spoilage microflora and resulting defects are greatly influenced by cheese variety. The susceptibilities of hard and semi-hard cheese varieties, such as Parmesan and Cheddar, to spoilage by microorganisms is somewhat limited by their relatively low moisture contents (<50%) and pH values (~5.0). Coliforms, Clostridium spp. and molds are examples of microbes...
that can proliferate in some hard cheeses. One example of cheese spoilage associated with bacterial contamination is the late gas blowing defect, which is caused by *Clostridium tyrobutyricum* (Cogan and Beresford, 2002). Late gas blowing is most common among brine-salted hard and semi-hard cheeses (e.g. Gouda, Edam, Emmental, Gruyere), but can also occur in some soft cheeses. *C. tyrobutyricum* contamination of cheese is thought to predominantly originate from the raw milk, which can become contaminated by fecal matter from cows or by silage (i.e. fermented forages). Improperly fermented silage can contain >100,000 *Clostridium* spores per gram. Corn silage appears to be less problematic than grass silage with regard to spore numbers. Improvement in the quality of grass silage, e.g. by using silage starters, can significantly improve silage quality, leading to a lower risk for transfer of clostridial spores into the raw milk.

Fecal matter is most likely to be the direct contamination source of spores in raw milk; a positive correlation exists between spore numbers in the fecal matter of dairy cows and the feeding of poor quality silage. Silage quality and milking hygiene are the most important factors contributing to the contamination of raw milk and therefore present potential control points for improving raw milk quality with regard to exclusion of *Clostridium* spores (Wiedmann *et al.*, 1999). Soft cheese varieties, such as cottage, Brie and Hispanic-style soft cheeses, permit the growth of a wider range of microorganisms as a result of their higher moisture contents (50–80%) and pH values (5.0–6.5) (Varnam and Sutherland, 1994; Farkye and Vedamuthu, 2002). Spoilage organisms of importance in soft cheeses include psychrotrophic Gram-negative rods such as *Pseudomonas*, *Alcaligenes*, *Achromobacter* and *Flavobacterium*, which can produce objectionable odors and flavors owing to the presence of lipolytic and proteolytic enzymes (Farkye and Vedamuthu, 2002).

**Cultured milks**

A wide variety of fermented milk products exist around the globe. In the USA, yogurt, sour cream and buttermilk are among the most widely consumed cultured milks. Microbial spoilage of yogurt and sour cream typically results from growth of yeasts or molds, although bacterial spoilage also can occur (Tamime and Robinson, 1999). Yeasts are a major cause of spoilage in cultured milk products because of their ability to grow at refrigeration temperatures and low pH values (Viljoen, 1998; Frohlich-Wyder, 2003). Product spoilage due to yeast contamination and growth may occur if good manufacturing practices are not followed. For example, poorly sanitized dairy processing equipment has been identified as a common source of cottage cheese contamination (Fleet, 1990). Fleet and Mian (1987) found yeast counts greater than 10^4 cells/ml in randomly sampled commercial yogurt. The predominant spoilage yeasts isolated from commercial yogurt samples were *Candida famata*, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*.

In a previous study, *Torulopsis candida* and *Kluyveromyces fragilis* were
the most frequently isolated yeast species in yogurt collected from retail outlets in Australia (Suriyarachchi and Fleet, 1981). Other yeast genera that were isolated from the yogurt samples include \textit{Rhodotorula}, \textit{Pichia}, \textit{Debaryomyces} and \textit{Sporobolomyces} (Suriyarachchi and Fleet, 1981). The primary yeast contaminants found by Green and Ibe (1987) in a similar survey of yogurt products were \textit{Candida lusitaniae}, \textit{C. krusei}, \textit{C. rugosa}, \textit{Kluyveromyces fragilis} and \textit{Saccharomyces cerevisiae}. Gas production by yeast fermentation and off-flavor development are among the defects associated with the presence of yeast cells at levels of approximately $10^5$–$10^6$ cells/g in packaged yogurt (Fleet, 1990). In addition to spoilage by yeasts, the presence of high numbers of thermoduric \textit{Bacillus} species in milk used to manufacture yogurt may produce bitter flavors in the finished product (Kosikowski and Mistry, 1997).

The high acid content in sour cream inhibits bacterial growth, although proteolytic activity from enzymes of heat-resistant bacteria (\textit{Bacillus} spp.) can result in bitter flavors. Coliforms (\textit{Escherichia}, \textit{Enterobacter}) and psychrotrophic organisms such as \textit{Pseudomonas} spp. are contaminants that may cause spoilage in cultured buttermilk (Mistry, 2001).

\textbf{Cream}

The dominant spoilage organisms found in conventionally pasteurized (high-temperature, short-time HTST) cream are \textit{Pseudomonas} spp. Other gram-negative genera found in pasteurized cream include \textit{Enterobacteriaceae}, \textit{Alcaligenes}, \textit{Acinetobacter} and \textit{Aeromonas} (Tekinsen and Rothwell, 1974; Phillips \textit{et al.}, 1981). As with pasteurized fluid milk, shelf-lives of cream products are limited when products are manufactured in processing environments in which post-pasteurization contamination by psychrotrophic \textit{Pseudomonas} and coliforms is not controlled. \textit{Bacillus} spp. and \textit{Corynebacterium} are among the Gram-positive organisms that have been isolated from pasteurized, refrigerated cream products (Phillips \textit{et al.}, 1981). The presence of psychrotrophic organisms (e.g. pseudomonads, \textit{Bacillus cereus}) in cream causes defects such as lipolytic spoilage and bitter taste due to proteolysis (Cousin, 1982).

\textbf{Butter and reduced-fat dairy spreads}

US Standards require that butter contain at least 80% milkfat (USDA, 1989). Dairy-based spreads are blends of milkfat and vegetable fats or reduced-fat (light) butter. Spreads are butter alternatives, ranging from full fat (72–80%) to very low fat (less than 30%) (Varnam and Sutherland, 1994). A wide range of spreads is available, and legal standards to encompass all categories have not yet been established. In the USA, reduced-fat or light butter must contain not more than 40% milkfat, and milkfat/butter blends must contain a 3:2 vegetable oil to milkfat ratio to total 80% fat content (USDA, 1993).

The water phase in butter, which exists in the form of small droplets, can support microbial growth (Muir and Banks, 2003). Butter normally contains
less than 16% water. Spreads have higher moisture contents than butter and, hence, are more susceptible to microbial growth. Similar to other dairy products, raw milk quality and the control of psychrotrophic contaminants throughout processing, handling and storage is of great importance to the keeping quality of butter and reduced-fat dairy spreads. Species of *Pseudomonas* (*P. fragi*, *P. putrefaciens*) can cause defects such as fruity aromas, proteolytic activity and rancidity (Cousin, 1982). Heat-resistant lipases and proteases produced by *Pseudomonas* spp. have been shown to cause butter rancidity (Kornacki et al., 2001). *Micrococcus* has been associated with lipolytic spoilage of butter. However, spoilage by *Micrococcus* can be controlled by storing butter below 5°C (Varnam and Sutherland, 1994).

Yeasts and molds can also cause spoilage of butter and dairy spreads. Mold growth on the surface of butter causes surface discoloration of the product and can also produce off-flavors. *Rhizopus, Geotrichum, Penicillium, Alternaria, Aspergillus* and *Cladosporium* are among the molds that have been associated with butter spoilage (Jay, 2000). Yeasts implicated in spoilage of butter during refrigerated storage include *Rhodotorula, Cryptococcus* and *Candida* (Fleet and Mian, 1987).

### 7.3 Current and emerging techniques for controlling spoilage microorganisms

#### 7.3.1 Sanitation practices on the farm and in the dairy plant environment

*Farm*

Milk secreted into a healthy cow’s udder is virtually sterile. While some microbial contamination of raw milk on the farm is to be expected, following good cleaning and sanitation practices during milking, cooling and storage will help to control microbial numbers. Sources of contamination during milk production and collection include udder infections, dirty udders and teats, and soiled contact surfaces on milking and cooling equipment. Milk from cows showing evidence of mastitis infections should be excluded from the bulk tank to prevent high bacterial and somatic cell counts. Cleaning of the udder and teats with water and a disinfectant prior to milking is a good practice to reduce bacterial contamination. Implementation of a systematic equipment cleaning and sanitizing routine consisting of surface soil removal, disinfection and drainage of the equipment helps reduce bacterial multiplication and prevent bacterial biofilm formation. Failure to remove soil and bacteria on a daily basis may contribute to the establishment of complex bacterial communities called biofilms, which are more difficult to eliminate from surfaces than free-living, vegetative bacterial cells.
Managing microbial spoilage in the dairy industry

Dairy processing plant
Sources of contamination that have been identified in dairy processing operations are pasteurizers, carton-forming mandrels, filling machine nozzles, packaging materials and airborne organisms (Ralyea et al., 1998; Gruetzmacher and Bradley, 1999; te Giffel, 2003). The most important steps in preventing contamination throughout the processing plant are the establishment and monitoring of a cleaning program and the adherence to good manufacturing practices. In the USA, the use of clean-in-place (CIP) systems is widespread. Even when processing equipment is cleaned and sanitized daily by CIP systems, bacteria may survive in areas where cleaning agents and chemical disinfectants are less effective, such as gaskets, dead-ends and corners of holding tubes. Biofilm accumulation on milk contact sites can enable bacteria to survive cleaning and disinfection routines, which can result in development of continuous reservoirs of contamination (Zottola and Sasahara, 1994; Austin and Bergeron, 1995; Mittelman, 1998; Somers et al., 2001).

The presence of post-pasteurization contaminants in the processing environment can cause rapid product spoilage, thereby limiting the expected product shelf-life (Schröder, 1984; Cromie, 1991). Gruetzmacher and Bradley (1999) found that shelf-life of fluid milk could be significantly increased by controlling and eliminating post-pasteurization contaminants, of which Gram-negative organisms are most predominant. Effective cleaning and sanitizing practices are essential for increasing shelf-life of fluid milk by reducing bacterial post-pasteurization contamination throughout processing.

The use of ultra-clean filling equipment is another method to prevent recontamination of pasteurized milk. Ultra-clean filling consists of packaging processed milk in a controlled environment in order to minimize the exposure to microbial contaminants. This method results in reduced post-pasteurization contamination and longer product shelf-lives relative to ambient packaging, although bacterial spoilage by heat-resistant organisms such as Bacillus spp. and Paenibacillus spp. ultimately limits shelf-life. Extended shelf-life of dairy products can be achieved by increasing pasteurization temperatures and eliminating or reducing the growth of typical post-pasteurization spoilage microorganisms and their reservoirs (Cromie, 1991). Aseptic packaging should be used for extended shelf-life products. Aseptic packaging consists of sterilizing the container and filling with a commercially sterile product under aseptic conditions (Bylund, 1995).

7.3.2 Refrigeration controls
Holding raw milk and dairy foods below the optimal growth temperature of microbial contaminants is an important practice in controlling spoilage on the farm and throughout processing. Cooling raw milk to 7°C or less immediately after milking inhibits the growth of mesophilic and thermophilic microflora that may be present in the milk. In modern dairies, raw milk is stored in bulk cooling tanks and transported to the plant in refrigerated milk.
tank trucks. Efficient cooling systems can enable less frequent bulk milk collections over wider geographic areas, which can result in longer holding times on the farm for raw milk. Throughout collection, the temperature of the chilled bulk milk should not exceed 10°C when milk from subsequent milkings is added to the tank (FDA, 2001). To prevent warm milk from raising the temperature of milk in the bulk tank, pretank cooling systems may be employed (Hayes and Boor, 2001).

Rapid cooling of milk after pasteurization is necessary to minimize quality loss. Storage temperatures of 4°C or less are recommended to preserve processed product quality and safety. The expected shelf-life of commercial fluid milk increases with decreasing temperature (Janzen et al., 1981). Although storage temperatures above freezing but below 4°C are optimal for processed fluid milk products, temperatures in the retail and home environment are variable and product shelf-life will reflect these variations.

7.3.3 Heat processing
Heat treatment serves the primary function of ensuring the safety of milk products. Other purposes of heat treatment include shelf-life extension and production of standardized cultured dairy products as a result of the reduction or elimination of competing microorganisms. Current methods for heat treatment of raw milk are pasteurization, ultrapasteurization and ultra-high temperature (UHT) processing.

The public health objective of milk pasteurization is to eliminate all non-spore-forming pathogens commonly associated with milk (FDA 2001). *Coxiella burnetti* is currently considered to be the non-spore-forming pathogen in milk that is most resistant to thermal destruction. Experiments to define the thermal destruction characteristics of *C. burnetti* are technically challenging, as, to date, the presence of this organism in a heat-treated milk sample is quantified only indirectly by assessing the presence and concentration of antibodies in a host animal that had been inoculated with the milk sample. The guinea pig is the host animal that has been used for monitoring residual levels of *C. burnetti* in heat-treated milk samples (Enright et al., 1957). Numbers of *C. burnetti* present in milk are referred to as ‘infective guinea pig doses’ as they are determined by identifying the highest ten-fold milk dilution that causes an inoculated guinea pig to have a significant rise (at least four-fold) in antibody titer to *C. burnetti* (Enright et al., 1957). The highest level of *C. burnetti* detected in milk of infected cows was 10000 infective guinea pig doses. Therefore, to provide an additional margin of safety, thermal destruction of 100000 infective guinea pig doses was selected as the goal for minimal pasteurization conditions (Enright et al., 1957).

Pasteurization also reduces the number of spoilage microorganisms in milk while maintaining sensory and nutritional qualities. In the USA, the FDA’s Pasteurized Milk Ordinance has established seven time and temperature combinations which can be used for milk pasteurization (FDA, 2001). The
standard time and temperature combinations used by the dairy industry are low-temperature long-time (LTLT), or ‘vat’, pasteurization and HTST pasteurization. Vat pasteurization is typically used for milk intended for yogurt or cheese production and consists of a minimum hold temperature of 63°C for 30 min. Pasteurization parameters for HTST milk consist of a minimum of 72°C for 15 seconds (FDA, 2001). Current shelf-lives of conventionally pasteurized fluid milk in the USA typically range between 14 and 21 days.

Ultrapasteurization consists of holding the milk at a minimum of 138°C for at least 2 seconds, with the objective of increasing product shelf-life beyond that achieved by conventional HTST processing by further reducing bacterial counts (FDA, 2001). Ultrapasteurization extends product shelf-lives to 60–90 days under refrigerated storage below 7°C, although spoilage times for ultra-pasteurized products are similar to those of conventionally pasteurized products after the product is opened (FDA, 2001). Bacterial spoilage due to post-pasteurization contamination does not limit the shelf-life of ultrapasteurized products unless process failures have occurred (e.g. filling or seaming faults). Spoilage of ultra-pasteurized products is mainly attributed to enzymatic activity due to the presence of heat-stable enzymes.

UHT treatment involves preserving milk by holding at a temperature of 140–150°C for 1 or 2 seconds (FDA, 2001). Heat may be applied indirectly through plate heat exchangers or directly by culinary steam injection to achieve the desired pasteurization temperature (Bylund, 1995). Direct heating methods are used more commonly than indirect methods for UHT processing in the dairy industry. Milk is then aseptically packaged in sterilized containers to prevent post-processing contamination. UHT milk has a shelf-life of approximately 3–6 months at ambient storage (~25°C) (Dunkley and Stevenson, 1987). No bacterial growth should occur when aseptically processed milk is tested for microbial quality by standard methods. Spoilage microorganisms associated with UHT-processed milk are heat-resistant thermophilic species, mainly *Bacillus stearothermophilus*, which causes flat-sour spoilage. To prevent high counts of *B. stearothermophilus* in UHT products, raw milk spore counts of this species should be low (Lewis, 1999).

*Factors that affect the effectiveness of heat treatment*

The effectiveness of heat treatment on the microbial quality of pasteurized milk products depends on raw milk quality, temperature and duration of the heat treatment, and the thermal resistance of microorganisms in milk. Raw milk should be cooled immediately after milking and maintained at 7°C or less until processing to retard microbial growth. To achieve the desired microbial reduction targeted by pasteurization parameters, raw milk should meet regulatory requirements. In the USA, these requirements are less than 300,000 cfu/ml for commingled milk prior to pasteurization (FDA, 2001). Microorganisms are more likely to survive thermal processes when numbers present in raw milk exceed regulatory bacterial limits.
As pasteurization time and temperature requirements are based on the heat treatment needed to kill the most heat-resistant pathogen found in raw milk, dairy processors must carefully control and record holding times and temperatures throughout processing. System controls, such as positive displacement pumps and flow controllers, are designed to divert any product that has not met a system’s required holding time and temperature.

Several factors affect the ability of microorganisms to resist thermal destruction, including physical and chemical properties of milk and the number and physiological state of the organisms in the microbial population (Jay, 2000). Water activity, pH, composition (e.g. protein and colloidal particles) and presence of antibiotics are among the physical and chemical properties of milk that affect microbial resistance (Jay, 2000; Hayes and Boor, 2001).

### 7.3.4 Use of carbon dioxide to control microbial flora

Carbon dioxide addition has been used to inhibit the growth of spoilage microorganisms in raw and pasteurized milk (Daniels et al., 1985; Roberts and Torrey, 1988). The use of carbon dioxide also has been investigated in other dairy products, including yogurt and cottage cheese (Chen and Hotchkiss, 1991; Loss and Hotchkiss, 2003), and is now broadly applied in the commercial cottage cheese industry in the USA. Carbon dioxide can be used in combination with refrigeration, pasteurization and high barrier packaging to further extend the shelf-life of processed milk products without negatively affecting quality (Loss and Hotchkiss, 2003). In refrigerated raw milk, carbon dioxide addition may be a valuable technique to control the growth of psychrotrophic microflora and to reduce the occurrence of heat-resistant microbial proteinases and lipases which decrease processed product quality (King and Mabbitt, 1982; Ruas-Madiedo et al., 1996; Espie and Madden, 1997). Carbon dioxide appears to retard the growth of coliforms and Gram-negative psychrotrophs, such as *Pseudomonas fluorescens*, by prolonging their lag phase (King and Mabbitt, 1982; Roberts and Torrey, 1988). Carbon dioxide has also been applied to pasteurized milk as a bacteriostatic agent (King and Mabbitt, 1982; Hotchkiss et al., 1999; Glass et al., 1999). Bacterial growth rates decrease with increasing concentrations of CO₂. However, it is critical to maintain CO₂ at levels that are below the sensory detection threshold and that do not promote toxin production or outgrowth of anaerobic spore-formers such as *Clostridium botulinum* (Hotchkiss et al., 1999). In pasteurized fluid milk held under refrigerated storage, carbon dioxide addition at moderate levels (~ 9.1–11.9 mM) did not appear to accelerate spoilage by *Bacillus cereus* or increase the risk of botulism from *Clostridium botulinum* (Hotchkiss et al., 1999; Werner and Hotchkiss, 2002).

Modified atmosphere packaging (MAP) consists of using elevated concentrations of carbon dioxide, with or without other gases, and a high barrier package to inhibit microbial growth and extend product shelf-life. Trace gases such as carbon monoxide, nitrous oxide and sulphur dioxide can...
be used, although carbon dioxide, nitrogen and oxygen are the most commonly used gases in commercial applications (Farber, 1991). The use of MAP to extend the shelf-life of dairy products has been primarily investigated with cheese (Chen and Hotchkiss, 1991; Fedio et al., 1994; Alves et al., 1996; Gonzalez-Fandos et al., 2000).

### 7.3.5 Microfiltration

Microfiltration is a form of membrane processing that can be used to improve dairy product quality and extend shelf-lives (Kosikowski and Mistry, 1990; Eckner and Zottola, 1991; Pafylia et al., 1996). Microfiltration provides a low-temperature processing alternative to thermal methods (e.g. ultrapasteurization) for reducing the number of bacteria in fluid milk or in whey (Eckner and Zottola, 1991; Bargeman, 2003). Low-temperature strategies such as microfiltration can produce products with flavor characteristics superior to those resulting from high-temperature processing, owing to the absence of cooked flavors and other off flavors (Hill, 1988). Microfiltration membranes typical range from 0.2 to 5 μm in pore size and have relatively low operating pressures (0.05–0.2 MPa) (Olesen and Jensen, 1989; Bargeman, 2003). Bacteria, which generally range from 1 to 3 μm in size, are retained by the microfilter. Fat globules are also concentrated by microfiltration; hence, the process is more suitable for non-fat milk products than for higher-fat products.

Bacterial counts and spore counts in microfiltered milk can be reduced by as much as 99.99% and 99.95%, respectively (Olesen and Jensen, 1989; Eckner and Zottola, 1991; Pafylia et al., 1996). Because spores of psychrotrophic strains of *Bacillus* spp. can survive pasteurization, elimination of these spores by microfiltration can reduce spoilage in milk products. The type of microfiltration unit employed, its design and its configuration influence the effectiveness of microbial retention (Eckner and Zottola, 1991). Applications of microfiltration that have been used in the dairy industry include the processing of extended shelf-life fluid milk products and the production of milk for cheese manufacture (van der Horst, 2001).

Microfiltration of milk intended for cheese manufacture may prevent the late gas blowing defect by elimination of *Clostridium tyrobutyricum* spores from the cheese milk (van der Horst, 2001). Microfiltration of milk also eliminates the spores of other spoilage organisms (e.g. *Bacillus* spp.) which can deteriorate cheese quality (Olesen and Jensen, 1989; Kosikowski and Mistry, 1990). The removal of milk fat by microfiltration poses a disadvantage in the processing of milk for cheese manufacture. However, skim milk produced by microfiltration may be mixed with cream to achieve the desired butterfat content for cheese making (Neocleous et al., 2002).

### 7.3.6 Nisin addition

The addition of the natural antibiotic nisin is a technology that has been explored and applied in dairy processing with the goal of ensuring product
safety and controlling microbial spoilage (Muir, 1996). Nisin has been found to inhibit growth of Gram-positive spoilage microorganisms and is approved for use in clotted cream and processed cheese in the United Kingdom (Muir, 1996). In the USA, nisin is approved by the FDA (Code of Federal Regulations, Anon., 2004) as an antimicrobial ingredient to inhibit the outgrowth of Clostridium botulinum spores in pasteurized cheese and processed-cheese spreads. Worldwide, nisin is approved as a food additive in nearly 50 countries, primarily for use in processed cheese and other dairy products (Delves-Broughton, 1990). The addition of nisin has also been demonstrated to be effective at controlling microbial growth in pasteurized fluid milk products stored at 10 and 20°C following a heat treatment of 117°C for 2s. Although the use of nisin to extend the shelf-life of milk products processed under such conditions has been suggested (Wirjantoro et al., 2001), its use as a preservative in fluid milk products has not been approved.

7.4 Future trends

The emergence of large-scale dairy production and processing through improved efficiencies and technologies has facilitated the trend towards consolidation in the dairy industry. Increased consolidation, from fluid milk processors to cheese manufacturers, demands the production of high-quality milk products that allow distribution over wide geographic areas. Future trends to deliver safe, nutritious, high-quality products are primarily concerned with ensuring product safety and controlling microbial spoilage. One of the major future trends is extension of conventionally pasteurized (HTST) fluid milk shelf-life to deliver products with enhanced freshness and microbial quality over longer periods of time.

Bacterial spoilage continues to be the most limiting factor in extending the shelf-life of conventionally pasteurized fluid milk products (Boor, 2001). Control and elimination of typical Gram-negative post-pasteurization contaminants in the processing environment have led to product shelf-life improvement. However, the presence of smaller numbers of heat-resistant, psychrotrophic Gram-positive bacteria has been uncovered as the next bacterial hurdle to further extend fluid milk shelf-life (Ralyea et al., 1998). Gram-positive psychrotrophs, such as Paenibacillus spp., Bacillus spp. and Microbacterium spp., can cause spoilage in milk products processed in dairy plant environments where Gram-negative post-pasteurization contamination has been controlled and longer product shelf-lives are expected (Champagne et al., 1994; Ralyea et al., 1998; Fromm and Boor, 2004). Owing to the relatively large variability in the microflora isolated from milk products collected from different processing environments, plant-specific strategies will be needed to identify contaminants and their reservoirs (Fromm and Boor, 2004).
7.4.1 Hazard Analysis Critical Control Point (HACCP) programs in the dairy industry

In the USA, the National Conference on Interstate Milk Shipments (NCIMS) implemented a voluntary HACCP pilot program for the dairy industry in 1999 with the objective of improving safety practices and product quality. Under the HACCP system, the establishment of prerequisite programs such as Good Manufacturing Practices (GMP) and Standard Sanitary Operating Procedures (SSOP) is required to help reduce potential food safety hazards. Several aspects addressed by these prerequisite programs, such as cleanliness of food contact surfaces, prevention of cross-contamination and safety of process water, have been specified in the current FDA Pasteurized Milk Ordinance (FDA, 2001). Therefore, the dairy industry has already recognized and addressed sanitation measures to reduce or prevent contamination (NAS, 2003). The critical control points likely to be included in a HACCP system for dairy processing operations are pasteurization time and temperature conditions and control of raw and processed product storage temperatures. Regulatory microbial standards will continue to be used by the dairy industry; however, microbiological critical control points are unlikely to be adopted in the absence of effective real-time microbial monitoring tools.

7.4.2 Emerging technologies and processes

Microfiltration and carbon dioxide addition are among the emerging technologies to improve microbial quality and extend dairy product shelf-life. The use of high hydrostatic pressure processing and addition of lantibiotics have also been investigated as methods to reduce the microbial load and extend the shelf-life of milk and cheese products.

High-pressure treatment is a non-thermal technique that consists of uniformly applying 200–1000 MPa of pressure to food, resulting in the destruction of bacterial cell membranes and cellular structures (Trujillo et al., 2000; Stabel, 2003). Pressures of 100–400 MPa are required to inactivate vegetative cells, while inactivation of bacterial spores may be achieved at higher pressures (Stabel, 2003). High-pressure treatment has been applied to milk for use in the production of fresh and ripened raw milk cheeses as an alternative to heat treatment (Trujillo et al., 2000; Needs, 2002). In addition to inactivating microorganisms in fluid milk, high-pressure treatment offers the advantage of only minimally affecting flavor characteristics. However, pressurization can affect the structure and functional properties of milk proteins and therefore can affect yield, proteolysis and water retention during cheese manufacture (Trujillo et al., 2000; Needs, 2002).

In addition to nisin, a number of other lantibiotics with potential applications in dairy products have been characterized. These bacteriocins are naturally-occurring peptides produced by bacteria that can inhibit the growth of a narrow or broad range of other bacteria (Ross et al., 2002). Lantibiotics produced by lactic acid bacteria, such as Lacticin 3147, have been suggested
as potential biopreservatives in cheese. Lacticin 3147 was demonstrated to influence the developing microflora of cheese by reducing the number of non-starter lactic acid bacteria (Ryan et al., 1996). By exerting greater control on the cheese microflora, manufacturers could potentially increase product uniformity and reduce spoilage. Variacin, a lantibiotic produced by Kocuria varians, was evaluated to determine its feasibility as an ingredient to control the growth of psychrotrophic Bacillus cereus in chilled dairy products (O’Mahony et al., 2001). Results of this study indicate that variacin in a spray-dried fermented ingredient form has potential applicability as an inhibitor of psychrotrophic B. cereus in contaminated chilled dairy products.

7.5 Sources of further information and advice

Further information related to the microbiology of dairy products, including spoilage microorganisms of concern to the dairy industry is provided in the reference book Dairy Microbiology Handbook: the Microbiology of Milk and Milk Products (Robinson, 2002). Further information with respect to current regulatory standards for milk products in various countries may be found at the following websites:

http://vm.cfsan.fda.gov/~ear/pmo01toc.html (US FDA Grade ‘A’ Pasteurized Milk Ordinance 2001Revision),

http://www.cfis.agr.ca/english/regcode/codes_tbl_e.shtml (Canadian Food Inspection System, CFIS; Canadian National Dairy Regulations and Code, NDRC),


7.6 References


MANAGING MICROBIAL SPOILAGE IN THE DAIRY INDUSTRY


Managing microbial spoilage in cereal and baking products

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8.1 Introduction

Cereals are contaminated with a wide range of bacteria, yeasts and filamentous fungi during crop growth, harvesting and during post-harvest drying and storage. The contamination levels during these phases and the level of management required are influenced by climatic conditions during cereal ripening and harvesting. Wet periods during ripening can result in significant infection with *Fusarium* species, which cause ear blight symptoms in a range of temperate cereals, reduce yield and grain quality and can result in trichothecene contamination, which impacts on post-harvest grain management and entry into the processing chain for bakery products. Wet harvesting conditions result in a significant sooty mould presence, which sometimes produces black-ended grain; this can have an impact on flour colour. Efficient drying is essential to prevent *Penicillium verrucosum* from becoming established, resulting in contamination with the class 2b carcinogenic mycotoxin ochratoxin A (OTA), especially in northern Europe.

Poor post-harvest management can lead to rapid deterioration in grain quality, decreasing dry matter content, nutritional quality and germinability. Mould contamination may also affect carbohydrate composition and storage proteins which are essential for conserving bread-making and malting quality. Furthermore, grain for human consumption or considered acceptable for animal feed may become contaminated with a range of mycotoxins for which legislation now exists (aflatoxins, ochratoxins) or is being considered (deoxynivalenol, fumonisins).
8.2 Microbial contaminants of cereals and bakery products

Table 8.1 summarises the key fungal species that occur in temperate cereals pre-harvest and during storage. Generally, pre-harvest fungal contamination is important as ripening grain is colonised by a range of phyllosphere fungi. Except for *Bacillus* species, bacterial contaminants are less important as the moisture content of ripened grain is not conducive to their growth and because of the high starch content. Populations of fungi and bacteria present in cereal grains can vary from between $10^7$–$10^9$ and $10^6$–$10^8$ cfu/g grain respectively at harvest. However, bacterial contamination, especially by *Bacillus* species, can cause problems later in the supply chain when products are made. Spoilage of grain occurs when drying is inefficient, resulting in pockets of heating because of fungal activity or heat and moisture from insect contamination. This can subsequently result in spontaneous heating, which may cause complete decay of the stored product (Magan et al., 2004).

Studies of milling of grain suggest that there is a 10–100 fold reduction in isolation of bacteria and fungi in flour (log$_{10}$ 6.5 to log$_{10}$ 3–4). During storage of flour for 12–48 months there is a further 10–100 fold decrease, especially at 10–25°C. When water availability conditions favour microbial growth, *Bacillus* species, amylolytic yeasts (*Saccharomycopsis, Pichia burtonii*) and filamentous moulds of the genera *Aspergillus, Penicillium* and *Eurotium* are important. In contrast, storage at –20°C results in very little change in microbial bacteria and fungi (Seiler, 1984, 1986).

Recently Schollenberger et al., (2002) have shown that *Fusarium* toxins can be present in wheat flour in some regions of Germany. They found that deoxynivalenol (DON) was the predominant trichothecene present, followed by nivalenol (NIV) and then zearalenone (ZEA). The degree of toxin contamination was related to ash content, suggesting that localisation of toxins may be primarily in the outer layers of the wheat grain, i.e. the bran portions. Although there is still a debate about the relative susceptibility and contamination

<table>
<thead>
<tr>
<th>Pre-harvest in wheat/barley/rye:</th>
<th>Airtight storage: Paecilomyces variotii, Scopulariopsis candida, Penicillium roqueforti, Candida spp., Bysschlamys fulva, Monascus ruber</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acid-preserved cereals: Penicillium roqueforti, P. glandicola, Aspergillus flavus, A. candidus, A. terreus, Monascus ruber</strong></td>
<td></td>
</tr>
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</table>
of conventional and organic production systems, this study suggested that DON content was significantly higher in flour from the former samples.

Moulds are particularly important in many bakery products, especially bread, which has a relatively high moisture content and thus water availability \((a_w = 0.94 \text{–} 0.97)\) with a pH of about 5.5–6. These conditions are conducive to growth by contaminating moulds during post-harvest processing. The bread and cakes most prone to spoilage by moulds are those that are sliced, pre-packed and wrapped in different polyethylene-based materials. About 90% occurs during cooling and wrapping operations (Spicher, 1980; Legan, 1993). The most common fungal spoilage species are from the genera *Penicillium*, *Aspergillus* and *Eurotium*. In yeast-raised wheat-based bread besides these species, *Cladosporium*, *Mucorales* and *Neurospora* can cause problems. However, *Penicillium* species are particularly important contaminants with 90% of loaves contaminated in N. Ireland by *P. viridicatum*. Storage temperature also has a significant impact on types and rates of microbial spoilage. At cooler temperatures *Penicillium* species are important, whereas at ambient temperatures (20–25°C) *Aspergillus* and *Eurotium* species, and very rarely *Rhizopus* (so-called bread mould) and *Neurospora* species (red bread mould) can occur because they produce copious amounts of amylase. Sourdough breads vary in pH from 4.5 (rye bread) to 5.1 (wheat bread) and are more likely to be spoiled by *Penicillium* species, especially *P. roqueforti* and *P. commune*. *P. roqueforti* is very acid tolerant (pH 3.0) and this may partly explain the prevalence of this species in spoilage of sourdough breads (Spicher, 1980).

Homemade breads have become popular in developed countries and can become spoiled by certain strains of *Bacillus subtilis* (*B. mesentericus*), so-called ropiness of bread. This may be evident as stringiness when breaking bread. The usual source is the flour and growth is favoured by holding of dough at favourable temperatures. Studies of partially baked soda breads showed that at pH 7–9 storage at room temperature could develop ropiness after 2 days, with the key species involved being *B. subtilis*, *B. pumilus* and *B. licheniformis* (Legan, 1993).

*Bacillus* spores are very heat resistant and when naturally present in cereal flour can survive in the interior of bread loaves (Kaur, 1986). They may thus cause spoilage problems and illness. Interestingly, recent studies have suggested that some *B. subtilis* strains isolated from the cereal rhizosphere may be beneficial by producing a battery of anti microbial compounds against a range of spoilage moulds (Földes et al., 2000). Attempts have been made to develop a rope-inducing potential (RIP) to predict spoilage. However, correlations between *Bacillus* spores in flour and ropiness showed no significant correlation, suggesting that different types of spores may be present, resulting in antagonism, which may influence the spoilage potential (Volavsek et al., 1992). The yeasts mentioned above can give rise to so-called chalky bread. Strains of *Hansenula anomala* produce an ethyl acetate smell, which can result in spoilage of bread (Legan and Voysey, 1991). Table 8.2 lists
some yeasts that have been found on British breads (Legan and Voysey, 1991).

Cakes usually contain humectants to enable the water activity to be maintained low enough to provide a reasonable shelf-life while conserving the moisture-eating properties of such products. This also makes them prone to microbial spoilage. They can sometimes undergo bacterial spoilage by *Pseudomonas* and *Bacillus* species and where cream fillings are used *Salmonella* can cause problems. However, because of the high sugar concentrations used, xerophilic and xerotolerant mould species are predominantly responsible for shortening of shelf-life. Common sources are the ingredients, especially sugar, nuts and spices. Even though the baking process destroys many microorganisms, subsequent surface contamination can occur when toppings are added or spore may be deposited from the atmosphere and from handling during the packaging process. The use of fruit and nuts can result in some cakes becoming spoiled by outgrowth from underneath these raw materials, which have been protected during the production process.

### 8.3 Current control techniques

#### 8.3.1 Cereal grain

Harvested cereal grains should be effectively dried prior to short-term storage on farm or longer-term storage in silos for processing. Provided grain is dried to a moisture content equivalent to $<0.70 \alpha_w$ (e.g. 14–14.5% moisture content for wheat/barley) then no spoilage problems should occur. However, as grain is sold on a wet weight basis, often loads containing different moisture contents are added to the same silo. Under warm autumnal conditions, pockets of wetter grain can result in the initiation of fungal activity, producing heat and moisture. This may end in complete spoilage of the grain bulk and sometimes spontaneous heating, resulting in a succession of fungi contaminating the grain and often leading to contamination with mycotoxins.
Food spoilage microorganisms

particularly ochratoxin (Magan et al., 2003). The environmental conditions of temperature and water availability that favour growth and mycotoxin production on grain have been identified for deoxynivalenol (wheat), ochratoxin (wheat, grapes), fumonisins (maize) and aflatoxins (groundnuts) (Sanchis and Magan, 2004). It has generally been found that the range of temperature and water contents favouring mycotoxin production are slightly narrower than those for growth/germination of the producing species (Table 8.3). This type of information is critical and useful in developing appropriate decision support systems for identifying high- and low-risk years for contamination of cereals.

A Hazard Analysis Critical Control Point (HACCP) approach to management of grain for prevention of mycotoxins entering food has been devised for cereals in relation to trichothecenes and ochratoxin (Aldred and Magan, 2004; Aldred, et al., 2004). This approach requires management commitment combined with a detailed assessment of the whole food chain to identify the critical control points (CCPs) where spoilage moulds and mycotoxins may enter the chain. This does require the implementation of Good Agricultural Practice (GAP) pre-harvest and effective identification and monitoring of CCPs. Figure 8.1 summarises the components of such a system for control of contamination with trichothecenes in temperate cereals.

8.3.2 Current techniques for control of microbial spoilage in bakery products and their limitations

Control of mould spoilage in bakery products can be achieved in various
ways. Generally, this is achieved by (a) restricting the access of the spoilage microorganisms in the product, (b) inactivating the microbial material and (c) inhibiting growth of spores and mould mycelium. However, if the microorganism gains access to the product, the objective turns into controlling its activity and growth on the food itself. For inactivating or inhibiting fungal growth in foods, several physical, chemical and biological measures can be taken.

The most common way to prevent or control mould growth in foodstuffs is by the use of antifungal agents. Antifungal agents are chemical substances that, when added to foods, tend to prevent or retard food spoilage by moulds. In practice, most are fungistatic and not fungicidal. Thus they stop germination and growth when present, but growth may occur from untreated pockets. Fungicidal compounds are more effective as they destroy the spoilage moulds directly. Of course the concentration of the antifungal agent to a large extent impacts on the safe shelf-life of a food product. Smid and Gorris (1999) suggested that ideally any antimicrobial substance should inhibit microorganisms in their initial lag phase of growth and not in the exponential log phase, since in the latter the necessary dosages of the agent would be too high and would most likely adversely affect the quality of the food.

At the present time mould spoilage of bread is generally prevented by the addition of food grade preservatives such as propionic, sorbic and acetic acids and their salts. These acids are generally recognised as safe (GRAS) (Liewen and Marth, 1985; Binstok et al., 1998). Sorbic acid, for example, has a half-life in the body of about 40–110 min and in normal conditions it is completely oxidised to CO₂ and H₂O (Liewen and Marth, 1985). However,
their potassium, sodium or calcium salts are the forms commonly used because of their higher water solubility and easier handling than their corrosive acids. The status and use of these materials are controlled in many countries by legislation that limits the type and concentration of preservatives that may be used. This can be affected by the bakery product or by other factors, such as whether or not the product is wrapped, and the type of wrapping used. In England and Wales, for example, sorbates are not permitted in bread but they are permitted for flour confectionery goods at levels of up to 1000 ppm (Seiler, 1984). In other countries such as Germany, Italy and the Netherlands, sorbic acid and its salts are approved in certain types of bakery products including bread. In the USA high-volume loaves similar to those produced in the UK have successfully been preserved using a spray of potassium sorbate applied immediately after baking (Killian and Krueger, 1983).

In the UK, as in many other countries, propionates are the chemical antimicrobial generally used to control moulds as well as bacterial (*Bacillus* spp.) spoilage of bread (Legan, 1993). Sodium, potassium or calcium salts, although more expensive, are less corrosive and easier to handle than the liquid acid. Their use is permissible at levels of not more than 0.3% (w/w) of propionic acid equivalent (Anon., 1984). Furthermore, propionates have little or no effect against yeast (Sauer, 1977) which make them highly suitable to control mould spoilage in yeast-raised bread.

However, the use of weak acids in bakery products has disadvantages. The low absolute efficacy of the propionates means that relatively high concentrations are needed in order to keep baked goods free of moulds for more than a few days (Lück and Jager, 1997). They are also fungistats and thus not lethal to spores, but delay the capacity for germination and growth. Also at such concentrations serious losses in volume and adverse effects on odour and flavour can occur. Using 0.2% calcium propionate, for example, a reduction of 5–10% of loaf volume occurs in commercial-scale baking because it reduces the yeast activity and alters the dough rheology. Sorbates have even greater adverse effects (Legan, 1993).

Incorporation of calcium propionate into bread at up to 0.3% concentration at pH 4.5 and 0.93–0.97$a_w$ effectively controlled a range of spoilage moulds in bread. However, at sub-optimal concentrations some stimulation was observed, and at pH 6, practically no control was achieved against *E. repens*, *P. verrucosum*, *A. ochraceus*, *P. coryolophilum* and *P. roqueforti* (Arroyo, 2003; Arroyo *et al.*, 2003, 2005). Sorbic acid and its salts are among the most thoroughly investigated of all preservatives. Studies with 0.3% (w/w) potassium sorbate on bread have also showed effective control of spoilage moulds at pH 4.5 and 0.93–0.97$a_w$ with the exception of *P. roquefortii*. However, at pH 6.0 practically no control was achieved with the recommended 0.3% treatment. Furthermore, when calcium propionate concentration was reduced to 0.03%, stimulation of *E. repens*, *P. coryolophilum* and *P. roqueforti* was observed. Recent studies with sponge cakes treated with sodium benzoate or calcium propionate at up to 0.3% concentration and 0.80-0.90$a_w$ and pH
6 or 7.5 also showed that four *Eurotium* species were only effectively controlled at pH 6 and 0.80–0.85\(a_w\). Over all other conditions growth was not significantly controlled (Guynot et al., 2002).

Weak acids are lipophilic acids that penetrate the cell membrane in the undissociated form. When the undissociated acid enters the cell a higher pH environment is encountered, the molecule dissociates, resulting in the release of charged anions and protons which cannot cross the plasma membrane. The high solubility, low taste and toxicity of weak organic acids make them highly suitable to be used in bread and bakery product preservation (Ray and Bullerman, 1982; Davison and Juneja, 1990). The pH of the environment and solubility of the acid often determines the foods in which these acids may be effectively used (Ray and Bullerman, 1982). In fact, because of their low \(pK_a\) value (4.19–4.87), these substances are effective antimicrobials in low pH substrates since this condition favours the uncharged, undissociated state of the molecule, which is freely permeable across the membrane.

Alternatives to chemical preservation include destroying or damaging the mould spores which gain access to the surface during the cooling and wrapping processes. This can be achieved using UV light, IR or microwave irradiation. These types of procedures have been used for sourdough bread in continental Europe (Seiler, 1984), but although effective, their bad publicity and the increased demand for minimally processed and ‘fresh’ products from the consumers limit their use. Furthermore, UV irradiation, for example, does not penetrate the product, so mould spores inside the loaf would probably not be affected.

Several other strategies have been followed to limit the rate of mould growth:

- Reformulation of bakery product recipes, e.g. by reducing the water availability but without adversely affecting the eating quality of the product or causing changes in volume, shape and texture.
- Use of novel ingredients such as raisin or prune juice concentrate that inhibit fungal growth (Sanders, 1991)
- Use of modified atmosphere packaging (MAP) or other active packaging techniques (Weng et al., 1999; Abellana et al., 2000).

MAP techniques are based on the fact that many moulds are oxygen dependent and highly sensitive to carbon dioxide (Porter et al., 1989; Farber, 1991); achieving environments with low \(O_2\) and high \(CO_2\) contents will protect the wrapped food against aerobic spoilage filamentous moulds, yeasts and bacteria. Further studies have been focused on the development of polymers that contain the preservative as an active packaging material: in this case the diffusivity of the preservative from the packaging to the food becomes very important (Han and Floros, 1998).

Ethanol has been used as a method of significantly increasing the shelf-life of bakery products as it can be added to the surface or in the packaging. Normally it can be added at 2% of product weight although efficacy against
yeasts can be variable (Seiler, 1989). Use of 1% ethanol released from an adsorbent pad extended mould-free shelf-life of packaged white bread to more than 60 days at 22°C. However, chalk moulds developed within 10 days, demonstrating that this approach was less effective against yeasts (Legan and Voysey, 1991). Smith et al. (1988), using commercial ethanol generators, showed that in vitro efficacy of ethanol against S. cerevisiae was related to $a_w$ and concentration of ethanol. With products such as apple turnovers ($0.93a_w$, pH 5), a shelf-life of 14 days was achieved with MAP ($CO_2:O_2 = 60:40\%$) at ambient temperature. However, visible swelling of packaging occurred due to growth of the yeast. Use of ethanol generators resulted in an extension to 21 days.

Ethanol vapour has, however, been used to control growth and toxin production by Clostridium botulinum in English-style crumpets ($0.99a_w$, pH 6.5) in high gas barrier packaging systems (Daifas et al., 2000). In these studies commercial ethanol vapour generators were used. Toxin was detected within 5 days in control samples while the ethanol treatment delayed toxicity for 10 days (2 g ethanol), while 4 or 6 g gave complete inhibition. This suggests that this approach could be effectively applied for control of such important foodborne illness-causing microorganisms.

Control strategies need to encompass the whole production process and thus effective hygiene in production facilities is critical to prevent microbial contaminants being deposited on freshly made product prior to packaging. Thus, cleaning and disinfection of surfaces and equipment are a vital part of a prevention strategy. The use of sanitisers that can effectively reduce the microbial load in production plants is therefore essential. This requires effective control of vegetative cells and spores, which are often particularly resistant to such products. McGrath et al. (1991) showed that quaternary-based ammonium products and a hypochlorite had differential efficacy against vegetative cells and ascospores of S. cerevisiae and P. anomala. Contact times of up to 30–60 min were examined to quantify survival. The survival curves can be used to predict sanitiser application system use in production plants. More research needs to be carried out on combinations of sanitisers to try to obtain a synergistic impact on such important microbial contaminants in these environments. This is important as the use of the same sanitisers can lead to acquired resistance when exposure occurs to sublethal doses of the products (Jones et al., 1989).

An area that has received much interest is that of tools for rapid diagnostics or predicting of quality and shelf-life of products without any microbial spoilage. The development of ERH CALC™, which is a software for predicting mould-free shelf-life of perishable bakery products (Campden and Chorleywood Food Research Association), has been useful, especially where new products are being developed. However, that this is not easy has been demonstrated when trying to predict shelf-life of bread and potential ropiness of bread. Direct correlations between contaminant loads and spoilage were not found (Volavsek et al., 2000). Sometimes complex interactions between
microorganisms can make this more difficult. Recently, attempts have been made to predict type of microbial spoilage and shelf-life by monitoring volatile fingerprints from bakery products and using bioinformatics approaches to determine spoilage. The use of such electronic nose systems has shown promise for use in bakery products and bread (Keshri et al., 2002; Vinaixa et al., 2004; Needham et al., 2005).

While organic acids and their salts are the most commonly used preservatives in bakery products, there is pressure from EU directives to reduce the use of these and instead use more natural preservatives such as antioxidants and essential oils either alone or in combination with packaging systems to stabilise shelf-life. Any reduction in the concentration of existing preservatives would result in significantly shorter shelf-life and more rapid moulding of bread, especially wrapped cut varieties. Consumers are demanding more natural bakery products with a minimum of preservatives. Thus the question arises as to whether alternative, more ‘natural’ additives would be effective, and economically acceptable and feasible.

8.3.3 Developing new methods for mould and mycotoxin control

**Synthetic antioxidants**

Phenolic-derived antioxidants have been screened for their possible antimicrobial efficacy. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and 2-tetra-butylhydroxybenzoic (TBHQ) are among them. Studies have been conducted using a range of antioxidants with different environmental factors to simulate conditions of bread, for example, screening of the inhibitory effect of antioxidants such as BHT, BHA, PG, propyl paraben, octyl galate at 0.95 $a_w$ and pH 6. This has demonstrated that common bakery product contaminants such as Cladosporium herbarum, Penicillium corylophilum, P. verrucosum and Aspergillus ochraceus were completely inhibited by propyl paraben, BHA and octyl gallate at 500 ppm. Importantly, these antifungal agents also at lower concentrations extended the lag phase prior to growth, which is indicative of extending shelf-life. Kubo et al. (2001) comparing the antifungal activity of three gallates, propyl (C3), octyl (C8) and dodecyl (C12), found that octyl gallate was the only active compound against four different fungal genera with a minimum inhibitory concentration (MIC) of 25 ppm. Recent studies have shown that mycotoxigenic spoilage fungi such as Fusarium species and Penicillium verrucosum are significantly inhibited by both parabens and BHA, and in some cases mycotoxin production is inhibited. Interestingly, combinations of antioxidants were sometimes more effective at lower concentrations than individual ones, indicating some synergy of efficacy (Etcheverry et al., 2002; Cairns and Magan, 2003; Reynoso et al., 2002; Torres et al., 2003).

Owing to their high p$K_a$ value (8.5), parabens are effective over a wider range of pH (3–8). Antimicrobial activity of parabens is related to the length
of the ester group of the molecule. As additives, parabens are applied as alkali solutions or as ethanol or propyl glycol solutions in fillings for baked goods, fruit juices, marmalades, syrups, preserves, olives and pickled sour vegetables (Belitz and Grosch, 1999).

**Essential oils (ESOs)**

In the past few decades great interest has emerged in the possible use of plant extracts and essential oils for food preservation. Essential oils are mostly derived from spices, i.e. dried aromatic products, obtained from different parts of the plant such as leaves (e.g. rosemary, sage), flowers (e.g. clove), bulbs (e.g. garlic, onion) or fruits (e.g. pepper, cardamon) (Shelef, 1983). Extracts and essential oils of many of these plants are now being screened for their antimicrobial effectiveness. *Aspergillus flavus*, one of the most toxigenic foodborne fungi that can contaminate flour used for bakery product production, has been reported to be inhibited by some of these plant derivatives. For example, Dwivedy and Dubey (1993), studying the antifungal activity of several umbelliferous plant essential oils against *Aspergillus* species, found an important fungistatic effect of *Trachyspermum* seed essential oil at relatively low concentrations (< 500 ppm). Azzouz and Bullerman (1982) established clove and cinnamon as the strongest antifungal agents against *Penicillium* and *Aspergillus* species. However, many of these studies neglected to simulate realistic environmental factors appropriate to the specific bakery product.

In some cases (Salmeron et al., 1990) stimulation of growth of the same *Aspergillus* species has been demonstrated when extracts of thyme and oregano were incorporated into nutritive media. Recently, very useful studies were carried out by Lopez-Malo et al. (2002) on dose–response curves for *A. flavus* in relation to vanillin, thymol, eugenol, carvacrol and citral or potassium sorbate/sodium benzoate. This showed that over a 60 day period it was possible to predict sensitivity of the mould to each ESO compound; this could be compared with organic acids. This showed that *A. flavus* had a higher sensitivity to thymol, eugenol, carvacrol, potassium sorbate and sodium benzoate at pH 3.5 than to vanillin or citral. MICs varied from 200 ppm for the organic acids to 1800 ppm for citral.

Screening a range (20+) of different plant essential oils for their activity against four spoilage moulds, *A. ochraceus, C. herbarum, P. corylophilum* and *P. verrucosum*, in a wheat-flour based medium at 25 °C has shown that at least 500 ppm was required for control of growth. Indeed, only clove, thyme, bay and cinnamon completely inhibited growth of all the species studied (Magan et al., 2003). Indeed lag phase prior to growth was also significantly increased in these studies. This is an important indicator of potential for improving shelf-life prior to any visible growth over a range of water activities and temperatures. Similarly, Patkar et al. (1993) found that 500 ppm of cinnamon ESO completely inhibited growth of the aflatoxigenic species of *A. flavus* growth in yeast extract broth and on agar for 7 days, whereas up to 1250 ppm of clove ESO was necessary to exert the same
inhibitory activity. Azzouz and Bullerman (1982) also reported a strong anti-mould activity of clove and cinnamon oil against several *Aspergillus* and *Pencillium* species.

*In vitro* screening of some 20+ essential oils for efficacy against *P. verrucosum* and *A. ochraceus* showed that only a few were effective on wheat-based media (Cairns and Magan, 2003). Generally, thyme, clove and cinnamon oils were found to be effective for both growth and ochratoxin control of these two species. Although many other studies have reported the strong effect of thyme and cinnamon ESO on the growth of mould species, results are not always similar. For instance, while 500 ppm of thyme oil completely inhibited growth of *A. ochraceus* on 2% wheat flour agar over 30 days (Arroyo et al., 2003), Paster et al. (1995) reported colonies on potato dextrose agar (PDA) of up to 35 mm of diameter in the presence of the same concentration of this ESO.

Özcan (1998) reported growth of *Aspergillus parasiticus* on czapek dox agar in the presence of 1% thyme (wild and black) oil. Although sensitivity to a certain plant essential oil may vary with the species studied, these differences of sensitivity may be attributed to the fact that composition of the essential oil can vary with the plant origin and extraction method. In fact, while in Özcan’s study thyme was a commercial essential oil obtained from Spanish plants, Paster et al. (1995) extract the thyme oil themselves from leaves of Israeli plants.

It is notable that while some work has been done on the potential of using mixtures of antioxidants, very few studies have been carried out to examine mixtures of antioxidants/ESOs, and mixtures of ESOs or deodorised ESOs. However, novel antimicrobials combined with established preservatives have been examined (Alzamora et al., 2003). They showed that by examining the MICs of vanillin, eugenol, carvacrol, thymol, sodium benzoate, sodium bisulphite and potassium sorbate in different concentrations they were able to develop fractional inhibitory concentrations (FICs) for plotting isobolograms to demonstrate the synergistic or antagonistic effect of combinations of these preservatives under different water availability, pH and time for *Aspergillus flavus* and *Zygosaccharomyces bailii*. This suggests that potential does indeed exist for exploiting mixtures of conventional and novel antioxidants/essential oils for use in bakery products.

### 8.4 In situ control of moulds in grain and bakery products using antioxidants/essential oils

Recent studies have been carried out to examine the potential of using some essential oils and resveratrol to control both *P. verrucosum* and *A. ochraceus* growth and ochratoxin production in grain used for processing (Table 8.4). This showed that quite high concentrations of ESOs were required for effective control of growth and ochratoxin (OTA) production. Recent studies with
resveratrol, an extract from grape skins, has shown that it is very effective at controlling growth and mycotoxin production by *Fusarium* species on wheat and maize (trichothecenes and fumonisins), and of *A. ochraceus* and ochratoxin in maize (Fanelli *et al.*, 2003).

The efficacy of antioxidants against spoilage moulds in some bakery products has been examined. These studies have shown that the concentrations required for inhibition of growth were generally higher than that shown to be effective *in vitro*. For example, propyl paraben had little effect on growth of four important spoilage moulds, even at 1000 ppm. This suggests that either the antioxidants are being bound by ingredients and thus less effective, or that it is difficult to get effective dispersion in the product, providing less direct contact with the contaminant moulds.

Essential oils have been examined in two ways for control of spoilage moulds in bread. The volatiles produced by the essential oils have been used in bread packaging to inhibit spoilage moulds, and attempts have been made to directly incorporate low concentrations with bread ingredients. For the former, Nielsen and Rios (2001) recently examined the efficacy of volatiles in MAP systems for control of rye bread spoilage fungi. Mustard essential oil in the volatile phase at 1–10 µg ml\(^{-1}\) was the most effective against spoilage fungi including *P. commune*, *P. roquefortii*, *Aspergillus flavus* and *Endomyces fibuliger*. Cinnamon, garlic and clove also had high activity in controlling growth *in situ* on slices of bread. Vanilla showed no inhibitory effects, and *A. flavus* was the most resistant of the species tested. Interestingly, the MIC varied with the active component (allylthiocyanate) and at least 3.5 µg ml\(^{-1}\) was required for fungicidal effects on the test fungi. However, introduction of small satchets or pads into packaging directly could be an effective way of enabling the slow release of volatiles of ESOs to control mould spoilage of bakery products, especially where this can be combined with modified atmosphere systems.

<table>
<thead>
<tr>
<th>Species</th>
<th><em>P. verrucosum</em></th>
<th><em>A. ochraceus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water activity</td>
<td>0.90</td>
<td>0.95</td>
</tr>
<tr>
<td>Clove</td>
<td>250</td>
<td>230</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>200</td>
<td>220</td>
</tr>
<tr>
<td>Thyme</td>
<td>260</td>
<td>235</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td><strong>Ochratoxin A production</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clove</td>
<td>240</td>
<td>200</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>260</td>
<td>210</td>
</tr>
<tr>
<td>Thyme</td>
<td>180</td>
<td>325</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>25</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 8.4 Inhibitory concentrations of essential oils and an antioxidant for 50% inhibition (PPM; LD\(_{50}\) values) of growth and ochratoxin production by *P. verrucosum* and *A. ochraceus* on gamma-irradiated wheat grain at different water availabilities at 15° C (Cairns and Magan, 2003; Cairns-Fuller, 2004)
When the ESO is incorporated directly into the substrate, the effectiveness appears to be lower. In fact, up to 1000 ppm of oregano ESO was necessary to completely inhibit growth of *A. flavus* in bakery products for 21 days (Basilico and Basilico, 1999) while only 100 ppm was required to completely inhibit growth of *Aspergillus niger* and several *Penicillium* species over a period of 6 days *in vitro*. Chemically, ESOs consists of a mixture of esters, aldehydes, ketones and terpenes. Although several studies have been carried out on the inhibitory effect of essential oil components (Sinha and Gulati, 1990; Bilgrami *et al.*, 1992; Mahmoud, 1994; Saxena and Mathela, 1996; Lachowicz *et al.*, 1998; Cosentino *et al.*, 1999), the role of these components in the antimicrobial activity of the oil is not clear. Various oil components may act synergistically in the antimicrobial activity while others can stimulate fungal spore germination (French, 1985).

In recent studies over 20 essential oils and 5 antioxidants were examined for control of growth of mycotoxigenic species such as *Fusarium culmorum* and *Penicillium verrucosum* on grain for flour and bakery production (Hope and Magan, 2003; Cairns and Magan, 2003). Only three essential oils (bay, clove and cinnamon oil) and two antioxidants (propyl paraben and hydroxymethylanisole) were found to be effective in controlling growth in the range 50–200 ppm and 0.995–0.955*aw* at 15 and 25 °C. At 500 ppm clove oil and BHA effectively controlled growth and both deoxynivalenol and nivalenol toxin production by *F. culmorum* at 15 and 25 °C. Similarly effective control of *P. verrucosum* and of ochratoxin was achieved at 500 ppm. However, at intermediate concentrations (100 ppm) stimulation of this mycotoxin was sometimes observed. Recent studies with antioxidants to control of fumonisin production by *Fusarium* section Liseola on naturally contaminated maize suggested that only very short-term control could be achieved regardless of storage water availability (Farnochi *et al.*, 2005).

Direct incorporation of the best ESOs into bread showed that efficacy was lower than on wheat flour-based medium, with 50% control of *E. repens* by cinnamon and clove, and only about 25% control of *A. ochraceus* by thyme. Practically no control of *P. verrucosum* and *P. corylophilum* with any of the best *in vitro* treatments was observed (Arroyo, 2003). However, recent studies by Suhr and Nielsen (2005) suggest that combinations of MAP and active packaging using mustard ESO may provide an approach for both rye and wheat bread. They showed that for *P. roqueforti* control O₂ absorbers were required for inhibition in rye bread because of its tolerance to elevated CO₂ levels. On wheat bread, *P. commune* was particularly tolerant of up to 90% CO₂ while the chalk mould species *E. fibuliger* was better controlled. The use of volatiles of mustard oil were least effective against *A. flavus* and *Eurotium repens* in wheat and rye bread respectively. However, by combining MAP and 2–3 µl mustard oil per slice completely inhibited growth of all the spoilage moulds on both types of bread. Results also suggested that the surface area and nature of the product influences the effectiveness and shelf-life extension of active packaging using such ESOs.
When an ESO is directly incorporated into the bakery product, less efficacy is observed, probably because specific components of the food product such as proteins or fats can bind essential oil components, inactivating them (McNeil and Schmidt, 1993; Smid and Gorris, 1999). The requirement for higher doses than that demonstrated to be required in vitro for incorporation into bakery products for effective control means that their typical odour is also noticeable. In some products this flavour may be desirable. For example, in tomato-based bakery products, the addition of basil can be used both as a flavouring and as an antifungal agent (Lachowicz et al., 1998). This has particular application in novel bread products where herbs and spices are often incorporated into the bread. The use of the volatiles produced by essential oils may be a more effective way of using low concentrations of ESOs in conjunction with MAP systems. This needs to be investigated further as an alternative approach to substitute for organic acids.

8.5 Future trends

Generally, in Europe consumers are very enthusiastic to have bakery products that contain natural preservatives, especially if the performance is equal to or better than existing preservatives. Some alternative ESOs and antioxidants are being used and have potential for enhancing flavour and aroma of products while effectively acting as preservatives and maintaining shelf-life of products. However, although consumers are prepared to pay a higher premium on organic or more natural food products, the economics may not always be acceptable. For example, calcium propionate is widely used in the bakery industry because it is effective and costs are relatively low at the maximum permitted levels (approx. 2000 mg/kg); some ESOs may cost twice as much if added at a level of only 250 mg/kg. In cakes, potassium sorbate is effective at similar concentrations. However, use of cinnamon oil would be cheaper while others such as bay leaf or clove oil would be four or five times more expensive. Resveratrol, which has also recently been shown to be very effective for control of mycotoxins in grain, is at present economically too expensive, although this may change in the future. There is probably a direct link between consumer acceptance, economics of use and actual efficacy of preservatives, antioxidants and ESOs.

Clear regulations exist on food additives with regard to the use of organic acids, and some antioxidants in bread and bakery products (e.g. 89/107/EEC; 95/2/EC; 97/77/EC; SI 1995 No. 3187). However, at the present time, although some ESOs can be used as food flavourings, there is no legislation with regard to their use as preservatives for controlling moulds in bakery products.
8.6 Sources of further information and advice

General texts which can be recommended include the following:

8.7 References


Managing microbial spoilage in cereal and baking products


9

Managing microbial spoilage in the meat industry

J. Samelis, National Agricultural Research Foundation, Greece

9.1 Introduction

Raw meat and poultry and most of their products are perishable owing to their biological and chemical composition. Chemically, the lean muscle tissue of domestic animals and birds, such as beef, pork, lamb and chicken, is composed of mainly water (71–76%), proteins (20–22%) and lipids (3–8%) (Lambert et al., 1991); average values for a typical adult mammalian muscle are reported as 75%, 19% and 2.5%, respectively (Lawrie, 1991). Meat also contains carbohydrates (1.2% = lactic acid, 0.9%; glycogen; 0.1%; glucose and glycolytic intermediates, 0.2%), miscellaneous non-protein soluble organic or inorganic substances (2.3%), and other minor components such as vitamins, pigments and flavor compounds (Lawrie, 1991). Biologically, these components are nutrients utilized by the meat microflora. The muscles of healthy animals and birds are essentially free of microorganisms, but they become contaminated at slaughter (Nottingham, 1982; Sofos, 1994; Gill, 1998). Complete avoidance of microbial contamination of meat is practically impossible. However, control of contamination during slaughter and further meat processing and handling operations is possible (Gill, 1986; Sofos, 1994). Such control is the most critical item of good manufacturing and sanitation practice (GMP, GSP) guidelines and Hazard Analysis Critical Control Point (HACCP) programs in order to produce meat products of high quality and safety (FSIS, 1996; NACMCF, 1998; Pearson and Dutson, 1999).

Since the early 1970s (Ingram and Dainty, 1971) there has been an increasing scientific interest in understanding the microbial ecology of meat and its associated chemistry of spoilage, and in establishing advanced technological methods to extend the shelf-life of raw and processed meat products. In addition, increased scientific awareness has been given to control meat-borne pathogens
in order to increase safety while preventing the meat industry from economical losses by delaying product spoilage (Lambert et al., 1991; Sofos, 1994; Mossel et al., 1995). As a consequence, research on the prevalence, characterization, evolution and interactions of different microorganisms during meat processing and storage has been intensified in recent years (Kotula and Kotula, 2000; Brown, 2000). Research has shown that from the great variety of microbial contaminants on fresh meat, only a fraction develops and eventually dominates the so-called ‘spoilage association’. This selection depends on the combined effect of intrinsic, extrinsic and processing factors affecting microbial survival, growth and competition in meat (Mossel and Ingram, 1955; Mossel et al., 1995). They include, among others, the type, pH, composition and texture of raw or processed meat, and mainly the storage temperature and the packaging atmosphere.

Refrigeration delays spoilage of perishable foods, such as meat, by increasing the lag phase and decreasing the growth rate of microorganisms, while packaging acts as a barrier to protect foods from microorganisms, and physical or chemical changes in their surrounding environment. Recent advances in the development of modified atmosphere packaging (MAP) technologies have led to food products of increased shelf-life, and to the evolution of fresh and minimally processed food preservation techniques (Smith et al., 1990; Farber, 1991; Church, 1994; Ohlsson, 1994; Molin, 2000). Particularly for meat, the partial removal of oxygen, with or without replacement with another gas in-package such as carbon dioxide (CO₂) or nitrogen, triggers major alterations in its microbial ecology and, thus, alters its pattern of spoilage also. Overall, in refrigerated meat products there is an inversion of the predominant spoilage flora from psychrotrophic Gram-negative bacteria (predominantly pseudomonads) to psychrotrophic Gram-positive bacteria (mainly lactic acid bacteria – LAB) when oxygen tension is reduced and/or CO₂ is increased in the packaging atmosphere. The fundamental microecological and biochemical changes associated with this shift in meat and poultry packaging conditions from aerobic to anaerobic or microaerophilic are well known and have been reviewed extensively (Gill, 1979, 1982, 1986; Grau, 1981; Mead, 1982, 1983; Dainty et al., 1983; Genigeorgis, 1985; Gill and Molin, 1991; Lambert et al., 1991; Dainty and Mackey, 1992; Sofos, 1994; Borch et al., 1996; Nychas et al., 1998; Labadie, 1999; Tewari et al., 1999; Brown, 2000; Molin, 2000; Rao and Sachindra, 2002).

Accumulation of scientific knowledge on microbial ecology and spoilage processes of meat and meat products has been the basis for introducing innovations in meat preservation. Recent research efforts have been focused on the use of additional hurdles to assist classical preservation and packaging technologies in controlling contamination and growth of spoilage and pathogenic microorganisms in raw and processed meat products. Such hurdles include, among others, decontamination technologies for fresh meat (Sofos and Smith, 1998; Huffman, 2002), as well as (bio)-preservation with the application of natural antimicrobials, protective cultures, active packaging
materials, irradiation, high hydrostatic pressure, other technologies and their combinations (Sofos, 1994; Holzapfel et al., 1995; Lee et al., 1996; Stiles, 1996; Hugas, 1998; Vermeiren et al., 1999, 2002; Farkas, 2001; Hugas et al., 2002; Quintavalla and Vicini, 2002; Roller, 2003; Devlieghere et al., 2004; Holley and Patel, 2005). Such technologies have drawn attention after it has been understood that meat production under GMP and GSP may not always provide sufficient control of microbial contamination across the processing line. Also, refrigeration combined with vacuum packaging (VP) or MAP cannot always prevent fresh or processed meat products from rapid microbial spoilage.

Another interesting field is the early detection of spoilage by advanced subjective methods (Dainty, 1996; Ellis and Goodacre, 2001; Gram et al., 2002), and the prediction of the microbiological shelf-life of meat products, based on mathematical models (McClure et al., 1994; McMeekin and Ross, 1996; McDonald and Sun, 1999). Microbial growth is correlated with shelf-life, provided the microbiological level above which the product is rejected. The latter, however, is difficult to specify since it depends on the biochemical capabilities of specific spoilage organisms (SSO) dominating in meat products under certain storage conditions rather than on total microbial populations (Gram et al., 2002). Levels of SSO and sensory data can be correlated with the catabolism of certain meat components that are key growth substrates for microbes (e.g. glucose) (Gill, 1976; Nychas et al., 1988) or with the formation of specific catabolic products by SSO (Dainty, 1996; Gram et al., 2002). Such metabolites have been proposed as potential chemical indicators of spoilage, whose determination by rapid methods would possibly result in early detection of spoilage (Jay, 1986; Dainty, 1996; Nychas et al., 1998) and assist models in providing safe shelf-life predictions (McMeekin and Ross, 1996; Pin and Baranyi, 1998). Models have also been designed and validated to predict the fate of meat-borne pathogens under different (bio-) preservation, active packaging and storage conditions. Models and strategies for pathogen control have recently been reviewed elsewhere (Blackburn and McClure, 2002; Samelis and Sofos, 2003a). They are not discussed here because they are beyond the scope of this book.

The present chapter aims at (i) providing an overview of current knowledge on the microbial ecology of fresh and processed meat products and the range of microorganisms involved in different types of spoilage; and (ii) emphasizing meat decontamination, (bio-) preservation, active packaging and emerging technologies to control meat spoilage organisms and extend product shelf-life, and the application of predictive models for these purposes.

### 9.2 Microbial ecology of fresh meat spoilage

Fresh meat and poultry are prone to microbial contamination and support microbial survival or growth due to their enriched nutrient composition,
high pH (5.5 to 6.5) and water activity (0.98 to 0.99) (Lawrie, 1991). Microbial contamination of raw meat results from processing, and starts during slaughter, when the carcass becomes contaminated with microorganisms residing on external surfaces, the gastrointestinal tract and lymph nodes of the animal, and in the plant environment. The main sources of contamination are the feces and the animal hides and bird feathers where high numbers of microorganisms may shed or attach, respectively, facilitating their transfer to the slaughterhouse (Nottingham, 1982; Mead, 1982; Sofos, 1994; Gill, 1998). Airborne contamination of carcasses is also important (Gustavsson and Borch, 1993; Rahkio and Korkeala, 1997). Owing to the diverse contamination sources and seasonal, farm- or plant-associated differences in microbial ecology and hygiene, the numbers and types of microorganisms found naturally on freshly slaughtered meat are very heterogeneous (Kotula and Kotula, 2000).

The initial number of spoilage microbes is one of the most critical factors affecting shelf-life of fresh meat and poultry. For example, the beef carcass surface may contain aerobic plate counts of $10^1$ to $10^7$ cfu/cm$^2$, but usually $<10^4$ cfu/cm$^2$ (Roberts et al., 1984; Gustavsson and Borch, 1993; Gill et al., 1998; Sofos et al., 1999a), most of which are psychrotrophic bacteria. Microbial indicators of hygiene, such as total coliform and *Escherichia coli* biotype I counts, were mainly $\leq 10^1$ cfu/cm$^2$ in US beef meat plants, owing to their operation under HACCP programs and the establishment of regulatory criteria (Sofos et al., 1999b). Similar low numbers of total Enterobacteriaceae, coliforms or *E. coli* were found in EU and Canadian beef plants (Gustavsson and Borch, 1993; Gill et al., 1998). Bacterial contamination on freshly slaughtered carcasses of small ruminants, such as lamb or poultry, is generally higher than that of beef, with mean aerobic plate and psychrotrophic counts being at a level of 5.0 log cfu/g or cm$^{-2}$, or higher (Mead, 1982; Prieto et al., 1991; Geornaras et al., 1995).

Another critical factor affecting microbial activity and rate of meat spoilage is the physical state of meat. This is determined by the meat form, such as carcass, wholesale or retail cut, comminuted and the processing treatment applied (Hedrick et al., 1994). Certain processing steps increase contamination by spreading the existing contaminants attached on the fresh meat surface to its entire mass or by introducing additional contaminants. For example, meat chopping or grinding results in greater microbial loads because of larger areas of exposed surface, more readily available water and nutrients, additional processing time, and contact with more sources of contamination such as equipment (Hedrick et al., 1994). This also enhances distribution of microbes in fresh ground meats, such as minced beef and lamb, which were found to contain total aerobic and psychrotrophic organisms at a concentration up to 6 log cfu/g (Emswiler et al., 1976; Jay and Margitic, 1981; Drosinos and Board, 1995a; Jay, 1996; Ajjarapu and Shelef, 1999). Therefore, efforts should be made to minimize initial microbial contamination to prevent fresh meat from rapid microbial deterioration.
Spoilage bacteria commonly found on fresh meat and poultry include *Pseudomonas*, *Acinetobacter/Moraxella*, *Psychrobacter*, *Aeromonas*, *Shewanella putrefaciens*, *Enterobacteriaceae*, *Brochothrix thermosphacta*, *Micrococcaceae*, *Clostridium* and lactic acid bacteria (LAB) of the genera *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, and *Weissella* (Dainty and Mackey, 1992; Borch et al., 1996; Broda et al., 1996; Garcia-Lopez et al., 1998; Kotula and Kotula, 2000; Björkroth and Holzapfel, 2003). Most contaminating bacteria are saprophytic Gram-negative, oxidase-positive rod-shaped *Pseudomonas* and related genera or Gram-positive catalase-positive cocci (*Micrococcus*) (Ayres, 1960; Barnes and Impey, 1968; Nortje et al., 1990; Gustavsson and Borch, 1993; Geornaras et al., 1996), while asexual yeasts (*Cryptococcus*, *Candida*, *Rhodotorula*, *Torulopsis*) may be present also (Dillon, 1998). However, as mentioned, few species dominate at spoilage. While the rate of spoilage is accelerated upon an overall high microbial contamination of fresh meat, the type of spoilage depends on the meat type and pH, and on the environmental conditions during storage (Gill, 1986; Lambert et al., 1991; Dainty and Mackey, 1992; Nychas et al., 1998).

The temperature and packaging atmosphere are the most important factors affecting microbial growth and selection during storage of fresh meat (Dainty et al., 1983; Mead, 1983; Gill and Molin, 1991; Borch et al., 1996; Nissen et al., 1996). Refrigeration selects for psychrotrophic species (Reuter, 1981; McMullen and Stiles, 1993) while it extends the lag phase and decreases the growth rate of all microorganisms, thus delaying or retarding their growth and preserving meat for limited times under aerobic storage conditions (Gill and Newton, 1977; Dainty and Mackey, 1992). Therefore, in addition to reducing microbial contamination, efforts should be made to maintain proper refrigeration temperature during processing, transportation and display of fresh meat and poultry. Improper holding temperature can be encountered in poorly maintained coolers all across the meat processing line and at home (Sofos, 1994).

The type of packaging is also critical for the type of meat spoilage because oxygen (air) is another strong selective agent affecting microbial growth and metabolism (Lambert et al., 1991; Dainty and Mackey, 1992; Mead, 1983; Nychas et al., 1998). In general, growth of all contaminating microorganisms, mainly bacteria, on meat occurs at the expense of one or more of its low molecular weight constituents, which are, however, attacked in various orders to produce different catabolic by-products depending on the species and the oxygen availability in-package (Table 9.1). Thus, depending on the affinity of each species to oxygen, bacteria differ in their competitive growth potential under aerobic or anaerobic conditions. Their relative spoilage potential depends on which groups or species predominate, and on their ability to form malodorous compounds, such as volatile amines, esters and H$_2$S, either aerobically or anaerobically (Table 9.1). As mentioned, in aerobically stored raw meat and poultry at low temperatures the spoilage flora includes aerobic putrefactive Gram-negative bacteria, predominantly pseudomonads (Gill and Newton,
Table 9.1 Main muscle constituents used as substrates for growth and main metabolic by-products of most important meat spoilage microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Substrates used for growth(^a)</th>
<th>Major end-products of metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Glucose(^1)</td>
<td>Amino acids(^3)</td>
</tr>
<tr>
<td></td>
<td>Amino acids(^3)</td>
<td>Lactic acid(^2)</td>
</tr>
<tr>
<td><strong>Acinetobacter/Moraxella</strong></td>
<td>Amino acids(^1)</td>
<td>Lactic acid(^3)</td>
</tr>
<tr>
<td><strong>Shewanella putrefaciens</strong></td>
<td>Glucose(^1)</td>
<td>Amino acids(^1)</td>
</tr>
<tr>
<td></td>
<td>Lactic acid(^1)</td>
<td>Amino acids(^1)</td>
</tr>
<tr>
<td><strong>Brochothrix thermosphacta</strong></td>
<td>Glucose(^1)</td>
<td>Amino acids(^2) (glutamate)</td>
</tr>
<tr>
<td></td>
<td>Glucose(^1)</td>
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<td><strong>Enterobacter</strong></td>
<td>Glucose(^1)</td>
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<td>Glucose 6-phosphate(^2)</td>
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<td>Amino acids(^3)</td>
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<td>Lactic acid(^4)</td>
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<tr>
<td><strong>Lactobacillus</strong></td>
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<td>Glucose(^1)</td>
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<td>Amino acids(^2)</td>
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\(^a\)The numbers in superscript indicate the order of utilization of this substrate.
1977; Shaw and Latty, 1982; Mead, 1982, 1983; Jimenez et al., 1997). When, however, raw beef, pork, lamb or poultry carcasses or retail meats are packaged under vacuum or MAP, LAB, B. thermosphacta, and enterobacteria dominate, with the former group governing the microbial association and the later two groups being of lower presence by decreasing the storage temperature and/or increasing the CO$_2$ concentration in-package (Christopher et al., 1979; Mead, 1983; Grau et al., 1985; Lambert et al., 1991; Ordonez et al., 1991; Dainty and Mackey, 1992; McMullen and Stiles, 1993; Kakouri and Nychas, 1994; Jimenez et al., 1997; Tsigarida and Nychas, 2001). This is because pseudomonads and other aerobic meat spoilage bacteria are more vulnerable to CO$_2$ than the respective Gram-positive bacteria, with LAB being the most resistant (Gill and Tan, 1980; Blickstad and Molin, 1983b; Farber, 1991).

The inhibitory effect of CO$_2$ against all bacteria is increased in fresh meat because of its increased solubility at refrigeration temperatures (Gill and Tan, 1980; Dixon and Kell, 1989). Vacuum-packaged meats contain increased CO$_2$ and decreased O$_2$ levels, but the concentrations do not vary as widely as those of MAP. Thus, while the microbial ecology of high-oxygen MAP fresh meat and poultry tends to be similar to that of air-packaged meats, vacuum-packaged and low-oxygen MAP fresh meat and poultry show similar microbial associations dominated by LAB (Dainty and Mackey, 1992; Nychas et al., 1998; Mano et al., 2000). The types of fresh meat spoilage in response to packaging and storage conditions, the main microbial species involved, and the chemistry of each type of spoilage, are discussed in more detail in the following paragraphs.

### 9.2.1 Spoilage caused by *Pseudomonas* and other Gram-negative bacteria

Spoilage of refrigerated, aerobically stored fresh meat and poultry is initiated at the expense of the limiting amounts of endogenous glucose, the rapid conversion of which to 2-oxo-glucurionate or glucuronic via the Entner–Doudoroff pathway offers a strong competitive advantage to *Pseudomonas* (Gill, 1976, 1982; Nychas et al., 1988, 1998). These catabolic products are not readily assimilated by most other meat spoilage bacteria and, thus, are reserved for use by pseudomonads as carbon and energy sources when glucose is depleted. In fact, glucose is the first preferably utilized low molecular constituent of the muscle tissues post-mortem by most bacteria, followed by amino acids, lactic acid and glucose-6-phosphate, depending on the species (Table 9.1; Lambert et al., 1991; Nychas et al., 1998). Hence, after the depletion of glucose in meat of normal pH, 5.5 to 5.8, the predominant *Pseudomonas* and closely related bacteria preferentially attack the amino acids, resulting in formation of volatile malodorous sulfides, esters and amines that eventually cause putrefactive types of meat spoilage (Freeman et al., 1976; Gill and Newton, 1977; Dainty et al., 1985, 1986, 1989a; Edwards and Dainty, 1987; Edwards et al., 1987).
Dark, firm and dry (DFD) meat, which has a high pH (>6.0) and is deficient in glucose, spoils more rapidly because deamination of amino acids by the aerobic spoilage flora starts earlier than in meat of normal pH (Erichsen and Molin, 1981). Even if stored under vacuum or high O₂-MAP conditions, DFD meat continues to spoil rapidly because its high pH selects for bacterial species with a higher spoilage potential than pseudomonads or LAB, such as \textit{Sh. putrefaciens}, \textit{S. liquefaciens} or \textit{B. thermosphacta} (Gill and Newton, 1979; Erichsen and Molin, 1981). The high pH of DFD meat is due to its reduced lactic acid content, since this meat is derived from stressed, exhausted or frightened animals before slaughter, conditions resulting in muscles that are deficient in glycogen to be converted into lactic acid during rigor mortis (Lawrie, 1991). Supplementation of DFD meat with glucose may delay spoilage by reducing the rate and lessening the offense of putrid off-odor formation in fresh meats stored under different conditions (Gill and Newton, 1979; Vanderzant et al., 1983; Lambropoulou et al., 1996).

The genus \textit{Pseudomonas} consists of five phylogenetic groups based on rRNA similarity studies (Palleroni, 1993), with the most important meat spoilage species being assigned to the first group (Group I) (Garcia-Lopez et al., 1998). This group includes both fluorescent (\textit{P. fluorescens} biovars A to D, \textit{P. putida} biovar A and \textit{P. lundensis}) and non-fluorescent (\textit{P. fragi} biovars 1 and 2) species. Their psychrotrophy and high affinity for oxygen have been suggested as the main reasons for the predominant growth of the above pseudomonad species in air-packaged or high-O₂ MAP fresh meats, since these properties presumably lead to a rapid glucose uptake (Gill and Newton, 1977; Gill, 1982; Gill and Molin, 1991). However, more recent studies have indicated that additional reasons to glucose oxidation may be more important for the natural selection of \textit{Pseudomonas} spp. on meat, which results in major differences in their predominance at spoilage. Indeed, while pseudomonads generally dominate on spoiled meats in the order \textit{P. fragi} > \textit{P. lundensis} > \textit{P. fluorescens} > \textit{P. putida}, (Garcia-Lopez et al., 1998), the glucose transport capacity (\(V_{\text{max}}\)) of these species is opposite (Nychas et al., 1998). More specifically, \(V_{\text{max}}\) was lowest in \textit{P. fragi}, highest in meat strains of \textit{P. fluorescens}, which were unable to oxidize glucose, and intermediate in strains of \textit{P. lundensis} and \textit{P. fluorescens} that oxidized glucose. Both \textit{P. fragi} and \textit{P. lundensis} and gluconate-positive \textit{P. fluorescens} have all shown a diauxic growth in meat juice associated with a sequential catabolism of glucose and lactate (Drosinos and Board, 1994). However, despite the fact that \textit{P. fragi} has a lower transport capacity, it grows more rapidly at low temperatures than \textit{P. fluorescens} and other fluorescent \textit{Pseudomonas} spp. (Gill and Newton, 1977; Gill, 1982; Lebert et al., 1998). Consequently, \textit{P. fragi} is predominantly isolated from spoiled meat, with an incidence ranging between 56.7 and 79% (Molin and Ternstrom, 1982, 1986). It is, therefore, evident that the dominance of \textit{P. fragi} over \textit{P. lundensis} and \textit{P. fluorescens} during aerobic meat spoilage may be due to glucose-independent metabolic capabilities, such as the ability of the former species, but none of the two later ones, to metabolize creatine and creatinine (Drosinos and Board, 1994).
Labadie (1999) suggested another potential reason for the increased adaptation and rapid dominance of *P. fragi*, namely its ability to rapidly use the very diverse sources of iron present in fresh meat to fulfil its important iron requirements for aerobic growth. This property of *P. fragi*, which is well documented in laboratory media (Champomier-Verges et al., 1996), may save from its cellular energy at low temperatures because the species is not in a need of synthesizing iron chelators or siderophores (green fluorescent pigments) to support growth on meat (Labadie, 1999). In contrast, such a need would exist in fluorescent *Pseudomonas*, and would require an extra expenditure of cellular energy. This may explain the greater generation time of *P. fluorescens* as compared with non-fluorescent *P. fragi* when grown in a meat medium at 2°C, which was 8.2 h and 7.6 h, respectively (Lebert et al., 1998).

Pseudomonads are also the main bacterial group responsible for spoilage of aerobically or high-O2 MAP stored poultry meat (McMeekin, 1977, 1981; Mead, 1982, 1983; Kakouri and Nychas, 1994; Kakouri, 1995; Jimenez et al., 1997). Although fewer numerical taxonomic studies are available, *Pseudomonas* spp. associated with poultry meat spoilage following attachment on poultry skin (Daud et al., 1979) appear to be similar to those found in red meats. Indeed, *P. fragi*, *P. lundensis*, *P. fluorescens* biovars A, B and C, as well as *P. lundensis*-like or *P. fluorescens*-like organisms were recently isolated from refrigerated poultry carcasses (Sundheim et al., 1998; Arnaut-Rollier et al., 1999). Sundheim et al. (1998) reported that none of the above species was dominant, although the *P. fluorescens* biovars constituted about 50% of the isolated pseudomonads. Conversely, the fluorescent *P. putida* has not been associated with poultry meat spoilage, while it generally has an incidence of less than 5% of the pseudomonad population on red meats also (Garcia-Lopez et al., 1998).

Other *Pseudomonas* species occasionally associated with meat spoilage include *P. stutzeri* and *Burkholderia (Pseudomonas) cepacia* (Garcia-Lopez et al., 1998). Most meat *Pseudomonas* preferentially utilize glucose and then lactate, although Molin (1985) reported that *P. fragi* utilized lactate while glucose was present in broth cultures incubated under aerobic or oxygen-limiting conditions. Nonetheless, the sequential or mixed glucose and lactate utilization is always followed by the use of amino acids (Table 9.1). With regard to their spoilage metabolites, pseudomonads produce dimethylsulfide, but not H2S, from amino acids, and this property differentiates them from Enterobacteriaceae and *Sh. putrefaciens* (Dainty et al., 1985, 1989a; Garcia-Lopez et al., 1998; Nychas et al., 1998).

*Shewanella putrefaciens* is an aerobic Gram-negative motile rod-shaped bacterium, whose taxonomic status is still uncertain (Garcia-Lopez et al., 1998). The species has been assigned to the genera *Pseudomonas* and *Alteromonas* since it resembles them in several physiological and biochemical properties. From a meat spoilage point of view, however, it is important that *Sh. putrefaciens* fails to grow on meat with pH < 6.0, while it can produce H2S by attacking sulfur-containing amino acids, such as cysteine and cystine,
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even when meat glucose is available (Gill and Newton, 1979; Dainty et al., 1989a). These conditions increase the importance of *Sh. putrefaciens* as a major spoilage agent in DFD meat (mainly pork) or in high-pH poultry skin or thigh meat, where accumulation of H$_2$S gives more offensive off-odors without *Sh. putrefaciens* being necessarily predominant at spoilage (McMeekin, 1977, 1981; Dainty et al., 1983; Dainty and Mackey, 1992). In addition, H$_2$S may interact with myoglobin to form sulfmyoglobin, which deteriorates the meat color by green discoloration (Gill and Newton, 1979). Thus, *Sh. putrefaciens* can have a greater spoilage potential on high-pH fresh meat, even when present at populations of, or less than, $10^7$ cfu/g and actually lower than the populations of predominant pseudomonads. Growth, and thus, spoilage potential of *Sh. putrefaciens* are significantly reduced at temperatures $\leq 2^{\circ}C$, and in meat of normal pH (Gill and Newton, 1979; Gill, 1986; Garcia-Lopez et al., 1998).

Other Gram-negative aerobic motile rods found, frequently or occasionally, in abattoirs and on spoiled meat are members of the genera *Alteromonas*, *Alcaligenes*, *Achromobacter*, and *Janthinobacterium* (Nortje et al., 1990; Garcia-Lopez et al., 1998). Their actual occurrence and role in meat deterioration is quite uncertain, at least when such conclusions are based on identification data from early studies. Indeed, *Sh. putrefaciens* was originally reported as *Alteromonas putrefaciens*, while *Achromobacter* used to be a diverse genus including, among others, aerobic non-motile non-fermentative rods, which were subsequently classified as *Acinetobacter*. Currently *Achromobacter* is not a taxonomically accepted genus, while the taxonomic status of *Alcaligenes* is also unclear. *Janthinobacterium lividum* has occasionally been associated with slime formation on roast beef, while it also produces violacein, a water-insoluble purple pigment (see review by Garcia-Lopez et al., 1998).

Gram-negative aerobic, but non-motile, rods of the non-pigmented genera *Moraxella*, *Acinetobacter* and *Psychrobacter*, and of the yellow-pigmented genus *Flavobacterium* may also constitute a minor to significant part of the spoilage flora of aerobically stored fresh meat (Gill and Newton, 1977; Nortje et al., 1990; Prieto et al., 1992; Geornaras et al., 1996). Currently, *Acinetobacter* consists of oxidase-negative rods, while *Moraxella* and *Psychrobacter* (formerly *Moraxella*-like organisms) include oxidase-positive rods, with *Psychrobacter immobilis* being the most commonly isolated species (Garcia-Lopez et al., 1998). *Acinetobacter johnsonii* and *Ac. lwofii* were found to be significant, or even predominant, on spoiled meat and poultry (Shaw and Latty, 1988; Gennari et al., 1992). Conversely, although members of the *Moraxella–Psychrobacter* group are frequently isolated as the main part of the carcass flora or the slaughterhouse flora, few isolates have been detected on spoiled carcasses (Prieto et al., 1992). Therefore, this group of organisms appears to have only low spoilage potential because of its inability to metabolize hexoses (Table 9.1; Prieto et al., 1992). *Moraxella* and *Acinetobacter*, however, are known to preferentially attack amino acids, without producing offensive metabolic by-products, and then lactate (Table
9.1; Gill and Newton, 1977). These bacteria have been found more frequently on poultry thigh than breast (McMeekin, 1977, 1981) and on fat surfaces (Shaw and Latty, 1988). Their presence in spoiling meat is considered more desirable in terms of defects than that of putrefactive *Pseudomonas* spp. and *Sh. putrefaciens* (Gill and Newton, 1977; Lambert et al., 1991).

Gram-negative rod-shaped bacteria, which are not aerobic but facultatively anaerobic, occur commonly on fresh meat and poultry and may cause spoilage also. They belong mainly to the large diverse family of Enterobacteriaceae and to *Aeromonas* (Dainty et al., 1983; Dainty and Mackey, 1992). The latter psychrotrophic genus, which includes the pathogen *Aeromonas hydrophila*, is normally outnumbered by *Pseudomonas* on fresh meats stored in air, but it can compete well in low oxygen packs (Mano et al., 2000). Several workers have detected enterobacteria of the genera *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Kluyera* (less commonly), *Proteus*, and *Serratia* on raw beef, lamb, pork, and poultry (whole or ground, fresh or marinated) products, as well as on offal meats (see review by Garcia-Lopez et al., 1998). In general, enterobacteria have an increased occurrence and role in spoilage of raw meat products that are of high pH, while they are more competitive against aerobic rods in vacuum than in air packs.

With regard to their meat spoilage potential, the most important Enterobacteriaceae are the species *Serratia liquefaciens*, *Hafnia alvei* and *Enterobacter* (*Pantoea*) *agglomerans*. Similarly to pseudomonads, they preferentially utilize glucose prior to degrading amino acids, with the release of amines, sulfides and H$_2$S. Their ability to produce H$_2$S, but not dimethylsulfide, significantly increases the severity of spoilage caused by Enterobacteriaceae, as it also does for *Sh. putrefaciens* (Dainty et al., 1985, 1989a). As a result, *S. liquefaciens*, *H. alvei* and other enterobacteria may become the main spoilage agents in DFD meat (pH > 6.0) under anaerobiosis by producing H$_2$S and greening (i.e., sulfmyoglobin) (Dainty and Mackey, 1992). In addition to producing malodorous compounds, the metabolism of enterobacteria and *Sh. putrefaciens* leads to the release of large amounts of ammonia, which contributes to spoilage off-odors (Dainty and Mackey, 1992), while *S. liquefaciens* produces acetic acid also. Therefore, Enterobacteriaceae can cause spoilage when they increase to a level of $10^7$ cfu/cm$^2$ (Grau, 1981). Ammonia is also produced by most pseudomonads in air-stored meat and poultry (Dainty and Mackey, 1992; Schmitt and Schmidt-Lorenz, 1992). However, while *Pseudomonas* spp. produce ethyl esters as one of their main spoilage by-products, Enterobacteriaceae may produce acids, alcohols, and acetoin/diacetyl, but not esters (Dainty et al., 1985, 1989a; Nychas et al., 1998).

### 9.2.2 Spoilage caused by lactic acid bacteria and related organisms

Lactic acid bacteria (LAB) that predominate on fresh meat and poultry packaged under vacuum or MAP belong to *Lactobacillus* (mainly to *Lact. sakei*/curvatus
group; Torriani et al., 1996), *Leuconostoc* and *Carnobacterium* (Collins et al., 1987; Shaw and Harding, 1989; Schillinger and Lucke, 1987a, b; Nissen et al., 1996; Stiles and Holzapfel, 1997; Björkroth and Holzapfel, 2003). Quite often members of other LAB genera, such as *Weissella* (mainly *W. viridescens* and *W. paramesenteroides*; Collins et al., 1993), *Lactococcus* (mainly *Lc. raffinolactis*), *Enterococcus* and *Pediococcus*, may constitute a significant part of the spoilage association (Schillinger and Lucke, 1987a,b; Borch et al., 1996; Jones, 2004). It should be noted that until the mid 1980s, most of these important meat spoilage LAB were unidentifiable, and thus, reported as atypical meat lactobacilli (streptobacteria or betabacteria) or atypical leuconostocs (Reuter, 1981; Hitchener et al., 1982; Shaw and Harding, 1984; Morishita and Shiromizu, 1986). However, recent advances in bacterial systematics based on molecular (genotypic) methods and polyphasic taxonomic approaches (Vandamme et al., 1996) have contributed to resolve their taxonomy. Genotypic characterization is normally combined with advanced biochemical methods, such as SDS-PAGE (Sodium dodecylsulfate polyacrylamide gel electrophoresis) of whole cell proteins or GC of cellular fatty acids (Vandamme et al., 1996), which can also differentiate between closely related meat LAB species (Samelis et al., 1995, 1998c; Torriani et al., 1996). Of course, molecular methods have been the basis for previous and more recent descriptions of novel LAB associated with fresh meat spoilage, such as *Lactobacillus algidus* (Kato et al., 2000) and *Lactobacillus fuchuensis* (Sakala et al., 2002) from vacuum packaged beef in Japan, and *Lactobacillus oligofermentans* from MAP poultry products in Finland (Koort et al., 2005).

During storage of vacuum-packaged or MAP fresh meats, LAB produce organic acids, mainly lactic acid, from endogenous glucose, which may reduce the pH. As a result, a fermenting type of spoilage occurs and is manifested by aciduric off-odors attributed to lactic acid, acetic acid and other volatile fatty acids (Table 9.1). This type of spoilage may be accompanied by gas and slime formation in-package and meat pigment discoloration (greening) at package opening due to H$_2$O$_2$ formation by certain obligatory or facultatively heterofermentative LAB, such as *Leuconostoc/Weissella* and *Lact. sakei/curvatus*, respectively (Schillinger and Lucke, 1987a,b; Borch et al., 1996). Moreover, when fresh meat is stored anaerobically, certain LAB such as *Lact. sakei* may cause or accelerate spoilage by formation of H$_2$S in-package, which results in malodorous off-odors and black spots on meat due to reaction of H$_2$S with the iron present in myoglobin (Shay and Egan, 1981; Egan et al., 1989). Overall, it should be stressed that LAB spoilage is far less offensive than putrid types of spoilage caused by Gram-negative meat-borne bacteria not only because of the shift from aerobic to vacuum or MAP conditions, but also because LAB are weakly proteolytic (Law and Kolstad, 1983). It should be also stressed that although 100% CO$_2$ in MAP is the best atmosphere to delay LAB growth and inhibit most other spoilage bacteria in fresh meats, it causes discoloration and, thus, some in-package O$_2$ is necessary to maintain the desired red color of myoglobin. Recent investigations (Sorheim
et al., 1997) to overcome this problem by the use of carbon monoxide (CO) instead of O2 in MAP meats seem promising, but they are still hampered by the toxicological concerns associated with CO.

Microbiological ecology studies have shown the existence of psychrotrophic LAB species that are highly specific to fresh meat and poultry. Typical examples are *Lact. sakei* (Champomier et al., 1987), and *Leuconostoc gelidum* and *Leuc. carnosum* (Shaw and Harding, 1989). These aciduric LAB species generally have an increased frequency of isolation among the diverse lactic flora present on freshly slaughtered meat, while they increase during anaerobic storage of refrigerated meats and most often predominate at spoilage. Also, the non-aciduric genus *Carnobacterium (carnis = meat)* is highly specific to fresh meat and mainly poultry. Indeed, all representative strains of the description study of the genus, including the most common species *Carn. piscicola* (currently reclassified as *Carn. maltaromaticum*; Mora et al., 2003) and *Carn. divergens*, were originally isolated from poultry meat (Collins et al., 1987). The specificity for meat of the above LAB species suggests that their natural selection and dominance may be due to one or more special physiological attributes and/or biochemical (metabolic) capabilities that increase their adaptation and competition against other LAB in fresh meat environments. Of course, important general properties, such as psychrotrophy, anaerobiosis, glucose fermentation and *in situ* production of bacteriocins, H2O2 or other antimicrobial substances, have been shown to contribute significantly to the predominance of *Lact. sakei, Leuc. gelidum, Leuc. carnosum* and *Carnobacterium spp.*, or of their selected Bac+ strains, on fresh and processed meat products (Schillinger and Lucke, 1989; Yang and Ray, 1994; Leisner et al., 1995, 1996; Hugas et al., 1998; Laursen et al., 2005). However, these properties cannot really explain why these LAB species are far more frequent and competitive on fresh meat and poultry compared to closely related species, such as *Lact. curvatus* and *Leuc. mesenteroides*, respectively. The latter two species and some weissellas, such as *W. paramesenteroides* and *W. viridescens*, have been reported to significantly increase their presence and competitiveness in processed meats, where curing salts, sugars, and additional nutrients or elements with spices and seasonings, are added (see Section 9.3). It appears, therefore, that *Lact. sakei, Leuc. gelidum, Leuc. carnosum* and *Carnobacterium* are less fastidious and specifically adapted for growth in fresh meat, where high pH but otherwise oligotrophic conditions or even starvation may prevail.

Especially for *Lact. sakei*, the most important meat spoilage LAB, it has been suggested that its ability to hydrolyze arginine provides this species with a major competitive advantage for growth in meat environments (Labadie, 1999). In broth cultures that contain little glucose (e.g. 0.5% or lower) but enough arginine, as normally occurs in fresh meat, *Lact. sakei* readily hydrolyzes arginine to support growth and, thus, ammonia is formed (Schillinger and Lucke, 1987b; Montel et al., 1991; Samelis et al., 1994a). This property represents one of the most reliable differentiating taxonomic criteria between
Lact. sakei and Lact. curvatus, and may be one of the main reasons for the dominance in fresh meat of L. sakei over its close relative, which is arginine-negative. In addition, arginine degradation by Lact. sakei may result in the formation of toxic amines, putrescine and spermine, which may also contribute to its dominance (Labadie, 1999). Arginine is also hydrolyzed by carnobacteria (Collins et al., 1987), but not by Leuc. carnosum or Leuc. gelidum (Shaw and Harding, 1989). However, meat leuconostocs, as well as Lact. sakei and carnobacteria, may utilize other amino acids or carbohydrates present in meat to support growth. For example, Stentz et al. (2001) reported that the high specific adaptation of Lact. sakei to meat may be enhanced through a new mechanism of ribose and glucose utilization regulated by PTS (i.e. the phosphotransferase system which is a multi-enzymatic complex responsible for sugar uptake).

Sugar uptake is of utmost importance for LAB proliferation on meat and the associated types of spoilage. Interestingly, most meat-specific LAB are either obligatory heterofermentative (Leuconostoc, Weissella, Carnobacterium), producing lactate, acetate and CO2 or ethanol from hexoses (glucose), or facultatively heterofermentative (Lact. sakei/curvatus), producing two moles of lactate from hexoses under optimal growth conditions (Kandler, 1983; Kandler and Weiss, 1986). Notably, these LAB are also capable of fermenting pentoses (e.g. ribose), unlike the obligatory homofermentative LAB (e.g. Lactococcus, Pediococcus, Enterococcus and many Lactobacillus spp., mostly dairy thermophilic) which are less numerous and rarely dominate in meat spoilage associations. Facultatively heterofermentative LAB, in particular, possess an inducible phosphoroketolase activated when pentoses (ribose) are present and catalyzing their conversion into lactate and acetate without gas formation (Kandler, 1983; Kandler and Weiss, 1986). It appears, therefore, that Lact. sakei, Lact. curvatus and other ribose-fermenting Lactobacillus, such as Lact. plantarum and Lact. pentosus, have the ability to switch their sugar metabolism from homofermentative to heterofermentative under glucose limitation (Kandler and Weiss, 1986; Borch et al., 1991). This switch results in significant production of acetate (Borch et al., 1991), as it is the case in meat (Nychas et al., 1988, 1998; Borch et al., 1996). Also, the above Lactobacillus groups as well as certain Weissella are able to produce both D(–) and L(+) lactate isomers from glucose (Kandler and Weiss, 1986; Collins et al., 1993). The Lact. sakei/curvatus group, in particular, possess a lactate racemase, which converts L-lactate to D-lactate. Conversely, leuconostocs produce solely D-lactate, while carnobacteria produce L-lactate (Collins et al., 1987, 1993; Stiles and Holzapfel, 1997). The greatest amount of L-lactate present in meat, however, is endogenous following rigor mortis. In contrast, D-lactate seems to be a sole by-product of LAB metabolism (De Pablo et al., 1989; Kakouri and Nychas, 1994; Nychas et al., 1998), although there is weak evidence that endogenous meat enzymes may also produce D-lactate. Nonetheless, under glucose limitation and/or aerobic storage, most meat spoilage LAB can oxidize L-lactate from meat or lactate and its catabolic precursor pyruvate, into acetate.
For all the reasons above, a marked decrease of L-lactate concentration accompanied by a significant increase in concentration of both D-lactate and acetate during storage of fresh meat, at different rates depending on whether meat is packed in air, vacuum or MAP, occur, and these changes are well documented (Ordonez et al., 1991; Borch and Agerhem, 1992; Kakouri and Nychas, 1994; Kakouri, 1995; Drosinos and Board, 1995a,b; Lambropoulou et al., 1996; Nychas et al., 1998). The increase in acetate is important because this acid may have a greater antimicrobial activity than lactate since it remains undissociated at higher meat pH values because of its higher pKₐ (Samelis and Sofos, 2003c). On the other hand, acetate may have a stronger negative impact than lactate on sensory quality of spoiling meat because of its sharper vinegar-like flavor.

*Brochothrix thermosphacta* is an important meat spoilage bacterium, which shows extreme morphological diversity, from coccobacilli and short rods to long cell filaments and chains, depending on the growth conditions (Gardner, 1981; Rattanasomboon et al., 1999). It is a Gram-positive bacterium, which possesses a fermentative metabolism and produces L-lactate from glucose. Morphologically and because of acid production, it resembles and may be confused with some LAB, such as carnobacteria; however, it differs in being catalase-positive. Moreover, *B. thermosphacta* displays variable biochemical properties that change its pattern of metabolism under different growth conditions. Anaerobically, *B. thermosphacta* produces L-lactate and ethanol as >85–90% of its end-products from glucose (Gardner, 1981; Grau, 1983; Blickstad and Molin, 1984). In contrast, aerobically, it produces L-lactate as well as a mixture of acetic, propionic, iso-butyrac, n-butyric, iso-valeric and n-valeric acids, acetoain, 2,3 butanediol, diacetyl, 3-methylbutanal, 2-methylpropanol and 3-methylbutanol in media with glucose, ribose or glycerol (Dainty and Hibbard, 1980, 1983; Gardner, 1981; Borch and Molin, 1989). It also utilizes glutamate aerobically, while it may produce small amounts of formic acid irrespective of gaseous atmosphere. Thus, *B. thermosphacta* has the ability to possess and activate various metabolic pathways for sugar breakdown. It generally behaves as a homofermentative bacterium under low oxygen and glucose availability, while it shifts to heterofermentative under high oxygen and/or glucose-limiting conditions.

How and why fresh meat has become the main ecological niche of *B. thermosphacta* is still unclear, given that this species has a very low incidence in farm samples (soil, hay, feces, etc.) (Labadie, 1999). The spoilage potential of *B. thermosphacta* in fresh meats is fairly high, especially when the meat has a pH > 5.8 and/or stored in air. The latter seems to be due to the in situ production of acetic and butyric acids, acetoain and alcohols, which significantly contribute to off-odor development. Acetic acid may also contribute to increase competition of *B. thermosphacta* against *Pseudomonas* spp. on meat, as it was observed in its mixed cultures with *P. fragi* in lamb juice stored at 4°C (Drosinos and Board, 1995b).
9.2.3 Spoilage caused by clostridia and other spore-forming bacteria

Psychrotrophic or psychrophilic members of the spore-forming genus *Clostridium* have been associated with an offensive type of spoilage of fresh vacuum-packaged meats (Dainty *et al*., 1989b; Kalchayanand *et al*., 1989), the so-called ‘blown pack’ (Broda *et al*., 1996). Spoilage is manifested by an excessive gas formation in the pack and an offensive cheese/dairy odor with or without sulfurous overtones at pack opening. Headspace analyses have shown the absence of oxygen and the presence of carbon dioxide and hydrogen plus butyl esters, butyric acid and mainly butanol, thus indicating a butyric type of fermentative metabolism by clostridia (Dainty *et al*., 1989b; Kalchayanand *et al*., 1989; Broda *et al*., 1996). Butanol is responsible for the ‘cheesy’ or ‘dairy’ off-odor, while a ‘sweet fecal’ off-odor is associated with the production of sulfurous compounds (Dainty *et al*., 1989b; Broda *et al*., 1996). In addition, when sulfurous compounds are present in the headspace, a green discoloration may develop on the surface of vacuum packaged meat owing to the formation of sulfmyoglobin (Broda *et al*., 1996).

The psychrotrophic clostridia associated with ‘blown pack’ fresh meat spoilage appear to be very heterogeneous, while they are widely distributed within meat species or countries. They have caused spoilage of beef in the UK (Dainty *et al*., 1989b) and USA (Kalchayanand *et al*., 1989), and of beef, lamb and venison in New Zealand (Broda *et al*., 1996). They resemble common mesophilic *Clostridium* spp. in producing butanol, but they differ in being saccharolytic and capable of growing at temperatures close to or slightly below the freezing point (–1.5 to 2 °C). Whether these uncommon clostridia should be characterized as psychrotrophic or psychrophilic is questioned, as it varies depending on the precise growth temperature range of each species (Broda *et al*., 1996). Taxonomic studies revealed that most of these clostridia represent new species, namely *Clostridium estertheticum* (Collins *et al*., 1992) and *Cl. algidicarnis* (Lawson *et al*., 1994) isolated from vacuum packaged beef and pork, respectively, in the UK, and *Cl. laramie* isolated from US beef (Kalchayanand *et al*., 1993). Similar strains isolated from ‘blown pack’ spoiled meats in New Zealand differed from the above species and were provisionally characterized as *Cl. difficile*, *Cl. beijerinckii* and *Cl. lituseburense* (Broda *et al*., 1996). However, more recent studies have led to the description of another three new species, the psychrophilic *Cl. gasigenes* from raw lamb (Broda *et al*., 2000a) and the psychrotolerant *Cl. frigidicarnis* from raw beef (Broda *et al*., 1999) and *Cl. algidixylanolyticum* from raw lamb (Broda *et al*., 2000b).

Soil particles attached to animal hides or present in feces seem to be the primary reservoir of ‘blown pack’ clostridia later introduced onto carcasses, reflecting the importance of dressing procedure hygiene for controlling their spread in meat packing plants (Broda *et al*., 2002). Also, heat shrink treatments of fresh meat in vacuum packs appear to accelerate ‘blown pack’ spoilage during refrigerated storage, probably by activating the clostridial spores present.
on meat (Bell et al., 2001). Notably, culturing and enumeration of meat spoilage clostridia on solid media is problematic because of their poor colony growth in the presence of selective agents or their outgrowth by the predominant LAB on non-selective media (Dainty et al., 1989b; Kalchayanand et al., 1989; Broda et al., 1996). Therefore, more advanced polymerase chain reaction (PCR) detection and molecular differentiation methods have been developed to assist in rapidly detecting and characterizing low levels of spoilage clostridia in meat (Broda et al., 2003a,b). ‘Blown pack’ spoilage remains a concern for the meat industry because it is not associated with temperature abuse of meat and, thus, it cannot be prevented by temperature control.

9.2.4 Spoilage caused by yeasts and moulds
Among the great variety of microorganisms that may contaminate raw meat and poultry, yeasts and moulds normally constitute a minor part of the total flora and have few opportunities to grow significantly and spoil the meat (Jay and Margitic, 1981; Dillon, 1998; Samelis and Sofos, 2003b). This is because yeasts and moulds proliferate at slow growth rates and, thus, compete poorly against psychrotrophic bacteria. Most meat yeasts, however, are more resistant than bacteria to dry or low water activity environments, low pH, high acidity and salinity, chemical preservatives and irradiation, while certain species are extremely psychrotrophic (Guerzoni et al., 1993; Dillon, 1998; Samelis and Sofos, 2003b). As a consequence, yeasts may opportunistically become important spoilage agents in fresh meat products, especially when bacterial growth is retarded due to the inhibitory effect of one or more of the above stress factors.

Yeasts, though, require oxygen for optimal growth, therefore their competitiveness and spoilage potential is reduced in fresh meats stored under VP/MAP, but may increase under aerobic packaging and low temperature storage. For example, asexual yeasts, mainly Cryptococcus laurentii var laurentii, predominated on lamb at –5°C (Lowry and Gill, 1984). Yeast numbers of $10^5$–$10^6$ cfu/g and yeast flora shifts resulting in prevalence of Candida lipolytica, Candida zeylanoides or Yarrowia lipolytica, in spoiled beef, poultry and retail meats may play a role in spoilage (Hseih and Jay, 1984; Viljoen et al., 1998; Chabela et al., 1999; Ismail et al., 2000). Yeast spoilage is manifested as surface slime, off odors and pigmented spots from their surface colonization. On properly cold-stored carcasses, the reduced environmental humidity dries out the meat surface to $a_w$ values < 0.95; under these conditions, fungal spoilage may also occur and potentially dominate (Mossel et al., 1995). Molds commonly associated with surface colonization of aerobically stored carcass meats include Thamnidium, Mucor, Penicillium and Rhizopus, as well as Cladosporium herbarum and Sporotorichum carnis, which cause ‘black spot’ and ‘white spot’ spoilage, respectively (Gill et al., 1981; Mossel et al., 1995).
9.3 Microbial ecology of processed meat spoilage

Processed meat products are produced from whole-muscle or comminuted meat, mainly pork, beef and poultry, or their mixtures with animal fats or plant oils. Meat is cured with sodium chloride plus nitrites and/or nitrates, while various other ingredients, such as seasonings, spices, flavorings, emulsifiers and preservatives, may be added (Tompkin, 1986; Brown, 2000). In terms of their technology, processed meats can be broadly differentiated into fresh-cured, cooked and dried-fermented. Fresh and cooked cured meats are sensitive to microbial spoilage and, thus, should be chill-stored under air or, better, vacuum or MAP. Cooked meats, in particular, are of two different types: (i) those that are heated and distributed in air-impermeable casings or cans and have an extended shelf-life, and (ii) those that are processed (e.g. peeled or sliced) post-cooking and re-packaged, which reduces their microbiological shelf-life (Tompkin, 1986; Brown, 2000). Conversely, dried or dry fermented meats are shelf-stable products that can be stored at ambient temperatures. They do not generally spoil microbiologically although sometimes sensory defects associated with the dominance of undesirable species due to process deviations may be caused (Brown, 2000; Lucke, 2000).

The shelf-life of processed meats is commercially defined as the storage time until the product develops organoleptic spoilage, and it depends mainly on the initial contamination level, the product type, the packaging method and the storage temperature (Blickstad and Molin, 1983a,b; Korkeala et al., 1987, 1989; Samelis et al., 1998a, 2000a,b). Their microbial contamination originates from the raw materials, or many other contamination sources in the plant, including the surface of equipment, air and personnel (Borch et al., 1988; Björkroth and Korkeala, 1996, 1997a; Björkroth et al., 1998). Specifically, while most bacterial contaminants in fresh or dried/fermented meats are normally acquired from the meat and fat (Samelis and Metaxopoulos, 1998, 1999), the respective flora of cooked meat products is mainly composed of post-processing contaminants since heat inactivates most bacteria present in the cured meat pre-cooking (Korkeala and Björkroth, 1997; Samelis et al., 1998a, 2000b). However, there are reported cases of heat-resistant vegetative cells that may survive heating to cause spoilage (Milbourne, 1983; Borch et al., 1988). Normal levels of post-process contamination of commercially manufactured, sliced or peeled, cooked meat and poultry products at packaging range from 10 to $10^3$ cfu/g or cm$^2$, parts of which are LAB (Borch et al., 1996; Samelis et al., 1998a, 2000a,b). The shelf-life of these products may be reduced to a few days under refrigeration, when the initial contaminants exceed $10^3$ cfu/g or cm$^2$. Of course, spoilage occurs even when the initial contamination is $\leq 10$ cfu/g (Korkeala and Björkroth, 1997; Hamasaki et al., 2003). Post-processing contamination can be avoided only in RTE meats that are heat-treated at high pasteurization or sterilization temperatures in hermetically sealed containers and then distributed.
The biodiversity of spoilage microorganisms found on processed meat and poultry products is similarly high to that of fresh meat and poultry. Thus, *Pseudomonas* and related genera, Enterobacteriaceae, *B. thermosphacta*, Micrococccaceae, *Bacillus* and *Clostridium*, yeasts and molds, and LAB (mainly *Lactobacillus*, *Leuconostoc*, *Weissella* and *Carnobacterium* and less frequently *Aerococcus*, *Enterococcus*, *Lactococcus* and *Pediococcus*) may all be among the initial contaminants (Blickstad and Molin, 1983a; Korkeala et al., 1988; Borch et al., 1996). Both the numbers and types of these contaminants are influenced from the microbiological quality of raw materials, the resident ‘factory’ flora and the plant hygiene (Holley, 1997b). Then, the storage conditions in combination with the product composition select for certain spoilage microbes, and affect their growth rate and activity (Samelis et al., 2000a). Overall, during manufacture and storage of cured meats, LAB, other Gram-positive bacteria and yeasts have an advantage to grow and predominate compared with Gram-negative bacteria. The combination of the microaerophilic conditions created by vacuum-stuffing, the presence of curing salt and nitrite and a reduced water activity resulting from the addition of sodium chloride and other humectants, such as phosphate salts and sodium lactate, favor growth of Gram-positive bacteria, mainly LAB (Nielsen and Zeuthen, 1983; Buchanan, 1986; Brown, 2000; Samelis et al., 2000a). Also, storage under refrigeration in vacuum or MA packages further contributes to favoring of psychrotrophic LAB, for the same reasons as those discussed for fresh meat and poultry.

When cured meats are fermented and dried, or simply chill-stored under anaerobic conditions, the meat proteins release water due to acidification caused by LAB, and, thus, the products lose moisture and their salt content increases (Samelis and Metaxopoulos, 1998; Samelis et al., 1998b; Lucke, 2000). Hence, the inhibition of acid- and salt-sensitive Gram-negative bacteria is enhanced, while the cured meat environment becomes more destructive for all bacteria. Under such conditions, yeasts may proliferate at the expense of certain metabolites, such as lactic acid, and may become a major part of the spoilage flora or may contribute in meat fermentation (Aggelis et al., 1998; Samelis and Metaxopoulos, 1998). For information on the beneficial role of LAB and other technological flora in fermented meat products the reader can refer to other reviews (Lucke, 2000; Samelis and Sofos, 2003b). Here, the different types of spoilage of processed meats in response to the product type and the packaging and storage conditions will be discussed.

### 9.3.1 Spoilage caused by *Pseudomonas* and other Gram-negative bacteria

Under normal circumstances, *Pseudomonas* are not important spoilage agents in cured cooked meat products because of their sensitivity to curing salts and heat pasteurization. Also, growth of aerobic *Pseudomonas* is not favored under vacuum or CO₂-enriched atmospheres, which are widely used for
packaging of processed meats, especially when the product is peeled or pre-sliced and re-packaged in the factory. When, however, the processed meat product is uncured and/or distributed unpacked or the peeling/slicing is performed in retail shops, aerobic conditions and lack of nitrites become important, and may allow for significant pseudomonad growth under refrigeration. In such cases, processors may use other antimicrobials to control *Pseudomonas* and other aerobic Gram-negative bacteria in fresh meat products as, for example, sulfite in British sausage (Banks *et al*., 1985). In conclusion, to date, if *Pseudomonas* predominate at spoilage of processed meats, this would be due to processing faults (e.g. no or insufficient curing, weak vacuum, unsuccessful package sealing) or to poor microbiological quality of raw materials or deficiencies in plant hygiene.

Certain cold- and mainly salt-tolerant genera or species of Enterobacteriaceae may cause spoilage of specific types of processed meats. For instance, some *Vibrio* spp., such as *V. costicola*, are known to spoil different varieties of raw cured hams and bacon (Gardner, 1982). Spoilage defects in bacon include internal taints such as bone or 'pocket' taints, from where *Vibrio* spp. as well as *Alcaligenes* and *Providencia* (formerly *Proteus*), mainly *Prov. alcalifaciens*, *Prov. stuartii* and *Prov. rettgeri*, have been isolated (Gardner, 1982; Garcia-Lopez *et al*., 1998). Enterobacteriaceae, mainly *Pr. bulgaris*, have been reported to cause putrefactive off-odors in Italian raw hams. Unless post-processing hygienic conditions are poor, Enterobacteriaceae are insignificant spoilage agents in vacuum-packaged cooked meat and poultry products. This is because their initial cross-contaminating populations, if any, are rapidly outnumbered by LAB, which are competitive during anaerobic refrigerated storage.

### 9.3.2 Spoilage caused by lactic acid bacteria and related organisms

As mentioned, LAB is the major bacterial group associated with the spoilage of processed meat and poultry products (Mead, 1983; Yang and Ray, 1994; Borch *et al*., 1996; Korkeala and Björkroth, 1997). The metabolic activity of LAB results in spoilage defects similar to those occurring in fresh meat and poultry; however, defects may be more severe because of the addition of carbohydrates (glucose, sucrose, maltodextrins, potato starch, skim milk, etc.) and other growth-enhancing ingredients (e.g. spices) in processed meat formulations (Nielsen and Zeuthen, 1983; Samelis *et al*., 1998a). Spoilage appears as ‘sour’ off-flavors and off-odors, milky exudates and frequently, slimy, swelling of the pack and/or greening (Niven and Evans, 1957; Egan, 1983; Korkeala *et al*., 1988; Ahvenainen *et al*., 1989; von Holy *et al*., 1991; Korkeala and Björkroth, 1997; Samelis *et al*., 1998a, 2000a,b). It is well documented that the dominance of LAB is unaltered by the chill temperature used, but their growth rate, and hence the rate of spoilage, are accelerated as the storage temperature increases (Zurera-Cosano *et al*., 1988; Korkeala *et al*., 1989; Samelis *et al*., 1998a; Samelis and Georgiadou, 2000). Also, increased concentrations of CO$_2$ in combination with NaCl may prolong the shelf-life
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of MAP products, given that growth of LAB is retarded and other spoilage bacteria and yeasts are inhibited (Blickstad and Molin 1983a,b; Laleye et al., 1984; Ahvenainen et al., 1989; Borch et al., 1996). Overall, the higher the oxygen concentration in-package or the higher the packaging film O2 permeability, the faster the growth of LAB and the greater the diversity of the microbial association due to co-growth of additional spoilage bacteria, such as B. thermosphacta, staphylococci and other catalase-positive cocci, enterobacteria and yeasts (Nielsen, 1983; Samelis et al., 2000a; Samelis and Georgiadou, 2000). Interestingly, a recent study (Cayre et al., 2005) reported a greater growth inhibition of B. thermosphacta in cooked meat emulsions packaged in high as compared with low oxygen permeable films, probably because of H2O2 production by the predominant LAB in the high-O2 samples. Nevertheless, microorganisms other than LAB may become important at spoilage when processed meat products are packaged in air, high-O2 MAP or under insufficient vacuum due to weak pumping out of air, or faulty sealing of the vacuum packs. Otherwise, the spoilage flora may exclusively consist of a mixture of LAB species, or even of a single type of LAB.

Numerous studies have examined the microbial ecology of processed meat products at spoilage (Kitchell and Shaw, 1975; Morishita and Shiromizu, 1986; Borch and Molin, 1988; Korkeala and Makela, 1989; Makela et al., 1992; Dykes et al., 1994a,b; Björkroth and Korkeala, 1996, 1997a; Björkroth et al., 1998; Samelis et al., 1998a, 2000a,b; Zhang and Holley, 1999; Barakat et al., 2000; Hamasaki et al., 2003; Cocolin et al., 2004; Santos et al., 2005). As mentioned, advances in polyphasic taxonomy have clarified the taxonomic position of most meat spoilage LAB reported as ‘atypical’ since the early to mid-1990s. Among the main LAB species are those dominating at spoilage of fresh meat, namely Lact. sakei, Leuc. carnosum and Leuc. gelidum, plus their closely related aciduric species Lact. curvatus, Leuc. mesenteroides subsp. mesenteroides, Leuc. citreum (an earlier synonym of Leuc. amelobiosum; Takahashi et al., 1992), W. viridescens and W. paramesenteroides. Additional species belonging to the above (Leuc. lactis, W. hellicena, W. halotolerans, W. minor, W. confusa) or other LAB genera (e.g. Carnobacterium, Lactococcus lactis, Lc. raffinolactis, Enterococcus faecalis) are isolated occasionally, but they are rarely at high numbers at spoilage (Barakat et al., 2000; Hamasaki et al. 2003; Cocolin et al., 2004; Santos et al., 2005). For instance, a recent study (Peirson et al., 2003a) found non-aciduric (alkalitrophic) LAB, namely Aerococcus viridans and Carn. viridans, to be responsible for a green discoloration observed in cooked cured bologna after 3 days of storage at 4 and 9 °C. It is the present author’s opinion, however, that the spoiled bologna above represents a rare case, and that green discoloration and other defects of terminally spoiled cooked cured meats are primarily caused by aciduric LAB, for reasons discussed below.

The effect of intrinsic (pH, moisture, salt, aw, etc.) and extrinsic (cooking and storage temperatures, gas permeability of packaging films, gas mixtures, etc.) factors on growth and competitive ability of LAB in cooked cured meat
products is well understood (Borch et al., 1996). However, relatively few studies on how the above factors in combination select the predominant genera/species of spoilage LAB exist (Dykes et al., 1996; Zhang and Holley, 1999; Samelis et al., 2000a; Vermeiren et al., 2005). This is, however, important to know, because the spoilage potential of LAB is not only dependent on their growth rate, but also on the specific metabolic activity of the different LAB species growing in processed meat products under certain storage conditions. Moreover, this is difficult to evaluate by comparing data from ecology studies of meat products processed in different plants at different manufacturing periods and stored under different temperature and packaging conditions. Therefore, Samelis et al. (2000a) compared the relative growth responses and the ability of different LAB species to specifically dominate the spoilage association of different cured meat products at 4 °C, sharing their processing plant environment, day of production and film packaging conditions. Growth of LAB under vacuum was more prolific in the order: ham > turkey breast fillet > smoked pork loin > pariza > mortadella > bacon, and ham > frankfurters, manufactured in two industrial meat plants. The Lact. sakei/curvatus group prevailed in all products, except the non-smoked, boiled whole-meats (ham and turkey breast fillet), where Leuc. mesenteroides subsp. mesenteroides predominated. Lact. sakei was the most prevalent species in smoked whole-meats (pork loin and bacon). Emulsion sausages (pariza, mortadella and frankfurters) contained a more diverse LAB flora. Leuc. carnosum and Leuc. citreum occurred in boiled, whole-meats and emulsion sausages, respectively, while W. viridescens was present in smoked meat products only. When ham and frankfurters were stored in air, growth of LAB was faster. The presence of oxygen resulted in a replacement of Leuc. mesenteroides subsp. mesenteroides by other Leuconostoc spp. in ham, and in a shift of the spoilage flora from homo- to heterofermentative LAB species in frankfurters (Samelis et al., 2000a).

Similar results were obtained with Greek processed meat products from other plants studied individually. Leuc. mesenteroides subsp. mesenteroides was the major gas- and slime-producing spoilage agent of unsmoked sliced ham and turkey breast fillets cooked in boilers and stored at 4 and 12 °C in vacuum (Samelis et al., 1998a, 2000b). In contrast, strains of Lact. sakei predominated in sliced, vacuum packaged, smoked, oven-cooked turkey breast fillets from the same plant at 4 °C. The spoilage flora of the ham and unsmoked breasts grew faster than that of the smoked breasts and was more diverse. Lact. sakei, W. viridescens and atypical Leuc. carnosum-like bacteria were also members of the former flora (Samelis et al., 1998a, 2000b). Interestingly, Leuc. carnosum has been the specific spoilage LAB of vacuum packaged sliced cooked ham in Finland also (Björkroth et al., 1998). The faster growth and greater presence of heterofermentative LAB in Greek cooked ham and unsmoked breasts led these products to undergo rapid and severe spoilage: the packs swelled and the cured meat developed strong sour odors and flavors and abundant slime within 2 weeks of storage at 4 °C. In contrast, smoked
turkey breasts developed mild, sour spoilage after four weeks at 4°C (Samelis et al., 2000b).

In another study (Samelis and Georgiadou, 2000), strains of the *Lact. sakei/curvatus* group, mainly non-slime-producing *Lact. sakei*, generally dominated the spoilage flora of taverna sausage, a traditional Greek smoked coarse-cut cured meat product, stored at 4°C and 10°C in air, vacuum and 100% CO₂. However, while the isolation frequency of *Lact. sakei/curvatus* strains from sausages stored anaerobically was as high as 92–96%, in air-stored sausages, leuconostocs, mainly *Leuc. mesenteroides*, were significantly present (14–21%). Also, *B. thermosphacta* and Micrococcaceae grew at low numbers during aerobic storage. LAB caused a progressive decrease of pH and an increase of the concentration of L-lactate, D-lactate and acetate in all sausage packs. L-lactate and mainly acetate increased more rapidly and to higher amounts aerobically, unlike D-lactate, which was higher anaerobically. Storage in air was the worst packaging method, resulting in greening and unpleasant off-odors associated with the high acetate content of the sausages, while 100% CO₂ had no significant effect on extending shelf-life compared with vacuum (Samelis and Georgiadou, 2000).

The above research data clearly indicate a high correlation between the types of naturally selected spoilage LAB in cured cooked meats and important factors, such as the product formulation and pH, moisture, salt (brine) concentration, cooking method, and packaging atmosphere and storage temperature. In particular, the effect of the product type on the synthesis of the LAB spoilage flora of vacuum packaged products from the same processing environment may be more important than anticipated. Whole-meat products are technologically very different from emulsion-type sausages. Their characteristics (higher moisture and lower salt content, lower cooking temperatures, absence of smoking) seem to be highly selective for *Leuc. mesenteroides* and other *Leuconostoc* spp. Increasing, however, the combined hurdle effect of pH, moisture-aw, salt in brine and smoke in whole or emulsion-type, smoked products, may favor the dominance of *Lact. sakei/curvatus* strains. With regard to the storage temperature, Zhang and Holley (1999) reported that at a normal cured meat pH of 6.0–6.5, *Leuc. mesenteroides* grew faster than *Lact. curvatus* at 6°C, but it did not outcompete *Lact. sakei* at this temperature. Also, Vermeiren et al. (2005) found that *Leuc. mesenteroides* subsp. *mesenteroides* grew faster than *Leuc. carnosum* in a model cooked ham at 7°C. These data are generally consistent with the findings discussed above about potential reasons for the prevalence of *L. sakei* in meat processing environments, followed by *Leuc. mesenteroides*.

As the most common and widely distributed meat spoilage LAB (Borch et al., 1996; Korkeala and Björkroth, 1997), *Lact. sakei* has gained much scientific interest in recent years. *Lact. sakei* (1) is considered the meat SSO used in models for shelf-life prediction (Devlieghere et al., 1999, 2000), (2) is one of the most promising protective cultures for meat biopreservation (Hugas, 1998), and (3) is the best-adapted LAB and most suitable Bac+
starter culture for sausage fermentations, at least in Europe (Samelis et al., 1994b, 1998b; Leroy and De Vuyst, 1999; Lucke, 2000). Suboptimal temperatures, high salt concentrations and increased amounts of dissolved CO₂ during MAP storage of meat reduce the growth rate, but may enhance long-term survival, of Lact. sakei (Devlieghere et al., 1998; Marceau et al., 2003).

Attempts have been made to follow spoilage LAB species recontamination throughout the entire processing line of cooked meat products (Borch et al., 1988; Björkroth and Korkeala, 1996, 1997a; Björkroth et al., 1998; Samelis et al., 1998a, 2000b). This is the best approach to specify the sources, sites and routes of contamination, and it is strain-accurate if based on advanced molecular typing techniques (Björkroth and Korkeala, 1996, 1997a). It should be stressed that the types of LAB present in raw cured meats pre-cooking are not necessarily similar to those that cross-contaminate the product post-cooking, e.g. during peeling and slicing operations. For example, neither Lact. sakei nor Leuc. mesenteroides were important in curing and tumbling of raw pork ham, as carnobacteria, mainly Carn. divergens, and B. thermosphacta dominated at this stage (Samelis et al., 1998a). Also, while Carn. maltaromaticum (formerly Carn. piscicola) and Carn. divergens along with B. thermosphacta were predominant on the raw cured turkey breast fillets prior to cooking, these species were not isolated from the cooked, vacuum-packaged sliced product during storage at 4 °C (Samelis et al., 2000b).

By checking the production line, processing errors leading to rapidly spoiling or unsafe meat products may be detected, which otherwise pass unnoticed. As mentioned, contamination of cooked meats is most likely to occur post-cooking; however, this should be proven and not simply assumed, as it is not always the case. Although LAB along with L. monocytogenes are generally considered heat sensitive, some strains may survive pasteurization at core temperatures <70 °C of certain deli meats, such as ham, bacon or country-style sausages, especially if they are entrapped in fat (Samelis and Metaxopoulos, 1999). For instance, an increased thermal tolerance of W. viridescens in ham (Milbourne, 1983) and an emulsion-type sausage (Borch et al., 1988) has been reported. Also, Lemay et al. (2002a) reported that the protective culture Lact. sakei Bactoferm-B2 could survive in a cooked chicken meat model as the meat itself and the binders provided protection from heat to the culture’s free cells, making their encapsulation unnecessary. Franz and von Holy (1996a) compared the heat resistance of meat spoilage strains of Lact. sakei, Leuc. mesenteroides and Lact. curvatus in vitro. It was found that the D values at 57, 60 or 63 °C of the former species were constantly higher than those of the latter, with Leuc. mesenteroides being intermediate in heat tolerance (Franz and von Holy, 1996a). Although all three species had D values below 60s at 57 °C and 35s at 63 °C (Franz and von Holy, 1996a), a high LAB contamination of raw tumbled pork hams and bellies, or sausage batters, pre-cooking may lead to survival of few cells post-cooking.
Indeed, Samelis et al. (1998a) isolated a group of atypical *Leuconostoc*-like bacteria that grew slowly and eventually dominated in whole cooked pork hams, which were stored at 4 or 12°C to serve as non-sliced controls. Although atypical strains could not be detected among the LAB isolates recovered from raw or freshly cooked ham, they appeared to survive cooking and proliferate during storage. Representative strains of these atypical leuconostocs are currently assigned to *Leuc. carnosum* by a polyphasic taxonomic approach (Samelis and Björkroth, submitted). Post-packaging thermal pasteurization was also shown to increase shelf-life of Vienna sausages; however, it did not prevent product spoilage caused mainly by leuconostocs and homofermentative lactobacilli that survived and grew in heat-treated samples (Franz and von Holy, 1996b).

In recent years, another novel type of spoilage caused by LAB has been observed in Finnish marinated meat and mainly poultry products, originally called ‘protein swell’ (Björkroth, 2005). Although meat is raw, such marinated products are considered processed meats because marinades contain salt, sugars, starch, acids (acetic, citric), tomato, plant oils, spices (paprika, cayenne, etc.), emulsifiers and preservatives (Björkroth et al., 2000; Björkroth, 2005).

‘Protein swell’ spoilage was first reported in raw tomato-marinated broiler strips in MAP and the main defect was an extensive bulging of packages caused by an atypical *Leuconostoc* sp. producing abundant gas, newly described as *Leuc. gasicomitatum* (Björkroth et al., 2000). Bulging was accompanied by an increase in pH, which most likely resulted from the buffering capacity of the meat following marination rather than from any degrading effect of LAB on meat amino acids as it was suggested initially (Björkroth, 2005). Strains of *L. sakei* and *L. curvatus* along with an unidentified heterofermentative *Lactobacillus* sp. (Björkroth et al., 2000), also newly described as *Lact. oligofermentans* (Koort et al., 2005), were co-present in the mixed LAB association at spoilage. As expected, *Leuc. gasicomitatum* was predominant in all marinated MAP broiler meat strips at 6°C taken on the sell-by date from different retail outlets, confirming its common occurrence in marinated poultry products (Susiluoto et al., 2003). However, neither non-marinated broiler meat/skin nor the marinades harbored *Leuc. gasicomitatum* or *Lact. oligofermentans*, indicating that poultry or ingredients in marinate are not their natural habitat (Björkroth et al., 2005). Thus, these novel LAB species are probably environmental contaminants, whose growth on meat may be enhanced by marination (Björkroth, 2005).

Another rare type of bacterial meat spoilage is the so-called ‘vein effect’ on dry-cured hams, which was recently suspected to be due to growth of strains of *Marinilactibacillus psychrotolerans* (Rastelli et al., 2005). This bacterium is actually an alkaliphilic, Gram-positive, catalase- and oxidase-negative, non-spore-forming rod, resembling with *Carnobacterium*. However, it can survive or grow at high salt concentrations, e.g. up to 25% and 12%, respectively (Rastelli et al., 2005).
9.3.3 Spoilage caused by spore-forming Gram-positive bacteria

Processed meat and poultry products may be contaminated with vegetative cells and/or spores of *Clostridium* spp. present on raw materials; however, their spoilage potential is generally low and mainly associated with uncured products (Kalinowski and Tompkin, 1999) or faulty cooking–cooling operations. Clostridia are sensitive to nitrite and other curing salts, while the typical mesophilic species cannot grow and compete against psychrotrophic LAB at refrigeration temperatures. Thus, mesophilic clostridia may cause spoilage of cooked meats only upon slow cooling rates and extended holding times of products under temperature abuse conditions post-cooking. Such processing errors may activate their heat-resistant spores, which may proliferate rapidly and spoil the product by causing blowing and butyric off-odors. For example, Juneja *et al.* (1994) reported that cooked beef must be cooled to 7.2°C in 15 h or less to prevent spore germination and growth of *Clostridium perfringens*. Conversely, psychrotrophic meat spoilage clostridia, such as *Cl. laramie* and *Cl. algidicarnis*, have been reported to cause ‘blown pack’ spoilage in refrigerated uncured vacuum-packaged roast beef and cooked hot dog rolls, pork and turkey breast (Kalchayanand *et al.*, 1989, 1993; Lawson *et al.*, 1994; Broda *et al.*, 1996; Kalinowski and Tompkin, 1999). However, their spores appear to be less heat-resistant than the spores of mesophilic clostridia (Broda *et al.*, 1996).

While of rare, sporadic occurrence, another unpleasant spoilage characterized by excessive blowing and eventually breaking of casings of cooked meat products is associated with the growth of aerobic spore-forming bacteria, such as *Bacillus* spp. These bacteria are readily present in seasonings and spices used in meat processing, and their spores may germinate following post-cooking errors similar to those discussed above. The use of irradiated spices and fast cooling rates, and avoidance of holding products for long under temperature abuse conditions post-cooking, are simple precautions to prevent this type of spoilage.

9.3.4 Spoilage caused by yeasts and molds

Yeast spoilage phenomena have been more prominent, but still occurring rarely, in processed meat products following progress in VP/MAP technologies (Samelis and Sofos, 2003b). In addition to sub-refrigeration temperatures and dryness increasing their competitiveness in fresh meat, yeasts are favored in processed meats by brining or addition of salt in the formulation, chemical preservatives and irradiation. Yeast spoilage of cured meat is normally delayed but it may be unmasked because *Pseudomonas* are inhibited while the dominant LAB do not cause offensive primary spoilage under aerobic conditions (Samelis and Metaxopoulos, 1998; Samelis and Georgiadou, 2000). In general, yeasts become the main spoilage agents only in cured meat products preserved by sulfite, such as fresh British sausage (Dalton *et al.*, 1984) or irradiation (Drake *et al.*, 1959), or when products are chill-stored aerobically (Samelis *et al.*, 2000a). Most common yeast spoilage species in unsulfited and sulfited

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fresh British sausages were *Debaryomyces hansenii* followed by *C. zeylanoides* and *Pichia membranifaciens* (Dalton et al., 1984). *Cryptococcus* and *Rhodotorula* were also present, but their proportion was reduced in sulfited sausages due to their lower tolerance to the preservative (Dalton et al., 1984). Similarly, *D. hansenii* and *Candida* spp., namely *C. catenulata*, *C. lipolytica*, *C. zeylanoides* and *C. saitoana* (formerly *Torulopsis candida*), along with *Trichosporon pullulans* were isolated from the surface of packaged frankfurters (Drake et al., 1959).

Apparently, when contamination is high before vacuum packaging of cooked RTE meats or the final product is in air-permeable films or following opening of vacuum packages and storage in domestic refrigerators, spoilage yeasts may grow to high numbers. Indeed, populations of unidentified yeasts of $10^6$–$10^7$ cfu/g grew in a traditional Greek country-style fresh pork sausage, stored aerobically at 3 and 12°C, with the higher storage temperature accelerating growth and the development of ‘malty’ off-odors (Samelis and Metaxopoulos, 1998). Also, when Greek ‘taueva’ sausage was stored in air-permeable packages at 10°C, more than $10^5$ cfu/g of yeasts developed in spoiled sausages after 18 to 30 days (Samelis and Georgiadou, 2000). The sausages developed unpleasant vinegar like off-odors, and a sticky surface slime due to surface-growing yeasts. Similar phenomena were also observed in various types of cooked meats stored at 4°C, but no yeast growth and associated spoilage were observed when the same products were stored at 4, 10 or 12°C in vacuum (Samelis and Georgiadou, 2000; Samelis et al. 2000a). The processing environment seems to be the most important contamination source of ready-to-eat (RTE) cooked meats with salt-tolerant yeasts. *Debaryomyces* and *Candida* were the most prevalent yeasts among 123 isolates from a Vienna sausage plant, while fewer isolates were *Rhodotorula*, *Yarrowia*, *Pichia*, *Galactomyces*, *Cryptococcus*, *Trichosporon* and *Torulaspora* (Viljoen et al., 1993).

### 9.4 Current and emerging technologies to control spoilage of raw meat and poultry products

Controlling microbiological contamination in slaughterhouses and meat packing plants remains based on operation under GMP, GSP and good hygienic practices (GHP), as well as implementation of prerequisite programs at farm level, such as good animal husbandry practices (Sofos, 2002), and effective HACCP systems (FSIS, 1996; NACMCF, 1998; Brown et al., 2000). However, an increased control may be achieved if decontamination and emerging technologies are applied as additional antimicrobial hurdles.

#### 9.4.1 Fresh meat decontamination

The fundamental concept to decontaminate fresh meat lies back in the early
1970s. The concept of ‘intervention’ was introduced by Mossel (1984), with the aim of increasing control of meat-borne enteric pathogens that could not be controlled sufficiently by hygienic meat processing and food service practices. Thus, he proposed spraying meat with organic acids as an additional safety barrier. To date, bacterial pathogens remain the main target organisms of meat decontamination technologies, which are approved and applied commercially in the United States, Canada and Australia, but not yet in the European Union, where regulators remain against their use in order to prevent the masking of deficiencies in plant hygiene (Sofos and Smith, 1998; Smulders and Greer, 1998; Huffman, 2002; Castillo et al., 2002).

Decontamination technologies include animal cleaning, knife trimming, chemical dehairing, steam vacuuming, spraying or rinsing of the animal carcass with water, pressurized steam or antimicrobial compounds (Sofos and Smith, 1998). Approved antimicrobials for use in solutions for meat and poultry decontamination include mainly organic acids (acetic and lactic acid), chlorine and trisodium phosphate (Hardin et al., 1995; Gill, 1998; Sofos and Smith, 1998; Castillo et al., 2002; Capita et al., 2002). Other antimicrobials, including hydrogen peroxide, ozone, acidified sodium chlorite, peroxyacetic acid and bacteriocins (e.g. nisin and pediocins), have been evaluated for decontamination purposes with variable results (Kim et al., 1999; Kemp et al., 2000; DeMartinez et al., 2002; Gill and Badoni, 2004). Decontamination interventions may be applied before carcass evisceration to prevent soil and bacterial attachment, and/or as the final step in the process, before chilling. Processors may employ more than one intervention, in sequence. This ‘multiple hurdle’ approach to decontamination results in microbiologically cleaner carcasses (Bacon et al., 2000).

In addition to reducing numbers of pathogens present on meat, decontamination interventions are effective against the natural spoilage flora as they reduce total meat surface contaminants by 1 to 3 logs (Smulders and Greer, 1998; Sofos and Smith, 1998). Furthermore, spraying with organic acids and other chemicals may exert residual antimicrobial effects on microorganisms that survive decontamination treatment or contaminate meat post-treatment. Thus, chemical decontamination may not simply reduce populations of contaminating microorganisms but may alter the composition of the resident microbial flora on meat, and may enhance growth of acid-resistant pathogens (Greer and Dilts, 1992, 1995) in the presence of lowered numbers of natural competitive flora (Jay, 1996). Also, the rate and pattern of spoilage of fresh meat may change during refrigerated storage because of potential alterations in natural flora. Indeed, a study by van Netten et al. (1994) showed that lactic acid decontamination affected the composition of the resident microorganisms recovered from an in vitro pork skin model. Mesophilic Enterobacteriaceae decreased 1–3 logs with a shift in the predominant microflora to Gram-positive bacteria and yeasts (van Netten et al., 1994). More recently, Samelis et al. (2002a) reported a similar shift of the natural flora from Gram-negative Pseudomonas-like bacteria to Gram-
positive LAB and yeasts in acid-containing meat decontamination runoff waste fluids, which suggests that long-term use of acid decontamination interventions may favor LAB while suppressing Gram-negative bacteria in meat plants.

Such shifts from decontamination, which could possibly contribute to increased control against putrefactive bacteria in fresh meats packaged in air or vacuum, have not been validated in studies with products (Kenney et al., 1995; Prasai et al., 1997; Dorsa et al., 1998a,b,c), because results are conflicting. For example, Dorsa et al. (1998b,c) reported no differences in microbial growth and shelf-life of ground beef originating from decontaminated meat tissue as compared to untreated meat tissue. A more recent study, however, found that sequential hot water and lactic acid decontamination treatments had profound effects on the spoilage flora during aerobic storage of fresh beef at 4, 10 and 25°C (Koutsoumanis et al., 2004). Acid treatments shifted the predominant microflora of stored meat in the direction of LAB and yeasts. In contrast, in untreated hot water-treated samples, the spoilage flora was dominated by pseudomonads, while LAB, Enterobacteriaceae and yeasts remained at lower concentrations during storage, and B. thermosphacta was detected in few samples periodically (Koutsoumanis et al., 2004). Thus, acid decontamination has the potential to prevent or delay putrid spoilage in fresh aerobically stored meats by changing the type, growth rate and metabolic activity of the spoilage flora. Acid decontamination may further monitor the spoilage pattern of fresh meat by selecting for certain LAB or other Gram-positive bacteria (e.g. gas-, slime-, H$_2$O$_2$- and/or H$_2$S-producers versus non-producers), but this issue has yet to be investigated. However, chemical decontamination may also induce microbial adaptation, resistance and cross-protection to food-related stresses, a concern associated mainly with pathogenic rather than spoilage bacteria (Samelis and Sofos, 2003a,c).

### 9.4.2 Natural antimicrobials

Natural antimicrobials are very attractive alternatives to chemical preservatives in minimally processed foods, including meat and poultry products (Roller, 2003; Devlieghere et al., 2004). The use of natural antimicrobials in combinations, or combined with new active packaging or emerging technologies (e.g. irradiation, high hydrostatic pressure) represents an ideal application of the ‘multiple hurdle’ concept in foods. Several combined hurdles at mild levels have the potential to increase the microbiological safety and shelf-life of food (meat) while sensorial qualities are preserved (Leistner, 2000). Research on the use of natural antimicrobials in perishable foods, such as meat and poultry, has been focused on pathogen control; however, effects against spoilage microorganisms have been evaluated also. Natural antimicrobials include, among others, organic acids and their salts, nisin and other bacteriocins, various active compounds of microbial or animal origin (e.g. lysozyme, chitosan, natamycin, lactoferrin), and plant essential oils, phenolic and smoke
compounds (Roller, 2003). In addition, the use of antagonistic strains of LAB or other food-grade microbes as protective cultures (Holzapfel et al., 1995) and the use of edible coatings or packaging materials containing natural antimicrobials (Scannell et al., 2000a,b) are of increasing commercial interest for the bio-preservation of meat and poultry products.

Organic acids
Apart from their use for carcass meat and poultry decontamination, organic acids and their salts can be effective as spraying or dipping solutions prior to packaging of meat retail cuts or as additives in ground meat and poultry to prolong shelf-life during refrigerated storage (Ismail et al., 2001; Samelis and Sofos, 2003c). Ouattara et al. (1997a) evaluated the inhibitory effect of organic acids upon six common meat spoilage bacteria, \textit{B. thermosphacta, Carn. piscicola, Lact. curvatus, Lact. sakei, P. fluorescens} and \textit{S. liquefaciens}, in culture broth at 20 °C. Except for benzoic and sorbic acids, which were of low activity owing to their low solubility in broth, all other organic acids totally inhibited bacterial growth at concentrations ranging from 0.1 to 1% (wt/vol). Inhibition by acids on a weight basis was acetic > propionic > lactic > citric, while on a molar basis the order was reversed (Ouattara et al., 1997a). \textit{Lact. sakei} and \textit{Lact. curvatus} were the most resistant and \textit{B. thermosphacta} and \textit{Carn. piscicola} were the most sensitive bacteria to the organic acids, while \textit{P. fluorescens} and \textit{S. liquefaciens} had an intermediate acid sensitivity (Ouattara et al., 1997a). \textit{B. thermosphacta} was also more sensitive than \textit{P. fragi} to a 3% lactic acid solution applied on fresh pork lean and fat stored aerobically at 4 °C (Greer and Dilts, 1995). Notably, the above meat spoilage bacteria and some important cold-tolerant pathogens tested were more resistant to lactic acid when attached to lean than fat tissue (Greer and Dilts, 1995).

The lag phase of total aerobic, psychrotrophic and coliform populations was reduced and, as a result, little growth (<1.5 log cfu/g) occurred during a 10-day storage of vacuum packaged ground beef patties at 4 °C following treatment with 5% lactic or fumaric acids (Podolak et al., 1996). Likewise, growth of gas-forming \textit{H. alvei} strains was reduced in ground beef stored at 7 or 15 °C, and no gas was produced after 9–10 days at 15 °C, when the beef trim was previously treated with combinations of 82 °C water plus a 2% lactic acid wash (Kang et al., 2002). Treatment of chicken breast with 1% acetic acid followed by packaging in a 70% CO$_2$/30% N$_2$ modified atmosphere produced significant decreases in total aerobic, LAB, Enterobacteriaceae and pseudomonad counts during storage at 4 °C (Jimenez et al., 1999). Control of spoilage bacteria with acetic acid resulted in chicken breasts that smelt slightly acidic and pleasant after 21 days of storage, while untreated breasts had developed off-odors by that time (Jimenez et al., 1999).

Organic acid salts also exert antimicrobial effects in fresh meat and poultry, while they differ from the acids in having limited lowering effects on muscle pH (Shelef, 1994; Sofos and Busta, 1999). This is also true for the salts of
weak lipophilic acids, such as benzoic and sorbic (Sofos, 1989; Brul and Coote, 1999). Potassium sorbate is well known to control the growth of *Lactobacillus* spp. and other spoilage bacteria in beef meat. Also, Sofos (1986) showed that the inclusion of 0.26% potassium sorbate in the formulation of uncured chicken and turkey breast products delayed initiation and rate of microbial growth and gas production by *Cl. sporogenes*.

Sodium lactate (SL), which is commonly used in commercially processed meat products (see Section 9.5.1), has also been used to control spoilage microorganisms in fresh whole or ground beef, pork and poultry (Zeitoun and Debevere, 1992; O’Connor et al., 1993; Shelef, 1994; Maca et al., 1997a; Eckert et al., 1997). Addition of 3% SL in ground pork reduced growth of total aerobic bacteria and extended pork shelf-life by about 12 days compared with controls, while it enhanced its flavor (O’Connor et al., 1993). Similar results were obtained when 3–4% SL was added to ground beef stored at 4°C for 3 days aerobically (Eckert et al., 1997) or for up to 28 days in vacuum packs (Maca et al., 1997a). Mixing of 3% SL with 0.2% sodium propionate increased inhibition of bacterial growth, while it also increased juiciness, decreased lipid oxidation and improved the color of ground beef (Eckert et al., 1997; Maca et al., 1997a). The above mixture had similar positive effects on the microbiological, sensory and chemical characteristics of roast beef top rounds (Maca et al., 1997b). Overall, the antioxidant activity of SL offers an additional technological advantage to its ability to slow microbial growth in meat systems (Nnanna et al., 1994).

**Protective cultures – bacteriocins**

During the past two decades, there has been an increasing interest in the application of bacteriocinogenic (Bac+) LAB as protective cultures for the biopreservation of meat and meat products (Holzapfel et al., 1995; McMullen and Stiles, 1996; Hugas, 1998; Devlieghere et al., 2004; Laursen et al., 2005). Numerous LAB species and strains have shown promise of producing sufficient bacteriocin amounts *in situ* in fresh meat and poultry to inhibit meat-borne pathogens, mainly *Listeria monocytogenes* (Schillinger and Lucke, 1989; Hugas et al., 1998; Juven et al., 1998; Bredholt et al., 2001; Amezquita and Brashears, 2002). With regard to spoilage control and shelf-life extension, relatively fewer studies have been conducted (Leisner et al., 1995, 1996; Castellano et al., 2004), which have shown limitations for the *in situ* application of Bac+ LAB in fresh meat and poultry. To be successful in biopreservation, a Bac+ LAB strain must survive during storage at refrigeration temperatures, compete with the relatively high indigenous microbial loads of raw meat, actively inhibit pathogenic and spoilage bacteria, and not alter the sensory properties of meat except under temperature-abuse conditions. Unfortunately, there may be a negative sensory impact of LAB that grow actively on raw meat at refrigeration temperatures, such as *Lact. sakei* and *Carnobacterium*, selected strains of which, such as *Lact. sakei* Lb706 and *Carn. maltaromicus (piscicola)* LV17 and UAL26 are otherwise antilisterial bacteriocin producers
(Leisner et al., 1995). Indeed, all these strains caused off-odors during storage of fresh beef at 2 or 7 °C under air or vacuum, while carnobacteria strains LV17 and UAL26 also caused discoloration (Leisner et al., 1995). In contrast, Leuc. gelidum strain UAL187 was shown to be a suitable Bac+ strain to be used as a protective culture in fresh beef stored in vacuum and then packaged aerobically for retail sale because of its slower growth and milder spoilage potential in situ (Leisner et al., 1995). In a later study, Leisner et al. (1996) reported that strain UAL187 could significantly extend the shelf-life of refrigerated (2 °C) vacuum packaged beef by suppressing growth of sulfide-producing Lact. sakei strains, which otherwise spoiled the meat by formation of distinct sulfide odors and greening. The growth of meat spoilage Lact. sakei was also inhibited in a meat slurry in co-culture with the Bac+ Lact. casei strain CRL705, which did not change significantly the pH of the slurry during vacuum storage at 4 °C for 21 days (Castellano et al., 2004). In conclusion, a need remains to isolate or genetically modify Bac+ LAB strains with strong antagonistic activities such as their negative impact on sensory characteristics of raw meat would be minimal upon their use as protective cultures (Devlieghere et al., 2004).

An alternative to protective cultures is the direct use in fresh meat and poultry of commercial or crude preparations of well-characterized bacteriocins, such as nisin, pediocins, enterocins, lactacin and lactocin S. These bacteriocins are known to be effective against a wide range of meat spoilage and pathogenic bacteria (Delves-Broughton and Gasson, 1994; Cintas et al., 1998); however, their application is often limited by their low production yield and high cost, and, thus, low commercial availability. Nisin remains the only bacteriocin that is commercially available, and this is a great advantage that has facilitated nisin research with raw meat and poultry. As mentioned, nisin has shown promise as a carcass meat decontaminant, while it may also be added as a biopreservative to improve the microbial stability and safety of chill-stored fresh meat products. Additional advantages of nisin are its broad antimicrobial spectrum and bactericidal mode of action (Stiles and Hastings, 1991), and its induced activity against Gram-negative bacteria when combined with refrigeration (Boziaris and Adams, 2000), other meat decontaminants such as trisodium phosphate (de Melo et al., 1998) or chelators, such as EDTA (Cutter and Siragusa, 1995; Shefet et al., 1995; Helander and Mattila-Sandholm, 2000). Potential limitations may also occur, including its low solubility and activity at pH > 6.0, limited diffusion in solid matrices, irreversible binding on meat lipids, phospholipid emulsifiers or other compounds, enzymatic degradation (Henning et al., 1986; Stiles and Hastings, 1991; Ganzle et al., 1999; Rose et al., 1999), and an inducible bacterial resistance to nisin (Ming and Daeschel, 1993).

Nonetheless, a number of studies have shown good retention of antimicrobial effects of nisin against spoilage flora in fresh meat and poultry (Shefet et al., 1995; Cutter and Siragusa, 1997, 1998; Ariyapitipun et al., 1999; Nattress et al., 2001, Nattress and Baker, 2003). In particular, nisin spray treatments or
treatments with nisin in calcium alginate gels have shown effectiveness in inhibiting growth of *B. thermosphacta* in whole or ground beef (Cutter and Siragusa, 1996a,b, 1997, 1998). Also, fresh beef pieces dipped in nisin (200 IU/ml) had lower total psychrotrophic counts, psychrotrophic and mesophilic Enterobacteriaceae, *Pseudomonas* and *Lactobacillus* counts, during vacuum storage at 4 °C for up to 56 days compared with the non-dipped controls (Ariyapitipun et al., 1999). Bacterial inhibition was increased further and the shelf-life of beef was extended when nisin was combined in dipping solutions with 2% polylactic or lactic acid (Ariyapitipun et al., 1999). Likewise, *B. thermosphacta* was inhibited for 27 days in pork juice at 2 °C and for up to 6 weeks on vacuum-packaged fat and lean pork tissue at 2 °C following addition of 125 µg/ml and 130 µg/cm² of nisin, respectively (Nattress et al., 2001). Again, the antimicrobial effect of nisin against *B. thermosphacta* and *Carnobacterium* was enhanced when combined with lysozyme at a 1:3 ratio (Nattress et al., 2001; Nattress and Baker, 2003). Thus, an increased susceptibility of nisin-treated cells to acid, other bacteriocins and lysozyme, and vice versa, occur when antimicrobials are used in combination (Kalchayanand et al., 1992; Ariyapitipun et al., 1999; Chung and Hancock, 2000; Nattress et al., 2001; Nattress and Baker, 2003).

**Antimicrobials of microbial or animal origin**

Spoilage microorganisms in fresh meat and poultry may be controlled by antimicrobials of microbial or animal origin, other than LAB bacteriocins. One example discussed above with nisin is lysozyme, which together appear to demonstrate synergy against meat spoilage LAB, such as *Lact. curvatus*, in laboratory media (Chung and Hancock, 2000). More recent studies reported the ability of lysozyme (125 µg/ml) to inhibit *B. thermosphacta* in pork juice at 2 °C, its inability to inhibit *Carnobacterium* sp. even when at 1000 µg/ml, and its ability to inhibit both bacteria on fat and lean pork when combined with nisin at 260 µg/cm² (Nattress et al., 2001; Nattress and Baker, 2003).

Other potent antimicrobials to control spoilage of raw meats include reuterin produced by *Lactobacillus reuteri*, which has shown activity against *L. monocytogenes* and *E. coli* O157:H7 in meat decontamination experiments (El-Ziney et al., 1999) and diacetyl (Williams-Campbell and Jay, 2002). Diacetyl (up to 100 µg/g) could be used only in combination with CO₂ to inhibit spoilage microorganisms in fresh ground beef, as in the co-presence of CO₂ the red color of beef was maintained and beef browning was prevented (Williams-Campbell and Jay, 2002).

**Plant antimicrobials**

Spices and herbs, whole or ground, essential oils, naturally occurring phenolic compounds in plants, and smoke are all known for their antimicrobial and antioxidant properties and have potential for use to improve shelf-life and safety of perishable foods, including meat (Nychas et al., 2003; Holley and Patel, 2005). It is beyond the scope of this chapter to list the numerous
essential oils, plant phenolics and smoke extracts that have been tested for antimicrobial properties, the methodological issues associated with testing, and their active compounds, mode of action and bacterial resistance development. The existing knowledge on these issues is summarized in the above recent reviews; here, selected applications of plant antimicrobials to control fresh meat spoilage will be discussed.

The essential oils from clove, cinnamon, pimento and rosemary were the most active among others against common meat spoilage, Gram-negative (*P. fluorescens* and *S. liquefaciens*) or Gram-positive (*B. thermosphacta*, *Carn. piscicola*, *Lact. curvatus*, *Lact. sakei*) bacteria, when added to culture media (Ouattara *et al.*, 1997b). The inhibitory effect of the above oils at 1/100 dilution correlated well with the presence of eugenol and cinnamaldehyde (Ouattara *et al.*, 1997b). There have been relatively few studies on the action of essential oils in situ in real foods, particularly fresh meat, most of which have been focused on control of meat-borne pathogens (Holley and Patel, 2005). When 0.8% (v/w) oregano essential oil was added to beef fillets, an initial reduction of 2–3 log cfu/g of all major spoilage groups was noted, with LAB being the most sensitive and pseudomonads the most resistant bacteria (Tsigarida *et al.*, 2000). Following storage of oregano-treated beef at 5°C, pseudomonads dominated but at lower yield counts in air or VP/MAP packages sealed with high O₂ permeability film, while in VP/MAP of low O₂ permeability film pseudomonads and LAB were inhibited in favor of *B. thermosphacta* (Tsigarida *et al.*, 2000). Oregano essential oil at 0.5% or 1% also inhibited bacterial growth in minced meat stored at 5°C under air and two MAP conditions, 40%CO₂/30%N₂/30%O₂ and 100% CO₂, compared with the control samples without oregano and more under 100% CO₂ (Skandamis and Nychas, 2001). In general, oregano essential oil delayed glucose and lactate consumption in whole or minced fresh meat under all storage conditions, while it inhibited meat proteolysis under air, and acetate formation under MAP, thereby exerting a synergistic effect with MAP in delaying microbial meat spoilage (Skandamis and Nychas, 2001, 2002). Likewise, a commercial herbal extract, Protecta II, had a bacteriostatic effect against the natural spoilage flora of fresh chicken broilers treated at a concentration of 2% (Dickens *et al.*, 2000).

It should be stressed, however, that the use in fresh meat of essential oils and other spice or herbal extracts as natural preservatives is limited mainly because of flavor changes, since effective antimicrobial doses may exceed organoleptically acceptable levels. Also, the lipophilic nature of many of these plant antimicrobials hampers their practical use because of low solubility and diffusion and enhanced interaction (binding) with meat lipids, reducing their efficacy (Nychas *et al.*, 2003; Devlieghere *et al.*, 2004; Holley and Patel, 2005).

### 9.4.3 Irradiation

Irradiation is the best-known intervention strategy that can ensure safety and
extend the microbiological shelf-life of fresh meat and poultry (Lee et al., 1996; Ahn and Lee, 2004). However, irradiation may also result in off-flavor and/or odor described as ‘barbecued corn-like’ or ‘bloody sweet’ and discoloration of fresh meat and poultry, while it may accelerate lipid and vitamin oxidation (Formanek et al., 2003; Ahn and Lee, 2004; Brewer 2004). The reasons and conditions under which irradiation reduces meat quality characteristics, and the measures applied to prevent or to lessen such problems, are discussed in numerous relevant studies and reviews (Lakritz and Thayer, 1992; Lee et al., 1996; Formanek et al., 2003; Ahn and Lee, 2004; Brewer 2004; Lacroix et al., 2004). In this chapter, only the effects of irradiation against meat spoilage microorganisms will be discussed.

Irradiation increases the shelf-life of fresh meat and poultry by (i) inactivating a great part of the initial contaminating spoilage flora, (ii) extending the lag phase of surviving cells, and (iii) selecting for Gram-positive microorganisms during refrigerated storage of meat, even when packaged in air. The immediate killing effects of irradiation on microbial cells attached to the meat, and its residual effects due to cell injury, depend on the irradiation type, dose and temperature, as well as on the type and packaging of meat. These factors simultaneously affect the negative impact of irradiation on meat quality, which must be monitored in order to achieve the best possible antimicrobial effects with the mildest possible sensorial defects. Overall, the higher the irradiation dose and temperature, the greater the microbial inactivation, but also the stronger the adverse effects of irradiation on the sensorial qualities of meat (Hanis et al., 1989; Mahrour et al., 2003). Therefore, low-dose ionizing radiation is preferred for use to control spoilage and pathogenic microorganisms in fresh meat and poultry. Adverse effects are consistently stronger when irradiated meats are stored in packages with air (oxygen) than in VP, or MAP without air. For example, while 10–20% oxygen in the package headspace enhanced the antimicrobial effects of irradiation, it adversely affected the organoleptic qualities and, thus, reduced the shelf-life of MAP, irradiated fresh pork, as compared with pork in MAP with 0% oxygen (Lambert et al., 1992).

Low-dose (e.g. <10 kGy) ionizing radiation can be very effective, especially when combined with anaerobic packaging of fresh meat and poultry. Indeed, the bacteriostatic effects of VP or MAP on the spoilage flora during refrigerated storage of meat are enhanced by previous irradiation. One main reason is that LAB and other Gram-positive bacteria that predominate in VP or MAP are naturally more resistant to irradiation than Gram-negative bacteria, and thus, gain an additional competing advantage in irradiated meat (Hastings and Holzapfel, 1987; Grant and Patterson, 1991a; Lambert et al., 1992; Farkas and Andrassy, 1993; Lacroix et al., 2004). Especially the combined use of MAP with irradiation is of increasing interest in the meat industry (Zhao et al., 1996). One concern is that pathogens may grow and/or produce toxins in irradiated meat and poultry stored in MAP because of a lack of competing bacteria and an apparent delay in development of the usual spoilage
warning signals (Lee et al., 1996). However, a recent study by Thayer and Boyd (2000) reported that the reduction of normal flora by irradiation did not correlate with a faster multiplication of post-irradiation contamination with *L. monocytogenes* on ground turkey during storage at 7°C in air-permeable pouches or MAP, as compared with the non-irradiated samples.

Lee et al. (1996) reviewed the effects of irradiation on shelf-life, safety and sensory quality of fresh beef, pork and poultry packaged in air, vacuum or MAP, as well as the effects of irradiation on packaging materials. Since that comprehensive review, several studies on irradiation of fresh meat and poultry have been conducted and reported similar results. In general, beef is more sensitive than pork to red pigment discoloration caused by irradiation, while poultry is more extensively studied than red meat relative to irradiation processing for several reasons: (i) minced tissue, intact muscle or whole carcasses can be readily irradiated and studied; (ii) the meat ranges from lightly pigmented breast muscles to more heavily pigmented leg muscle for a range of color effects; (iii) chickens are readily obtained and handled on a laboratory scale; and (iv) improved methods of preserving eviscerated chicken carcasses would be of value in the distribution of poultry (Lee et al., 1996).

An additional reason is public concern of a high risk of *Salmonella* contamination in poultry, which can be readily reduced by irradiation (Lee et al., 1996; Zhao et al., 1996; Mahrour et al., 2003).

Ionizing radiation doses may range from 0.5 to 10 kGy, with doses ≤ 5 kGy being most commonly applied. In particular, gamma irradiation doses of 1.5–3.0 kGy may have good antimicrobial effects against various types of meat spoilage bacteria without causing significant reductions in meat quality (Grant and Patterson, 1991b). For example, the levels of aerobic and facultative mesophiles increased during 4 weeks of storage at 5°C from approximately 6.5 and 4.0 logs to >8 logs in samples of vacuum-canned mechanically deboned chicken meat (MDCM) irradiated at 0 and 1.5 kGy, respectively (Thayer et al., 1995). However, the respective populations in MDCM samples irradiated at 3 kGy increased from approximately 3.0 to 5.2 logs. Thus, initial reductions of spoilage flora in MDCM were >3 logs following irradiation at 3 kGy, which is the maximum level approved by the FDA for poultry. Irradiation of raw ground turkey decreased the total bacterial plate counts (TPC) and increased the lag phase and consequently the length of time to spoilage (Thayer and Boyd, 2000). More specifically, a dose of 2.5 kGy extended the time for the TPC to reach 10^7 cfu/g from 4 to 19 days of storage at 5°C compared with that for non-irradiated turkey in air permeable packs (Thayer and Boyd, 2000). While under a radiation dose of 2.5 kGy, TPC remained lower than 7 and 5 log cfu/g after 28 days of storage at 5°C in packages containing 25% and 50% CO₂, respectively (Thayer and Boyd, 2000). Similar results were also found by Abu-Tarboush et al. (1997), who reported that a dose of 2.5 kGy was adequate to extend the shelf-life of aerobically stored chicken at 4°C by 12 days.
As mentioned, in addition to reducing TPC, low-dose ionizing radiation may have strong selective effects on the spoilage association of irradiated fresh meat and poultry. Indeed, numerous studies have shown that irradiation favors the growth of LAB, *B. thermosphacta* and other Gram-positive bacteria in fresh meats, especially under VP or MAP (Lambert *et al.*, 1992). For example, the microflora of irradiated MAP pork was almost exclusively composed of LAB, predominantly *Lact. sakei/curvatus* (Grant and Patterson, 1991a,b; Lambert *et al.*, 1992). Conversely, the typical Gram-negative psychrotrophic flora of fresh pork declined by 2 log cycles and most Enterobacteriaceae were inactivated following irradiation to 1 kGy only (Lambert *et al.*, 1992). Likewise, a shift in the microbial ecology of MDCM during 5°C storage from Gram-negative rods in non-irradiated samples to Gram-positive streptococci in samples irradiated to 3 kGy was reported (Thayer *et al.*, 1995). A dose of 2.5 kGy was enough to destroy coliforms and enteric pathogens such as *Salmonella, Campylobacter* and *Yersinia* in fresh meat (Abu-Tarbush *et al.*, 1997). Also, radiation doses of 1.0–2.5 kGy were sufficient to eliminate *P. aeruginosa* from broiler carcasses, while higher doses of 2.5–5.0 kGy were required to eliminate *S. marcescens* (Hannis *et al.*, 1989). Finally, *Sh. putrefaciens*, a main SSO in aerobically stored or VP fresh meat, was shown to be very sensitive to irradiation, with an average D_{10} value of 0.11 kGy on MDCM irradiated at 5°C (Thayer and Boyd, 1996). The presence or absence of air (oxygen) and the type of meat (hamburger, ground beef round, ground pork and ground turkey breast) did not significantly alter resistance of *Sh. putrefaciens* to gamma irradiation (D_{10} value = 0.18 kGy). Based on these data, Thayer and Boyd (1996) suggested that a radiation dose of 1.5 kGy, which is the minimum dose approved for poultry in the USA, should eliminate *Sh. putrefaciens* from meats.

Meat spoilage yeasts are generally more resistant to irradiation than bacteria and, thus, are expected to predominate in fresh meat treated at high irradiation doses (Samelis and Sofos, 2003b). Indeed, Johannsen *et al.* (1984) reported enhanced growth of yeasts on minced beef irradiated at 2.5 kGy as due to bacterial inhibition by irradiation and considered yeasts as possible spoilage flora; however, no sensory evaluation data were presented to support this possibility. Also, while yeasts were not detected in unirradiated fresh poultry, *Y. lipolytica, C. zeylanoides* and *T. beigelii* were isolated from the corresponding irradiated samples, with the former species being present in high numbers post- irradiation owing to its apparent higher resistance (Sinigaglia *et al.*, 1994). More recently, Abu-Tarboush *et al.* (1997) reported that yeasts of the genera *Candida* and *Saccharomyces* started to grow after 12 days of storage at 4°C in chicken meat irradiated at 5.0, 7.5 or 10 kGy, but not in samples treated at doses <5 kGy. Thus, despite their higher resistance to irradiation, yeasts may not predominate in fresh meats irradiated at doses that allow bacterial survivors to recover and establish competitive growth.
9.4.4 High hydrostatic pressure

High hydrostatic pressure (HHP) is a new mild physical preservation method that has a high potential for use in meat processing (Hugas et al., 2002); therefore, in-plant commercial applications of HHP are increasing despite the still high cost of this emerging technology. HHP is very attractive because it is a non-thermal process that can inactivate both spoilage and pathogenic microorganisms in meat without causing major adverse effects on meat quality (Hugas et al., 2002). Compared with irradiation, the effects of HHP at usual pressure values in the range of 350–600 MPa on the sensorial qualities of meat are restricted to mild color changes (e.g. loss of redness) and changes in the activity of proteolytic muscle enzymes, such as cathepsins B and H and aminopeptidase B (Homma et al., 1994; Jung et al., 2003).

Meat-borne microorganisms are all sensitive to HHP; however, similarly to irradiation, Gram-negative bacteria are generally more sensitive than Gram-positive bacteria. For example, the growth of *P. fluorescens*, a common meat spoilage agent, in culture broth was reduced by 5 log cycles following pressurization at 200 MPa for 10 min at 5°C, while under the same time/temperature conditions at 400 MPa the organism was inactivated (Lopez-Caballero et al., 2002a). Also, when HHP at 300 MPa for 15 min at 5, 20, 35 and 50°C was applied on ground pork patties, pseudomonads and enterobacteria were inactivated at higher levels than LAB and staphylococci, with a significant shelf-life extension being apparent (Lopez-Caballero et al., 2002b). Likewise, when HHP values of 408, 616 and 888 MPa were applied for 10 min at ≤28 °C on raw ground chicken, the estimated microbial spoilage times (e.g. >7 log cfu/g) were extended to 27, 70 and >98 days, respectively, at 4°C (O’Brien and Marshall, 1996).

The types of bacteria that survived best in the HHP-treated chicken samples were the facultatively anaerobic psychrotrophic bacteria *Carn. divergens* and *S. liquefaciens* (O’Brien and Marshall, 1996). In a more recent study (Yuste et al., 1999), combinations of HHP (350, 400, 450 and 500 MPa), time (5, 10, 15 and 30 min) and temperature (2, 10 and 20°C) were applied on vacuum-packaged mechanically recovered poultry meat (MRPM), which was then stored at 2°C for 2 months. In most treatments, pressurization at 2°C gave the best results against total mesophilic counts, which remained as low as 2.3 log cfu/g after 60 days of 2°C storage in samples HHP-treated at 500 MPa for 30 min at 2°C (Yuste et al., 1999). Thus, in all the above cases, the pressurization temperature appeared to be the most critical factor influencing the HHP effectiveness, which may also be influenced by the type of meat product. Overall, microbial inactivation by HHP on meat products is greater at higher pressurization temperatures (Lopez-Caballero et al., 2002b), although the opposite occurred in MRPM (Yuste et al., 1999). Nonetheless, Yuste et al. (2002a) reported that HHP processing (350 and 450 MPa for 5 or 15 min) at subzero (–20°C) temperatures did not extend shelf-life of MRPM, as HHP did at temperatures above 0°C (Yuste et al., 1999).
9.4.5 Combined effects
Based on the hurdle technology concept (Leistner, 2000), significant progress has already been made by using classical preservation technologies in combination, such as refrigeration combined with heating, acids, curing salts or anaerobic packaging, in the meat industry. In recent years, combinations of newly applied technologies have received the most attention. As such, natural antimicrobials in combination with irradiation have shown promise in extending the shelf-life of fresh meat and poultry, an issue recently reviewed by Ouattara and Mafu (2003). For example, combinations of irradiation (2 kGy), pH reduction to 5.6 by ascorbic acid and water activity reduction to 0.96 with SL extended the shelf-life of vacuum packaged minced meat stored at 2°C by a factor of three (Farkas et al., 1996). Similarly, Bhide et al. (2001) showed that treatment of sheep/goat meat with propionic acid (1%), lactic acid (2%) and acetic acid (2%) pre-sensitized total spoilage flora and Bacillus cereus to a following irradiation (1–3 kGy) step, as their reductions were greater than in the irradiated meat without acids. More recently, Mahrour et al. (2003) reported that marination of chicken legs in natural plant extracts prior to being irradiated at 0, 3 or 5 kGy had an additive effect with irradiation in reducing bacterial growth and controlling proliferation of TBC and Salmonella during storage at 4°C in air packages.

Another promising combination of hurdles to extend the shelf-life of fresh meat is HHP combined with bacteriocins against both vegetative bacterial cells (Kalchayanand et al., 1994, 1998) and spores (Kalchayanand et al., 2003). For example, the inclusion of pediocin AcH (3000 AU/ml) in broth suspensions of meat spoilage bacteria Lact. sakei, Leuc. mesenteroides, S. liquefaciens and P. fluorescens contributed to achieve an 8-log cycle viability loss when pressurized at 345 MPa at 50°C, but this loss was not achieved in the absence of the bacteriocin (Kalchayanand et al., 1998). Likewise, the presence of nisin in a meat model system pressurized at 400 MPa for 10 min at 17°C significantly reduced the numbers of slime-producing meat LAB and E. coli post-pressurization and inhibited the growth of HHP-nisin-injured cells for up to 61 days at 4°C (Garriga et al., 2002). Also, an HHP treatment of MRPM at 450 MPa at 20°C in the presence of 200 ppm nisin resulted in higher than 5 and 7 log reductions of total mesophilic and psychrotrophic bacteria, respectively (Yuste et al., 2002b). While a combination of HHP (345 MPa, 60°C, 5 min) with a nisin–pediocin mix (5000 AU/g) extended the shelf-life of roast beef for 7 days at 25°C or 84 days at 4°C by controlling growth of Cl. laramie spores (Kalchayanand et al., 2003).

9.4.6 Active packaging
As mentioned, new packaging technologies, such as MAP, have greatly contributed in prolonging the shelf-life of fresh meat and poultry by exposing the product and its microbial flora to an altered composition of gases, enriched in CO2 and reduced in oxygen. Active packaging represents a novel technology
aiming at enhancing MAP benefits while reducing safety concerns and potential risks from MAP, such as the possibility that *L. monocytogenes* and other facultatively anaerobic pathogens may have a longer time to grow before detectable spoilage of the meat occurs (Genigeorgis, 1985; Garcia de Fernado *et al.*, 1995; Marth, 1998). Thus, while in typical MAP a pre-selected atmosphere is introduced at sealing and thereafter in-package conditions change randomly in response to the passive properties of the packaging material, and the activity of meat and its flora, in active packaging conditions continue to change dynamically inside the pack. The most applied active packaging system is the oxygen scavenger, removing the residual oxygen from the headspace and/or absorbing oxygen diffusing through the packaging material during storage (Vermeiren *et al.*, 1999; Devlieghere *et al.*, 2004). A new, more advanced type of active packaging is the incorporation of natural antimicrobials, such as organic acids and their salts, bacteriocins, essential oils, chitosan and triclosan, to control microbial surface contamination of foods (Padgett *et al.*, 1998; Scannell *et al.*, 2000a; Quintavalla and Vicini, 2002; Vermeiren *et al.*, 2002; Mauriello *et al.*, 2004; Holley and Patel, 2005). So far, most of these investigations have been done in culture media, while activity of the antimicrobial films may be much reduced when in contact with a food surface. Especially with fresh meat and poultry products, available data are still limited and somewhat conflicting. For example, a low-density polyethylene film containing 1g/kg Triclosan did not effectively reduce spoilage bacteria and *L. monocytogenes* on vacuum packaged chicken breasts stored at 7°C, despite the fact that it had a strong antimicrobial effect in culture media (Vermeiren *et al.*, 2002). Conversely, these bacteria were reduced by approximately 1 log cfu/g within 24 h in pork steak and ground beef packaged in a polyethylene film coated with a bacteriocin from *Lact. curvatus* and stored at 4°C (Mauriello *et al.*, 2004). Also, *B. thermosphacta* populations were by approximately 1.5 to 2.5 logs lower during 4°C storage of beef carcass tissue sections vacuum packaged in a polyethylene-based plastic film with incorporated nisin, as compared with the control packs (Siragusa *et al.*, 1999).

### 9.5 Current and emerging technologies to control spoilage of processed meat and poultry products

Plant operation under GMP, GHP and GSP and effective HACCP programs is also necessary for the production of processed meat and poultry products of good quality and safety. The microbiological stability of most of these products is primarily based on cooking treatments following stuffing in natural or artificial casings, while, as indicated, curing and, for many product types, smoking and drying further contribute to extend shelf-life. However, research and experience have shown that GMP and strict hygienic measures during peeling or slicing cannot guarantee complete avoidance of post-process
contamination with certain spoilage and pathogenic bacteria, such as LAB (Holley, 1997a,b; Samelis et al., 2000a) and L. monocytogenes (Samelis and Metaxopoulos, 1999; Centers for Disease Control and Prevention, 1999, 2002). Thus, additional hurdles are needed to be introduced at packaging, and act at this stage or in-package to control spoilage of processed meat and poultry products. Applications in meat products of hurdles that are the most investigated and promising are presented below.

9.5.1 Natural antimicrobials

Organic acids

The addition of organic acids or their salts as preservatives in high-pH processed meat and poultry products has been extensively investigated and numerous commercial applications are in place (FSIS, 2000; Samelis and Sofos, 2003c). Recent research has been focused on the use of sodium or potassium lactate, sodium acetate, sodium diacetate and potassium sorbate or benzoate to inhibit L. monocytogenes during refrigerated storage of vacuum-packaged RTE meats (Blom et al., 1997; Mbandi and Shelef, 2002; Stekelenburg, 2003; Samelis and Sofos, 2003c). Post-processing contamination with L. monocytogenes can be controlled by either the inclusion of organic acid salts in cured meat formulations prior to cooking (Samelis et al., 2002b), or by dipping or spraying the products with acid or salt solutions prior to vacuum packaging (Samelis et al., 2001). Because meat spoilage bacteria are also inhibited by organic acids or salts (Ouattara et al., 1997a; Peirson et al., 2003b), their use as antilisterial agents in RTE processed meat products can delay growth of natural flora and, thus, extend shelf-life in addition to increasing safety. Indeed, significant inhibitory effects against both L. monocytogenes and total spoilage bacteria were observed during refrigerated (4°C, 120 days) storage of laboratory-made sliced pork bologna dipped in 3 or 5% lactic or acetic prior to vacuum packaging (Samelis et al., 2001). The growth of the background flora was also delayed in vacuum-packaged beef bologna containing 2.5% SL and/or 0.2% SDA (Mbandi and Shelef, 2002), and in pork frankfurters containing 1.8% SL plus 0.25% SA, SDA or GDL (Samelis et al., 2002b), with their combinations showing the most effect at 4–5°C. A more recent study showed that the combination of 1.8% SL with 0.25% SDA was the treatment that controlled growth of LAB in commercially manufactured pork bologna more effectively at 4°C, while at 10°C LAB were inhibited more effectively in combination with 0.125% GDL (Barmpalia et al., 2005). Also, the addition of SL (2% in pure form) suppressed growth of inoculated B. thermosphacta by 4 log cfu/g in an acidified (pH 5.0) model chicken sausage stored at 22°C for 14 days (Lemay et al., 2002b).

Meat spoilage LAB exceeded 7 to 8 log cfu/g in commercial Greek frankfurters formulated without antimicrobials or with 1.8% SL, or 1.8% SL plus 0.25% SA, and stored for up to 90 days at 4 or 12°C in vacuum packages
All other microbial types remained <5 log cfu/g during storage. However, the growth of LAB during storage at 4 °C was significantly less and more delayed in frankfurters with SL than SL + SA, while it was the greatest in control samples at both storage temperatures. Frankfurters with SL + SA had the highest pH after 90 days of storage at 12 °C. Also, in-package gas and slime formation and off-odors at opening decreased in the order SL + SA (good at 90 days) < SL (acceptable at 75–90 days) < control (spoiled at 30 days) frankfurters at 12 °C. Decreases in pH, sensory scores and LAB flora shifts at spoilage followed similar patterns in frankfurters at 4 °C; however, terminal pH values were higher and sensory defects were milder during storage (Samelis and Kakouri, 2004).

It appears, therefore, that the inhibition of most important aciduric meat spoilage LAB, *Lact. sakei* and *Lact. curvatus*, in processed products cannot be complete owing to their natural resistance to lactate and acetate. Indeed, Stekelenburg and Kant-Muermans (2001) reported that inoculated *Lact. curvatus* grew well in all different hams with organic acid salts, with growth being delayed for 1–2 weeks in hams with 2.5 to 3.3% SL. However, after 3–5 weeks (e.g. 21–35 days) the *Lact. curvatus* populations had exceeded the cut-off level of 7 log cfu/g, without adverse effects on sensorial quality of the hams (Stekelenburg and Kant-Muermans, 2001). More recently, Stekelenburg (2003) showed that the addition in frankfurters of 2–3% of a solution containing a mixture of 56% potassium lactate and 4% SDA inhibited the development of *Lact. sakei* inoculated on vacuum packaged frankfurters stored at 4 °C. *Lact. sakei* was mainly inhibited by the addition of lactate and its water activity lowering effect, resulting in a shelf-life extension of 75–125%. In contrast, the growth of *Lact. sakei* and *Lact. curvatus* was not inhibited in frankfurters formulated with 0.1% SDA or in cooked ham with 0.1–0.2% SDA, respectively (Stekelenburg and Kant-Muermans 2001; Stekelenburg, 2003). However, 0.2% SDA inhibited growth, while 0.1% SDA consistently delayed growth, of *Cl. laramie* in cooked uncured turkey breast (Kalinowski and Tompkin, 1999). SDA inhibition was strain-dependent, while a concern was that some psychrotrophic clostridia utilized lactate added as SL to the product (Kalinowski and Tompkin, 1999).

Given that the sensitivity of meat spoilage bacteria to organic acids and their salts is primarily species-dependent (Houtsma *et al*., 1993; Ouattara *et al*., 1997a), the type of predominant LAB and the associated spoilage defects may be affected from their use in RTE meat products. For example, alkalitrophic LAB, such as *Carnobacterium* and *Aerococcus*, and certain *Weissella* and *Leuconostoc* spp. (e.g. *W. hellenica, Leuc. carnosum*) are acid-sensitive, particularly to acetate but, as indicated above, *Lact. sakei, Lact. curvatus* as well as *Leuc. mesenteroides, W. viridescens* and other LAB are not (Collins *et al*., 1987, 1993; Samelis *et al*., 1994a; Ouattara *et al*., 1997a; Peirson *et al*., 2003b). Therefore, addition of SA or SDA along with SL may be among the main processing factors resulting in low selection of carnobacteria, *Leuc. carnosum* and *W. hellenica* in vacuum-packaged cooked meat products during...
refrigerated storage, although these LAB may dominate in whole pork and poultry or in sausage batters at a pre-cooking stage (Samelis et al., 1998a, 2000a,b). For example, in commercially manufactured Greek taverna sausages where Lact. sakei/curvatus strains comprised more than 90% of the spoilage flora in vacuum packs, acetate levels of 133 mg/100 g were detected in the sausages before storage, indicating addition of ca. 0.1% SA in the batter (Samelis and Georgiadou, 2000). In agreement, Samelis and Kakouri (2004) observed a shift of the LAB flora from gas- and slime-producing species, mainly Leuconostoc spp., in terminally spoiled vacuum-packaged frankfurters formulated without antimicrobials to Lact. sakei/curvatus strains in frankfurters formulated with 1.8% SL and 0.25% SA.

In conclusion, SL (2–3%) is necessary to be added in processed meat products, as it is effective in inhibiting non-LAB bacteria and more effective than SA or SDA in retarding growth of aciduric LAB. SL acts primarily by reducing \( a_w \) in meat muscles or emulsions (Houtsma et al., 1993), while its activity is significantly enhanced in the presence of SA or SDA. When combined with 0.25% SA or preferably 0.1–0.2% SDA, SL may extend shelf-life by favoring selection of acetate-tolerant, non-gas-forming LAB species with a mild spoilage potential in addition to moderately suppressing LAB growth during extended storage. Levels of SDA higher than 0.2% may have a negative influence on sensory quality. Combinations of organic acid salts seem to be more important for the shelf-life of processed RTE meat and poultry products under temperature abuse conditions than refrigeration.

**Protective cultures – bacteriocins**

In recent years, the application of protective cultures in processed meat and poultry products has been a great research issue due to the need to control risks associated with post-processing contamination and growth of L. monocytogenes in RTE deli meats (Devlieghere et al., 2004). Therefore, numerous studies have proposed a great number of strains from various LAB species as potent protective cultures for cured cooked meats. Among them, Bac+ strains of commonest meat-borne LAB, such as Lact. sakei and Leuc. carnosum producing sakacins and leucocins, respectively (Hugas et al., 1998; Budde et al., 2003), and pediocin-producing Pediococcus sp. (Degnan et al., 1992), seem to be the most suitable for providing in situ inhibition of L. monocytogenes. In addition, meat-borne Bac– strains of Lact. sakei (Bredholt et al., 2001; Vermeiren et al., 2004), Lact. casei/paracasei (Amezquita and Brashears, 2002) and Lact. alimentarius present in the commercial biopreservative FloraCarn L-2 (Andersen, 1995; Kotzekidou and Bloukas, 1998) have shown good in situ competitiveness against L. monocytogenes and Salmonella. With regard to the potential of protective cultures to control spoilage and extend shelf-life of vacuum packaged RTE meat products, published studies are fewer and their results are rather conflicting. For example, Kotzekidou and Bloukas (1996, 1998) reported good inhibition of spoilage flora, and as a result, a 7-day and 19-day shelf-life extension of refrigerated
vacuum packaged Greek cooked ham and frankfurters preserved with *Lact. alimentarius* (FloraCarn L2), respectively. In contrast, this commercial biopreservative could not prevent spoilage associated with well-adapted, very competitive ropy-slime producing *Lact. sakei* in Finnish frankfurters (Björkroth and Korkeala, 1997b). However, others reported that competitive strains of *Lact. sakei* with a mild spoilage potential are suitable for use as protective cultures to inhibit *Leuc. mesenteroides* and *B. thermosphacta* and, thus, to delay spoilage in addition to controlling *L. monocytogenes* in Belgian cooked meat products (Vermeiren et al., 2004). Earlier, Yang and Ray (1994) demonstrated the need to control the Bac+ *Leuc. carnosum* and *Leuc. mesenteroides* strains that caused spoilage of processed meats in the USA by early accumulation of gas and purge in vacuum packages. This reflected that the presence of Bac+ LAB in processed meats is not always an advantage. To control this type of spoilage, addition in the product at packaging of a low level of nisin-producing *Lactococcus* strains was recommended (Yang and Ray, 1994).

As an alternative to protective cultures, addition of bacteriocin preparations in RTE processed meat products has been evaluated as a means to control spoilage bacteria, with nisin having received the most attention (Yang and Ray, 1994; Scannell et al., 1997; Davies et al., 1999; Gill and Holley, 2000, 2003) for the same reasons discussed with fresh meat. Specifically, Davies et al. (1999) reported that concentrations of nisin (Nisaplin) between 6.25 and 25 µg/g inhibited growth of LAB in vacuum-packaged bologna-type sausage formulations for 4 to 5 weeks at 8°C, with higher inhibitory bacteriocin concentrations required in formulations with an increased fat content. As with fresh meats, a nisin–lysozyme (3:1) combination at 500 mg/kg further combined with 500 mg/kg EDTA inhibited growth of inoculated *B. thermosphacta, Lact. curvatus* and *Leuc. mesenteroides* for 4, 3 and 2 weeks, respectively, on vacuum packaged ham and bologna stored at 8°C (Gill and Holley, 2000). However, more recent investigations by Gill and Holley (2003) showed that such interactions between nisin, lysozyme and EDTA may indeed result in increased synergistic inhibitory effects against meat spoilage and pathogenic bacteria under conditions of nutrient limitation on meat surfaces, but not on growing cells in nutrient broth. Thus, new protocols for reliable screening of natural antimicrobials is required when dealing with growing cells in food (cured meat) systems (Gill and Holley, 2003). Nevertheless, combinations of nisin with organic acids or salts seem to be more effective than those with lysozyme for the biopreservation of processed meat products. Scannell et al. (1997) reported that the antimicrobial activity of organic acid salts increased upon addition of nisin in fresh sausage formulations, while similar results were obtained by combining lacticin 3147, another lantibiotic similar to nisin produced by *Lc. lactis*, with 2% SL (Scannell et al., 2000b). Addition of nisin (500 IU/g) or lacticin (2500 AU/g) with 2% SL resulted in higher inhibition of total aerobic counts, *Salmonella* and *L. innocua* than sodium metabisulfite and thus, they may function as alternatives to this
traditionally used chemical preservative in fresh British sausage. Also, addition of nisin (5000 IU/ml) in 3 or 5% acetic acid or SDA or 3% potassium benzoate, did not permit growth of *L. monocytogenes* and total aerobic flora for at least 75 days of storage of sliced vacuum packaged bologna at 4°C (Samelis *et al.*, 2005).

**Antimicrobials of microbial or animal origin**

In addition to lysozyme discussed above in combination with bacteriocins, another promising natural antimicrobial for the preservation of processed meats is chitosan (Roller, 2003). Chitosan is commercially made by alkaline deacetylation of chitin, which is an abundant constituent of crustacean shells and fungi (Roller, 2003). Chitosan (0.6%) combined with low levels (170 ppm) of sulfite retarded growth of spoilage microorganisms in fresh British sausage more effectively (3–4 log cfu/g) than high levels (340 ppm) of sulfite alone, and thus, the product remained acceptable for up to 24 days at 4°C (Roller *et al.*, 2002). It should be noted, however, that moderate concentrations of NaCl present in cured meat products may neutralize the antimicrobial character of chitosan, lysozyme and many LAB bacteriocins such as sakacin K (Leroy and De Vuyst, 1999; Devlieghere *et al.*, 2004). Therefore, the actual effectiveness of these antimicrobials in processed meat systems may be significantly reduced by the curing salts, in addition to other factors (e.g. low diffusion, binding on lipids, enzymatic degradation, bacterial resistance development) hampering their application.

**Plant antimicrobials**

Whole or ground spices and herbs and smoke from wood are traditionally used in processed meat and poultry products to provide the desired flavor, aroma and color characteristics, as well as for their antioxidant and antimicrobial properties. To increase convenience for their use and to exclude potential carcinogenic compounds formed during hot smoking, patented mixtures of spices/herbs, herbal extracts or essential oils and liquid smoke are commercially available and of increasing use in the meat industry. Certain spice constituents, such as manganese, enhance growth and competition of LAB in fermented meat products. For all these reasons, studies on the inclusion of plant antimicrobials in cured meat formulations to inhibit spoilage bacteria are limited (see review by Holley and Patel, 2005), as they are included anyway. One early study has shown that addition of 4% garlic in an Egyptian fresh sausage exerted a bacteriostatic effect on the natural flora, specifically on *Lact. plantarum*. More recently, the essential oil of mustard was shown to cause significant reductions in aerobic mesophilic bacteria and LAB when added in a cooked and acidified model sausage made from mechanically deboned chicken meat (Lemay *et al.*, 2002b). Nevertheless, the main current interest is to treat RTE meats with plant essential oil dips or sprays before packaging to control *L. monocytogenes* during storage (Singh *et al.*, 2003).
9.5.2 Irradiation

Compared with fresh meat and poultry, there has been less scientific and commercial interest in applying irradiation to processed meat and poultry products. This is mainly because the shelf-life of these products can be extended by other more consumer-friendly preservation methods, such as curing, heat pasteurization, fermentation, addition of organic acid salts or other natural antimicrobials and, more recently, high-pressure technologies. Following, however, the recent deadly listeriosis outbreaks in the USA due to consumption of RTE meats, such as frankfurters and deli turkey meat (Centers for Disease Control and Prevention, 1999, 2002), the interest in irradiation has been renewed to eliminate *L. monocytogenes* (Murano et al., 1999; Lucore et al., 2000; Sommers et al., 2004). Similarly to fresh meat, ionizing radiation at usual doses of 1–3 kGy can be very effective in eliminating microorganisms from processed products, but may also affect their quality factors, including color, lipid oxidation and generation of volatile sulfur compounds and hydrocarbons (Fan et al., 2004; Sommers et al., 2004; Zhu et al., 2004).

From a microbiological point of view, irradiation is not expected to alter the spoilage association of VP or MAP processed meats since it may select for LAB, which would become dominant anyway. However, it may extend the product shelf-life by eliminating initial LAB contaminants or extending their lag phase when applied singly or in combination with organic acid salts in meat formulations. For example, Kuo and Chen (2004) reported that irradiation at 5 kGy alone, or at 3 kGy in combination with 2% sodium lactate completely inhibited LAB growth in vacuum-packaged Chinese sausages stored at 25°C. The only apparent micro-ecological change that irradiation may deliver in fresh or cooked meats is an increased contribution at spoilage of yeasts due to their radiation resistance. Indeed, yeasts were significant in the spoilage of irradiated (2–5 kGy) frankfurters (Drake et al., 1959). *Candida zeylanoides* and *Tr. beigelii* were the most resistant yeasts in irradiated (3 kGy) British fresh sausage, whereas *Debaryomyces hansenii* was reduced by 1.5 kGy (McCarthy and Damoglou, 1993). The greater sensitivity of *D. hansenii* to irradiation, as compared with *Candida* spp., was also observed in frankfurters (Drake et al., 1959). Notably, *C. zeylanoides* is the yeast that could better sustain the combined effects of irradiation (3 kGy) and sulfite in British fresh sausages stored at 4°C for 14 days (McCarthy and Damoglou, 1993). McCarthy and Damoglou (1996) reported that *D* subscripts 10 values of the above yeasts at higher (>2 kGy) irradiation doses increase in sausage as compared to phosphate-buffered saline. This indicates a protective effect of meat proteins and polysaccharides to irradiated yeast cells (McCarthy and Damoglou, 1996). Recently, an irradiation dose of 5.7 kGy is reported to inhibit yeasts in addition to the complete inhibition of *L. monocytogenes* in a pre-prepared meat meal (Foley et al., 2001).

9.5.3 High hydrostatic pressure

There is an increasing interest in HHP as a powerful tool to control post-
processing contamination of processed meat and poultry products with both spoilage and pathogenic microorganisms (Hugas et al., 2002). HHP has the ability to inactivate bacteria in the same way as heat, but with generally less impact on cured meat quality. For example, when vacuum packaged cooked sausages were pressurized at 500 MPa for 5 or 15 min at a mild temperature of 65 °C, immediate reductions in total bacteria and LAB were about 4 log cfu/g (Yuste et al., 2000). HHP inactivated psychrotrophs and enterobacteria similarly to pasteurization at 80–85 °C for 40 min, and as a result, insignificant or no bacterial growth occurred in the HHP-treated sausages during storage at 2 or 8 °C for 18 weeks (Yuste et al., 2000). Likewise, HHP at 500 MPa for 10 min significantly affected initial counts and reduced growth of total and psychrophilic bacteria, LAB and enterococci in vacuum-packaged cooked pork ham and smoked pork loin during refrigerated storage for 4 weeks (Karłowski et al., 2002). HHP at 600 MPa for 6 min at 31 °C inhibited Enterobacteriaceae and yeasts and delayed growth of LAB in sliced cooked ham, dry cured ham, and marinated beef loin during storage at 4 °C for 120 days; in addition, safety risks associated with *Salmonella* and *L. monocytogenes* were reduced (Garriga et al., 2004). Thus, as a general conclusion, an HHP treatment of 600 MPa for 6–10 min at 30 °C can reduce bacteria by 5–6 logs and provide an equivalent microbial inactivation effect to post-process heat pasteurization of several different meat products (Hugas et al., 2002; Karłowski et al., 2002; Garriga et al., 2004). Physicochemical characteristics of sensitive meat products, such as sliced cooked ham, are not significantly affected by the above HHP treatment after 8 weeks of storage, while increases in the color lightness may be observed in smoked products, such as pork loin (Karłowski et al., 2002).

Although few studies are yet available, the resistance of meat spoilage yeasts to HHP seems to be less than their resistance to irradiation. For example, an HHP treatment of 300 MPa at 25 °C or 250 MPa at 45 °C, inactivated *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* in meat spaghetti sauce, with inactivation being enhanced by mild heat treatment and increased acidity (Pandya et al., 1995). Another recent study showed that *Saccharomyces cerevisiae* was more sensitive than Gram-positive bacteria *Ent. faecalis* and *Staph. aureus* in Marengo, a low-acid stew pork product, treated at 400 MPa for 30 min at 20 and 50 °C (Moerman, 2005). In general, HHP processing of neutral-pH meat products cannot rely on pressure alone as a pasteurization/sterilization process, but another physical agent like heat is needed (Moerman, 2005; Devlieghere et al., 2004).

### 9.5.4 Active packaging

Active packaging of sliced cooked meat products in films coated with natural antimicrobials is of increasing interest. The direct contact of the active compounds with the cured meat surface, and/or their partial migration from the film onto the meat, could inactivate or inhibit growth of post-processing...
contaminating *L. monocytogenes* and spoilage bacteria. Results of some recent studies are promising. Use of films with immobilized bacteriocins, nisin (Nisaplin) and lacticin 3147, reduced LAB growth and extended shelf-life of sliced cooked ham stored in MAP at 4°C, while it also reduced levels of *L. innocua* and *Staph. aureus* by ≥ 2.0 and 2.8 logs, respectively (Scannell *et al.*, 2000a). In another study (Ouattara *et al.*, 2000), antimicrobial films were prepared by incorporating acetic or propionic acid into a chitosan matrix, with or without addition of lauric acid or cinnamaldehyde, and used to pack cooked bologna, ham and pastrami. While naturally occurring LAB and surface-inoculated *Lact. sakei* were not affected by the above antimicrobial films, the growth of enterobacteria and surface-inoculated *S. liquefaciens* was delayed or completely inhibited during vacuum storage of products at 4 or 10°C (Ouattara *et al.*, 2000). Inhibition was greater on drier meat product surfaces, such as bologna, due to slower release of the acids from the chitosan-based film, and with films supplemented with 1% (w/w) cinnamaldehyde (Ouattara *et al.*, 2000).

In conclusion, packaging films with incorporated organic acids, bacteriocins or essential oils may be effective in improving shelf-life and safety of processed meat products. However, at present, their commercial use is not allowed by the EU legislation and their permission for use requires changes in definitions of food additives and packaging materials and methods.

### 9.6 Detection and prediction of meat spoilage

Traditional methods to detect food (meat) spoilage are the sensory evaluation based on panelists, accompanied by determination of total or selective microbial plate counts. However, classical microbiological methods are slow, while sensory analyses have also the disadvantage to detect signals of spoilage with delay, even when expert panels are employed. The less the expertise of the panelists, the more delayed the detection of spoilage and the less the remaining product shelf-life during which corrective action could be taken. Traditional methods give retrospective results, and this can be a major drawback within the modern meat industry as monitoring procedures, such as HACCP systems, need to give results in real time to enable corrective actions to be taken as soon as possible. Therefore, in recent years, there has been an increasing interest in rapid methods for detection of meat spoilage, and in effectively incorporating such data to models aiming at providing accurate shelf-life predictions.

#### 9.6.1 Rapid methods for detection of meat spoilage

Several rapid methods for detecting meat spoilage have been proposed and some of them are commercially applied. They rely on different principles and indicator measurements, and can be broadly categorized into
microbiological, chemical/biochemical and miscellaneous. Rapid microbiological detection methods usually employ advanced molecular techniques, such as PCR, or better quantitative, real-time PCR, to isolate, characterize and quantify total or specific spoilage microorganisms, such as LAB, on meat products (Venkitanarayanan et al., 1997a). Total microbial contamination of animal meat or poultry carcasses can also be evaluated by rapid microbial ATP bioluminescence assays, since the light density from reaction of luciferin/luciferase with ATP is correlated with microbial levels present on meat (Siragusa et al., 1996; Cutter et al., 1996). It should be stressed, however, that the assay should differentiate the microbial from non-microbial ATP.

A novel, fascinating field toward an early detection of spoilage may be based on extraction/detection of compounds, such as N-acyl homoserine lactones (AHLs), which are quorum sensing signals excreted by potential spoilage bacteria, such as _H. alvei_ and other Enterobacteriaceae, during storage of meat (Gram et al., 2002; Bruhn et al., 2004).

Chemical/biochemical detection of spoilage is based on methods that rapidly and accurately quantify the concentration of certain endogenous food constituents that are converted by microorganisms, or of certain metabolites which accumulate due to microbial catabolism (Dainty, 1996). Next, the concentration of these chemical indicators found in meat is correlated with increases in populations of specific spoilage organisms (SSO), mainly bacteria, during storage. Dainty (1996) reviewed the most important compounds that may be used as spoilage indicators in meat, namely the endogenous glucose and end-products of microbial growth. End-products include, among others, gluconic and 2-oxogluconic acids, l- and d-lactic acids, acetic acid and other volatile fatty acids (e.g. n-butyric acid, 2,3-butanediol, n-butyrate), biologically active amines (e.g. tyramine) and numerous volatile compounds (e.g. ethanol, indole, hydrogen sulfide, diacetyl, acetoin, acetone, methyl ethyl ketone, dimethyl sulfide). The SSOs that most actively produce the above compounds were discussed in Sections 9.2 and 9.3 of this chapter. For instance, the decreases in d-glucose and increases in acetate during vacuum or MAP 3°C or 10°C storage of poultry breast (normal pH meat) and thigh (DFD meat) were suggested to play a role as indicators of incipient spoilage (Kakouri and Nychas, 1994). A more recent study (Byun et al., 2003) reported that volatile basic nitrogen (VBN) and d-glucose contents showed the best correlation with bacterial counts in fresh beef and pork stored aerobically at 0 and 4°C. Conversely, the correlation of meat pH, l-lactate and amine contents, thiobarbituric acid values and electrical conductivity with bacterial counts was poorer than that of VBN and glucose (Byun et al., 2003).

Meat spoilage can be also detected by measuring the difference in rate of proton efflux from and influx into bacterial cells (Seymour et al., 1994), or the time for fluorescein diacetate hydrolysis or resazurin reduction (Venkitanarayanan et al., 1997b). Also, volatile organic compounds in spoiling meat measured using proton transfer reaction mass spectrometry (Mayr et
al., 2003) or an electronic nose (Ellis and Goodacre, 2001) are new tools for real-time detection of spoilage. Another rapid, reagent-less and non-destructive method that has shown promise is Fourier transform infrared (FT-IR) spectroscopy (Ellis et al., 2004).

### 9.6.2 Shelf-life model development for prediction of meat spoilage

For years, research into food spoilage has been related to commercial advantage and hence has been supported to a lesser extent. Today, however, as the food industry is moving ahead, there is a need for accurate shelf-life predictions. The financial losses due to recalls of products spoiled before their expiry date must be reduced. On the other hand, unwarranted disposal of wholesome food because of an overly conservative shelf-life estimate means a lot of money, and must also be reduced. In particular, the meat industry is in need of shelf-life models of high reliability because most of its products are subject to rapid microbial deterioration (McDonald and Sun, 1999).

As mentioned, to predict the shelf-life of perishable meat products based on mathematical models, it is necessary to define the microbiological level above which the product is rejected, which is a difficult task without knowing its ecology. For example, the aerobic spoilage of fresh meats due to pseudomonad growth occurs soon after their counts exceed 10^7 cfu/g, while 10^5–10^7 cfu/g of yeasts are known to cause spoilage in various types of processed meats. In contrast, LAB populations as high as 10^8 cfu/g may not cause noticeable spoilage in vacuum packaged fresh or processed meat products (Schillinger and Lucke, 1987a; Korkeala et al., 1987, 1989; Samelis and Georgiadou, 2000). Thus, it is preferable to evaluate shelf-life by considering the ‘minimum spoilage level’ of the SSO which are most likely to establish a spoilage domain (Gram et al., 2002) rather than simply using a pre-estimated threshold of total bacterial population density, e.g. >10^7 cfu/g, as the criterion (Zamora and Zaritzky, 1985). A need also remains to correlate levels of SSO in fresh or processed meats with sensory evaluation scores, which more directly associate with the spoilage defects, as well as the levels of SSO and sensory data with various chemical indicators of spoilage discussed above.

To predict shelf-life accurately, it is essential to describe mathematically the microbial ecology of a given complex food, such as meat, in a way that the resulting model could give reliable predictions on the growth kinetics of most important spoilage organisms under certain manufacturing or storage conditions. On this basis, shelf-life models should overcome two major problems. The first is the shelf-life definition: from a commercial point of view, shelf-life is the storage time until consumers reject a food (meat) because of an unacceptable off-odor/off-flavor or appearance. However, this point may be from a few hours to several days later than the storage time required for the dominant spoilage flora to reach a maximum acceptable level, depending on its composition and the severity of the sensory defects
caused by its catabolic products. Thus, to predict shelf-life, the population density of the SSO must be correlated with an early detection of the biochemical changes caused by them and contributing to spoilage.

The second problem is the existing difficulty to apply predictive modeling in practice due to several reasons summarized by McMeekin and Ross (1996). Two of the most important ones are: (i) the complexity of food systems in terms of heterogeneity in their composition, especially when they are in solid state, and (ii) the variations in numbers and types of the microorganisms naturally present in a food, and their possible interactions. To date, most food models have been validated by experimental data derived after growth of the target organisms in sterile, synthetic liquid media or food extracts (e.g. meat juices) under precisely controlled conditions, and mostly in pure culture. This can easily lead to model weakness when testing conditions vary differently, in a better or worse manner than of the model design. A need therefore exists for another type of predictive model in structured foods harboring mixed microbial associations, such as meat products (Dens and Van Impe, 2001).

Also, the difficulty in predicting microbial growth under fluctuating cold storage temperatures, as frequently happens with meats (Gill, 1996), must be considered in models.

Several workers have developed promising mathematical models to describe the individual or mixed-culture growth kinetics of important meat spoilage bacteria under different sets of parameters affecting growth (e.g. temperature, pH, water activity, glucose and lactate concentration) within the normal range found in meat (Pin and Baranyi, 1998; Martens et al., 1999; Malakar et al., 1999; McDonald and Sun, 1999; Lebert et al., 2000; Wijtzes et al., 2001). As expected, the culture pH has been shown to have a strong effect on the maximum specific growth rate ($r_{\text{max}}$) of both *Lact. curvatus* and *Enterobacter cloacae*, with the latter organism being clearly more acid/pH-sensitive than the former (Martens et al., 1999). In contrast, glucose had no effect on $r_{\text{max}}$ of the above bacteria, while the effect of lactate *per se* was minor compared with that of pH (Martens et al., 1999). With regard to interactions between *Lact. curvatus* and *Enterobacter cloacae*, the most important factor when *Lact. curvatus* dominated was the pH, while it turned to be substrate limitation when *Enterobacter cloacae* was dominant (Martens et al., 1999). However, the pH had decreased to inhibiting levels for *Enterobacter cloacae* only after *Lact. curvatus* reached $10^8$ cfu/ml (Malakar et al., 1999). Given that in non-fermented foods (meats) such high populations exist only when the product is spoiled or about to spoil, Malakar et al. (1999) opined that microbial interactions can be neglected when predicting shelf-life. Pin and Baranyi (1998) concluded otherwise after modeling the evolution in mixed culture of all main bacteria genera commonly found in aerobically stored meat; they found a constant high predominance of *Pseudomonas*, reflecting the strong interaction of these bacteria against all other competitors.

There are relatively few studies that have modeled microbial growth and predict shelf-life of cured meat products in the literature (Aggelis et al.,
1998; Devlieghere et al., 1999, 2000; Cayre et al., 2003). In their novel model approach, Aggelis et al. (1998) described the specific growth rate of the main types of spoilage microbes as a function of the specific destructive effect of a raw sausage ecosystem (e.g. the combined effect of pH, moisture and salt). More recently, Devlieghere et al. (2000) incorporated SL as an additional (to temperature, water activity and CO₂) determinative factor of shelf-life in a cooked meat model, to account for the wide commercial use of SL as an antimicrobial in cured meat formulations.

9.7 Future trends

Research on the microbiology and the associated microbial spoilage of fresh and processed meat and poultry will be intensified given the high importance of muscle foods for human nutrition and the great evolution of the meat industry in recent years. To date, the safe operation of big meat slaughterhouses, packing or processing plants, and centralized packaging centers of retail meat cuts in developed countries (Tewari et al., 1999) is a necessity in order to avoid unsafe or spoiled products, their recalls and great economical losses. Following urbanization in most countries, there is a need for transportation of large amounts of food products from centralized production and processing locations to distant markets at urban centers. In addition to this fact being one of the main potential reasons for pathogen emergence and transmission of foodborne disease (Samelis and Sofos, 2003a), it underscores the need for production of food (meat) products with an extended shelf-life.

So, the following research fields are expected to gain more scientific interest in the near future, with their respective major tasks: (i) a better mapping of the ecology and dynamics of meat spoilage microorganisms and the identification of new species or taxa specific to meat environments; (ii) a better understanding of the biochemical capabilities and metabolism of known or newly isolated microbial species and their associated physiology of spoilage in order to provide clear answers on their specific occurrence and predominant growth in meat products and plants; (iii) advancement of existing meat packaging and preservation technologies, and development and industrial application of novel technologies based on the combined use of new packaging materials, gas mixtures, emerging physical methods and/or natural antimicrobials; (iv) development of more effective methods and techniques for the rapid subjective detection of meat spoilage, and establishment of better links with the respective objective methods based on sensory panels; (v) development and application of more effective shelf-life-predicting models which will better consider the structured (solid) state of meat and its mixed microbial associations; and (vi) critical use of the existing and new research data to design and apply more effective shelf-life monitoring systems in the meat industry.
To achieve the above tasks, certain new tools recently incorporated in meat research will be useful and promising. For example, recent advances in molecular culture-dependent and culture-independent (e.g. DGGE) characterization methods (Yost and Nattress, 2000; Björkroth and Holzapfel, 2003; Cocolin et al., 2004; Takahashi et al., 2004) may assist in providing an improved mapping of the microbial consortia and dynamics in spoiling meats and in the detection of unknown important species or species interactions and successions. Advances in laboratory equipment for organic analyses of foods will contribute to improve chemical detection of spoilage by isolating and on-line characterizing more catabolic by-products, as well as compounds associated with the ‘quorum sensing’ mechanisms of meat spoilage microorganisms (Gram et al., 2002). Also, biochemical analyses can assist to isolate and characterize active compounds specifically present in natural antimicrobials, as for example thymol and carvacol in oregano essential oil (Lambert et al., 2001), and then mix to re-synthesize antimicrobials that lack negative sensory effects on meat of the original crude antimicrobial. Finally, the increasing rate of technological achievements in food processing and computer sciences can ideally serve to promote novel intelligent ideas on how to delay microbial spoilage and monitor shelf-life of commercial meat and poultry products.

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Managing microbial spoilage in the meat industry


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Part III

Spoilage yeasts
10

Zygosaccharomyces and related genera

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10.1 Introduction

Food and beverage spoilage yeasts often show some degree of substrate specialization, and species of Zygosaccharomyces and related genera (i.e. Lachancea, Torulaspora and Zygotorulaspora) are usually the yeasts that colonize and spoil high-sugar and high-salt products such as fruit juices and their concentrates, dried fruit, honey, jams and preserves, soft candy, salad dressings, soy sauce and sugar syrups as well as wine (Pitt and Hocking, 1997; Kurtzman, 1998a, b; Barnett et al., 2000; James and Stratford, 2003). Growth of these species can be slow, with an apparently good product leaving the manufacturer only to become spoiled after several weeks or months on the grocery store shelf. The most obvious problem seen for spoiled products is gas build-up in the container. The gas, which is carbon dioxide, results from slow fermentation of sugars (e.g. glucose, fructose) in the products. The carbon dioxide build-up may be sufficient to cause the distortion or even explosion of either the product, e.g. fondant-filled chocolate cream eggs, or the product packaging. Glass containers may shatter, resulting in serious injury, especially of the eyes (Grinbaum et al., 1994). Other types of spoilage caused by these species include the generation of taints, odors and off-flavors, and the development of hazes due to substantive yeast growth. Yeast spoilage is not known to lead to human infections or formation of toxic products, but changes in product composition might result in concurrent growth by pathogens or microorganisms that produce toxins.

In this chapter, methods for isolation, quantitation, identification and control of spoilage yeasts will be presented. Newly developed molecular methods for species identification will be discussed along with traditional methods based on phenotypic characterization.
10.2 Products affected

The list of products spoiled by Zygosaccharomyces, Torulaspora and the recently described genera Zygotorulaspora and Lachancea (Kurtzman, 2003), here referred to collectively as the Zygosaccharomyces complex, has remained fairly constant over many decades, and typically includes high-sugar foods and beverages, as well as occasional high-salt products such as soy sauce (Table 10.1). Walker and Ayres (1970) used the term osmophilic for yeasts that grow in high sugar concentrations and the term osmoduric for species that survive but do not grow in high sugar concentrations. With regard to Zygosaccharomyces, Z. bailii, Z. bisporus and most notably Z. rouxii are all recognized as being highly osmophilic. Indeed, Z. rouxii is the most osmophilic yeast species known, with some strains reported to grow in products with a water activity ($a_w$) as low as 0.62 (Walker and Ayres, 1970; Tilbury, 1980). While not as osmophilic as Z. rouxii, Z. bailii is nevertheless still capable of growing at $a_w$ as low as 0.80 at 25°C (Pitt and Hocking, 1997). Most of the other species in the Zygosaccharomyces complex also commonly spoil foods and beverages, but many are found in products with a somewhat lower sugar concentration.

10.2.1 Honey, syrups, soft candy

Fabian and Quinet (1928), as well as Lochhead and Heron (1929), were among early food microbiologists to recognize that fermentation of stored honey was often caused by species of Zygosaccharomyces, with less frequently implicated species being members of Saccharomyces, Schizosaccharomyces and Candida. Fabian and Quinet (1928) made the interesting observation that honey is quite hygroscopic and will absorb up to a third of its weight in moisture, thus increasing the likelihood of spoilage. Lochhead and Heron (1929) showed the presence of sugar-tolerant yeasts in flower nectar and in nectar from beehives, suggesting that these are the sources of the spoilage organisms. Although spoilage of honey was a problem in earlier times, it is infrequent today.

Fabian and Hall (1933) reported maple syrup underwent spoilage from Zygosaccharomyces species. These authors determined that the moisture content of fresh syrup ranged from 26.3 to 36.5%. Syrup with a moisture range of 32.7–34.6% was susceptible to yeast spoilage, which became evident after 3–4 weeks when syrups were inoculated with spoilage yeasts. Molasses (Hall et al., 1937), cane syrup (Shehata, 1960) and soft center chocolates (cf. Walker and Ayers, 1970) undergo fermentative spoilage when contaminated with Zygosaccharomyces species.

The identification of Zygosaccharomyces species causing spoilage of honey and other high-sugar products has been problematic when using phenotypic tests. On the basis of strain comparisons from nuclear DNA reassociation, Z. bailii, Z. mellis and Z. rouxii were identified as the species of Zygosaccharomyces responsible for spoilage of high-sugar products.
Table 10.1  Products commonly spoiled by species of *Zygosaccharomyces* and related genera

<table>
<thead>
<tr>
<th>Species</th>
<th>Products</th>
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<tbody>
<tr>
<td></td>
<td>Honey, other</td>
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<tr>
<td></td>
<td>high sugar</td>
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<td></td>
<td>Dried fruit</td>
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<td></td>
<td>Fruit juices,</td>
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<tr>
<td></td>
<td>concentrates</td>
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<tr>
<td></td>
<td>Soft drinks</td>
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<tr>
<td></td>
<td>Wine, spirits</td>
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<tr>
<td></td>
<td>Salad dressings,</td>
</tr>
<tr>
<td></td>
<td>condiments</td>
</tr>
<tr>
<td></td>
<td>Low sugar, high salt</td>
</tr>
<tr>
<td><em>Lachancea cidri</em></td>
<td>+</td>
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<tr>
<td><em>L. fermentati</em></td>
<td>+</td>
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<tr>
<td><em>L. kluyveri</em></td>
<td>+</td>
</tr>
<tr>
<td><em>L. thermotolerans</em></td>
<td>+</td>
</tr>
<tr>
<td><em>L. waltii</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Torulaspora delbrueckii</em></td>
<td>+</td>
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<tr>
<td><em>T. franciscae</em></td>
<td>+</td>
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<tr>
<td><em>T. globosa</em></td>
<td>+</td>
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<tr>
<td><em>T. microellipsoides</em></td>
<td>+</td>
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<tr>
<td><em>T. pretoriensis</em></td>
<td>+</td>
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<tr>
<td><em>Zygosaccharomyces bailii</em></td>
<td>+</td>
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<tr>
<td><em>Z. bisporus</em></td>
<td>+</td>
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<tr>
<td><em>Z. kombuchaensis</em></td>
<td>+</td>
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<tr>
<td><em>Z. lentus</em></td>
<td>+</td>
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<tr>
<td><em>Z. mellis</em></td>
<td>+</td>
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<tr>
<td><em>Z. rouxii</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Zygotorulaspora florentinus</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Z. mrakii</em></td>
<td>+</td>
</tr>
</tbody>
</table>

1 Not all species of each genus are known to cause spoilage. Data from Kurtzman and Fell (1998), Kurtzman *et al.* (2001) and Steels *et al.* (2002).
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(Kurtzman, 1990). Prior to the DNA comparisons, Z. mellis was often regarded as a synonym of Z. rouxii.

10.2.2 Dried fruits and fruit juices

Dried fruits, because of their relatively high sugar and moisture contents, are susceptible to spoilage by yeasts. Mrak et al. (1942) reported a diverse yeast flora on California dates, with the predominant species belonging to the genus Zygosaccharomyces. Baker and Mrak (1938) made similar observations for dried prunes with the primary spoilage yeasts again identified as Zygosaccharomyces species. Spoilage began when the prunes contained over 22% moisture.

Fruit juices are susceptible to spoilage by a large number of yeasts but Z. bailii, Z. rouxii, Z. mellis and the recently described Z. lentus (Steels et al., 1999) are the primary species that spoil juices and juice concentrates (Walker and Ayres, 1970). However, other species of Zygosaccharomyces, as well as species of Torulaspora (e.g. T. delbrueckii), have been isolated from fruit juices and these species can also be found in other sugary products (Kurtzman, 1998a, b).

10.2.3 Beverages

Beverage spoilage can be caused by a variety of yeasts, some of which are members of the Zygosaccharomyces complex. For example, Sand and van Grinsven (1976) reported that Z. bailii caused spoilage of soft drinks and Z. rouxii was isolated from a spoiled, commercially produced canned tea drink (Kurtzman, unpublished). Although wine spoilage is often caused by Brettanomyces bruxellensis, wines are not immune to spoilage by species of Zygosaccharomyces and Torulaspora. In particular, sweet and sparkling wines are susceptible to spoilage by Z. bailii and related species (Rankine and Pilone, 1973; Loureiro and Malfeito-Ferreira, 2003). On the basis of strain isolation histories (Kurtzman and Fell, 1998), Z. bailii, Z. lentus, Z. rouxii and T. delbrueckii are the primary species of the Zygosaccharomyces complex isolated from spoiled wines (Table 10.1).

10.2.4 Salad dressings

Spoilage of salad dressings has been a longstanding problem for the food industry. Although some spoilage can be attributed to species of Bacillus and Lactobacillus, the primary spoilage organisms are species of Zygosaccharomyces, especially Z. bailii (Kurtzman et al., 1971; Kurtzman and Smittle, 1984). Both spoiled and unspoiled dressings typically have a pH range of 3.4–4.2, thus suppressing most bacterial growth. When spoiled, the yeast count of these products ranged from 5 to 165 500 viable cells/g of product (Kurtzman et al., 1971). Most likely, the low counts recorded for a few spoiled samples reflect cell death following the spoilage outbreak.
10.3 Detection, enumeration and isolation of spoilage species

In heavily spoiled products, the spoilage organisms can often be detected by bright field light microscopy at a magnification of 400–500. Yeasts, as well as bacteria, are readily stained for microscopic observation by using crystal violet. A small drop of 0.5% crystal violet may be mixed directly with the product on a microscope slide or mixed with a drop of product that has been diluted 1:10 with distilled water. Since there are occasions when viable microorganisms are not recovered from spoiled product, microscopic detection provides a clue to the identity of the spoilage organism.

A variety of media have been proposed for isolation of species from the *Zygosaccharomyces* complex. Pitt and Hocking (1997) recommended dichloran rose bengal chloramphenicol (DRBC) medium for both molds and yeasts. The advantage of this medium is that the spreading growth of mold colonies is restricted, allowing more accurate colony counts on crowded plates. Plates with this medium must be incubated in the dark to prevent formation of photo-induced inhibitors. Species of *Zygosaccharomyces*, *Torulaspora* and related genera usually grow well at 25°C on standard YM agar (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 20 g agar, 1 liter water, pH 5 to 7); consequently, this medium can be used for detection as well as enumeration by standard plate count methods. A sample diluent of 0.1% peptone water is effective. Spread plates, rather than pour plates, are recommended because the increased aeration of surface growth favors recovery and subsequent growth of the yeast cells. Because of its near neutral pH, YM agar also favors growth of bacteria, which can be inhibited either by adjusting the pH to 4.5 with hydrochloric acid or by adding antibiotics. Use of antibiotics is favored over pH adjustment, and the choice of antibiotics varies. Tetracycline (30 mg/l) or a combination of chlortetracycline and chloramphenicol, each at a concentration of 100 mg/l, are often effective (Koburger and Marth, 1984; Kurtzman and Smittle, 1984). Other plating media typically used by food microbiologists include malt extract agar (MEA) and tryptone glucose yeast extract agar (TGY), the latter often in preference to YM agar.

For rapid, presumptive detection of preservative-resistant species such as *Z. bailii* and *Z. bisporus*, two media routinely used are acidified MEA (MAA, i.e. malt acetic agar) and acidified TGY (TGYA) (Pitt and Hocking, 1997). In both cases, the basal medium (MEA or TGY) is supplemented with 0.5% acetic acid. Yeast colonies are usually visible within 3–7 days after sample plating, and more than one yeast species can be present in spoiled products, which may not be initially evident because of similarities in colony appearance. In the case of *Zygosaccharomyces* species (e.g. *Z. bailii*), Hocking (1996) reported that the time required to detect their presence in products could be reduced by incubating agar plates at 30°C, instead of at 25°C. Because some species are osmophilic (e.g. *Z. bailii*, *Z. bisporus*, *Z. rouxii*), diagnostic plating can include use of a high osmotic medium comprised of YM or TGY.
agars supplemented with 40% (w/v) glucose. If these media are used, the sample diluent should be high osmotic as well, e.g. 0.1% (w/v) peptone water with 40% glucose.

A number of specialized detection media have been developed for species of the *Zygosaccharomyces* complex with reports of varying degrees of effectiveness (Pitt and Hocking, 1997; Beuchat and Cousin, 2001; Beuchat *et al.*, 2001; Loureiro and Malfeito-Ferreira, 2003). Working stock cultures of species from the *Zygosaccharomyces* complex can be temporarily maintained by growing on YM or 5% (w/v) ME agar at 25°C for 2–3 days followed by storage for several months in the refrigerator before the need to retransfer. Longer-term storage should be by lyophilization or freezing in the vapor phase of liquid nitrogen.

### 10.4 Classification of spoilage species

The genus *Zygosaccharomyces* was described by Barker in 1901 and *Torulaspora* by Lindner in 1904 (cf. van der Walt, 1970). Members of both genera show multilateral budding typical of *Saccharomyces*. Species of *Zygosaccharomyces* typically have ‘dumbbell’-shaped asci that arise from conjugation between two independent cells (Fig. 10.1a). There are often two ascospores per conjugant, but this may vary with one conjugant being empty or with a single ascospore and the other conjugant with up to three ascospores. Species of *Torulaspora* are often characterized by asci with a tapered protuberance (Fig. 10.1b). Ascus morphology is not always typical for a genus and it is sometimes difficult to separate species of *Zygosaccharomyces*, *Torulaspora*, *Zygotorulaspora* and *Lachancea* from one another and from

![Fig. 10.1](image_url)

**Fig. 10.1** (a) Ascospore formation by *Zygosaccharomyces bailii*. Asci of *Zygosaccharomyces* often have ‘dumbbell’ configurations (arrow) with two ascospores in each conjugant. (b) Ascospore formation by *Torulaspora delbrueckii*. A characteristic of the genus *Torulaspora* is the formation of asci with tapered projections (arrow). Bar = 5 µm for both figures.
Saccharomyces. Because of this difficulty, van der Walt (1970) combined Torulaspora and Zygosaccharomyces with Saccharomyces, but Yarrow (1984) later separated the three genera, once again along the lines of their original morphological descriptions.

James et al. (1994, 1997) demonstrated from phylogenetic analysis of 18S ribosomal DNA (rDNA) that Z. bailii, Z. rouxii and several other Zygosaccharomyces species formed a distinct clade that was well separated from the Torulaspora delbrueckii clade as well as from the Saccharomyces cerevisiae clade. This work also demonstrated that not all species classified from phenotypic criteria as Zygosaccharomyces or Saccharomyces grouped in their respective genera as now recognized from molecular characterization. Kurtzman and Robnett (1998) provided a similar appraisal of species relationships from analysis of domains 1 and 2 (D1/D2) of large subunit (26S) rDNA. However, neither the 18S nor the 26S rDNA datasets, either alone or in combination, provided sufficient resolution to define individual genera. Kurtzman and Robnett (2003) compared the ca. 90 species in what they termed the ‘Saccharomyces complex’ from a multigene dataset, and resolved the group into 14 clades, which were interpreted as genera. Five of these clades were described as new genera (Kurtzman, 2003). Three of the newly circumscribed genera, Zygotorulaspora, Torulaspora and Lachancea, received species that were previously classified as Zygosaccharomyces (Fig. 10.2). This work has clearly demonstrated that ascus morphology and other phenotypic characters do not always distinguish genera. In view of previous assignments and reassignments of species to genera, the results from sequence analysis are not surprising.

10.5 Identification of spoilage species

Molecular methods offer the most reliable means for identification of species assigned to Zygosaccharomyces and related genera, and we recommend DNA sequence comparisons. Sequences for the D1/D2 domains of large subunit rDNA are available in GenBank for all known species (Kurtzman and Robnett, 1998, and subsequent submissions), and a Blast Search will provide rapid identification. One might also use non-coding ITS rDNA sequences, which are available for many species (James et al., 1996; Kurtzman and Robnett, 2003). In fact for certain species groups, e.g. Saccharomyces sensu stricto (Fischer et al., 2000; Liti et al., 2005), ITS sequences may provide better resolution than D1/D2 rDNA sequences. Amplification and sequencing of yeast rDNA (e.g. 18S, 26S or ITS) can be done rapidly with currently available technology and new species are quickly recognized from their absence in the database. Indeed, it was through the use of rDNA sequencing that two new species of Zygosaccharomyces, namely Z. kombuchaensis (Kurtzman et al., 2001) and Z. lentus (Steels et al., 1999), were recently identified. Furthermore, there is some evidence to suggest that Z. bailii isolates may represent a
complex of closely related species (James et al., 1996; Kurtzman unpublished), and similar complexes may be detected for other species, e.g. Z. rouxii (James et al., 2005).

At present, there are no fully developed nonsequencing molecular methods available for identification of all known species assigned to the Zygosaccharomyces complex, but these diagnostic methods, some of which are discussed in Chapter 2, are under development in many laboratories and will become available for general use in the near future. In one example, restriction fragment length polymorphisms (RFLP) of rDNA were successfully used to separate Z. kombuchaensis from Z. lentus (Kurtzman et al., 2001). However, an RFLP database must be developed for other known species of the Zygosaccharomyces complex. Similarly, amplified fragment length polymorphism (AFLP) typing, a PCR-based (polymerase chain reaction) fingerprinting method developed by Keygene (Wageningen, The Netherlands),
originally for screening plant cultivars with differing agronomic traits (Vos et al., 1995), has also been tested on a number of Zygosaccharomyces species, including different strains of Z. bailii. It has been extremely useful for both strain and species discrimination (van der Vossen et al., 2003; MacKenzie et al., 2005). Again, a comprehensive database needs to be developed before this method can be fully exploited for typing and tracking spoilage yeast strains.

For those wishing to identify species from standard tests, a key is provided in Table 10.2. Methods for preparing media for fermentation, assimilation, morphological examination and ascosporulation were given by Yarrow (1998).

**Table 10.2** Key to species of the genera *Zygosaccharomyces*, *Zygotorulaspora*, *Torulaspora* and *Lachancea*

<table>
<thead>
<tr>
<th>A</th>
<th>Zygosaccharomyces. Asci are conjugated, some with a ‘dumbbell’ configuration (Fig. 10.1a).</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(a) Maltose fermented; growth with 16% NaCl + 5% glucose</td>
</tr>
<tr>
<td></td>
<td>Z. rouxii</td>
</tr>
<tr>
<td></td>
<td>(b) Maltose fermented; growth absent with 16% NaCl + 5% glucose</td>
</tr>
<tr>
<td></td>
<td>Z. mellis</td>
</tr>
<tr>
<td></td>
<td>(c) Maltose not fermented</td>
</tr>
<tr>
<td>2</td>
<td>(1) (a) Sucrose fermented; trehalose not assimilated</td>
</tr>
<tr>
<td></td>
<td>Z. kombuchaensis*</td>
</tr>
<tr>
<td></td>
<td>(b) Sucrose fermentation and trehalose assimilation</td>
</tr>
<tr>
<td></td>
<td>not as described above</td>
</tr>
<tr>
<td></td>
<td>(2a)</td>
</tr>
<tr>
<td></td>
<td>Z. lentus</td>
</tr>
<tr>
<td>3</td>
<td>(2) (a) Trehalose assimilated</td>
</tr>
<tr>
<td></td>
<td>Z. bailii</td>
</tr>
<tr>
<td></td>
<td>(b) Trehalose not assimilated</td>
</tr>
<tr>
<td></td>
<td>Z. bisporus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Torulaspora. Asci form tapered protuberances (Fig. 10.1b):</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(a) Growth at 37°C strong and rapid</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(b) Growth at 37°C slow, weak or absent</td>
</tr>
<tr>
<td></td>
<td>T. delbrueckii</td>
</tr>
<tr>
<td>2</td>
<td>(1) (a) Galactose assimilated</td>
</tr>
<tr>
<td></td>
<td>T. franciscae</td>
</tr>
<tr>
<td></td>
<td>T. pretoriensis</td>
</tr>
<tr>
<td></td>
<td>T. globosa</td>
</tr>
<tr>
<td></td>
<td>(b) Galactose not assimilated</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>Lachancea. Asci not as in A or B; m-inositol required for growth, or if not required, growth absent with 0.01% cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(a) Trehalose assimilated</td>
</tr>
<tr>
<td></td>
<td>L. waltii</td>
</tr>
<tr>
<td></td>
<td>(b) Trehalose not assimilated</td>
</tr>
<tr>
<td>2</td>
<td>(1) (a) Growth absent with 0.01% cycloheximide</td>
</tr>
<tr>
<td></td>
<td>L. kluyveri</td>
</tr>
<tr>
<td></td>
<td>(b) Growth present with 0.01% cycloheximide</td>
</tr>
<tr>
<td></td>
<td>L. thermotolerans</td>
</tr>
<tr>
<td>3</td>
<td>(2) (a) m-Inositol not required for growth</td>
</tr>
<tr>
<td></td>
<td>L. fermentati</td>
</tr>
<tr>
<td></td>
<td>(b) m-Inositol required for growth</td>
</tr>
<tr>
<td></td>
<td>L. cidri</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D</th>
<th>Zygotorulaspora. Asci not as in A or B; m-inositol not required for growth; growth present with 0.01% cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(a) Trehalose assimilated</td>
</tr>
<tr>
<td></td>
<td>Z. florentinus</td>
</tr>
<tr>
<td></td>
<td>(b) Trehalose not assimilated</td>
</tr>
<tr>
<td></td>
<td>Z. mrakii</td>
</tr>
</tbody>
</table>

*Although standard phenotypic tests fail to distinguish between Z. kombuchaensis and Z. lentus (Kurtzman et al., 2001), Steels et al. (2002) recently reported that the two species could in fact be distinguished from one another, as Z. kombuchaensis, unlike Z. lentus, is sensitive to sorbic acid.*
and Kurtzman et al. (2003), and descriptions of individual species can be found in The Yeasts, a Taxonomic Study, 4th edition (Kurtzman and Fell, 1998) with descriptions of more recently described species provided by Steels et al. (1999) and Kurtzman et al. (2001). The species pairs Zygosaccharomyces kombuchaensis/Z. lentus and Torulaspora franciscae/T. pretoriensisis are unresolved by the following key. Initial identification to genus requires that ascospores are produced because there are no genus-specific physiological tests currently known. Ascosporulation often occurs on YM, 5% ME or RG or corn meal agar media at 15 or 25°C after 1–4 weeks.

10.6 Characteristics of spoilage species

Species of the Zygosaccharomyces complex ferment and assimilate relatively few sugars (Kurtzman and Fell, 1998). However, for those sugars that are fermented, Zygosaccharomyces species, in contrast to other yeasts, are unique in that they appear to metabolize fructose in preference to glucose, a phenomenon referred to as fructophily (Emmerich and Radler, 1983). This ability has important spoilage implications, as the growth rate of Z. bailii is often enhanced if the level of fructose present in a product exceeds 1% of total product composition.

None of the species from the Zygosaccharomyces complex grow at 45°C and only a few strains of Z. fermentati, T. globosa and T. pretoriensis grow at 42°C. While all members of this species complex can grow sparingly at 5–7°C, Z. lentus, unlike other Zygosaccharomyces species, will grow slowly at 4°C (Steels et al., 1999). Although no incidences have yet been documented, such ability nevertheless raises the possibility that Z. lentus could potentially pose a realistic threat as a spoilage agent of chilled products. The species show little tolerance to elevated temperatures with ascospores more heat resistant than vegetative cells. Put et al. (1976) reported survival of Z. bailii ascospores for 10 min at 60°C, but none of the ascospores was viable after 20 min at this temperature. Extensive studies of heat resistance (given as D values) for Zygosaccharomyces and other yeasts under various buffer conditions were provided by Put et al. (1976) and Shearer et al. (2002).

However, species of the Zygosaccharomyces complex show great tolerance to acidity, to common food preservatives and to high osmotic conditions. For example, Z. rouxii was reported to grow over the pH range of 1.8–8.0 in a medium containing 46% glucose (English, 1954), and Z. bailii is noted for its resistance to sorbic, benzoic, acetic and propionic acids, as well as to SO₂ (Ingram, 1960; Pitt, 1974; Hammond and Carr, 1976; Tilbury, 1980; Neves et al., 1994). Remarkably, Z. bailii showed a minimum inhibitory concentration (MIC) of 1000–1400 mg/l of benzoic acid at pH 3.5 (Warth, 1986). Many of the Zygosaccharomyces species show tolerance to high osmotic conditions, and strains of Z. bailii and Z. rouxii were found to grow in products with an aw as low as 0.80 for Z. bailii, and 0.62 for Z. rouxii (Walker and Ayres, 1970; Tilbury, 1980; Pitt and Hocking, 1997).
10.7 Conditions for spoilage

Prevention of spoilage by species of the Zygosaccharomyces complex requires strict sanitation in manufacturing plants, use of high-quality ingredients and scrupulous attention to use of sanitized packaging and proper pasteurization, when the product is amenable to this latter process. This group of spoilage yeasts is particularly aggressive because of their resistance to commonly used preservatives, and because relatively few yeast cells are required to initiate spoilage. For example, *Z. bailii* has been shown to be capable of causing soft drink spoilage from an initial inoculum of only one viable cell per container (van Esch, 1987; Davenport, 1996; Pitt and Hocking, 1997). Consequently, monitoring of ingredients and equipment is essential for quality products, and rapid species identification is a key part of this process. For example, three groups of organisms are commonly used as microbial indicators of sanitation: yeasts and molds, lactobacilli and aerobic bacteria. High numbers of these groups are indicative of poor sanitation and potential spoilage problems. The presence of *Z. bailii* and other aggressive spoilage organisms is unacceptable at any level. Since most yeasts, molds and bacteria die quickly in acidic products, the products usually contain <10/g of these organisms. Any increase in numbers >10/g indicates a sanitation problem (Kurtzman and Smittle, 1984).

Product contamination is often traced to plant sanitation with yeast cells surviving and flourishing in fill lines, pumps and drains. The source of contaminating cells is often uncertain, but the natural habitat of *Z. bailii*, and perhaps other osmophilic species, appears to be in mummified fruits from orchards (cf. Walker and Ayres, 1970). Consequently, besides being brought in on raw ingredients (e.g. fruits), contaminating yeasts may be windblown into manufacturing plants and become entrenched in hard to clean areas. Furthermore, there is also some evidence to suggest that spoilage yeasts, as well as yeasts with the potential to cause spoilage (e.g. *Candida davenportii*), may be introduced into food and beverage processing facilities via insect vectors such as bees and wasps (Stratford et al., 2002).

10.8 Strategies for control of spoilage

As noted in the previous section, control of spoilage by species of the Zygosaccharomyces complex is dependent on maintaining good manufacturing plant sanitation and use of high-quality ingredients. Plant sanitation includes thorough cleaning and sanitizing of pipes, fill vessels, pumps and filtration equipment. Because of blind ends in pipes and pumps and leakage in gasket assemblies, this is not an easy task.

Changes in the types of preservatives used for product stability represent another approach to prevention of spoilage because commonly used preservatives, such as acetic, sorbic and benzoic acids, often do not prevent
spoilage even when used at the maximum levels legally permitted. For example, Ludovico et al. (2003) showed that 320–800 mM acetic acid is required to induce cell death in Z. bailii.

Some plant-derived secondary metabolites have shown promise for inhibiting spoilage organisms. Araújo et al. (2003) tested essential oils from Mediterranean Lamiaceae species and discovered that citral showed significant inhibitory activity against Z. bailii, T. delbrueckii and several other spoilage yeasts. Vanillin, a major constituent of vanilla beans, is another plant-derived compound with significant activity against spoilage yeasts (Fitzgerald et al., 2004). Chitosan glutamate, a derivative of chitin, was shown by Roller and Covill (1999) to inhibit several common spoilage yeasts. A strain of Z. bailii was inhibited by 0.1 g/l of this compound in a 32 day storage test at 25°C, whereas 5 g/l was required to inhibit Saccharomyces ludwigii.

In addition to use of new types of chemical preservatives, preservation is also being effected by novel physical treatments of food products. El Halouat and Debevere (1996) used a combined treatment of chemical preservatives and modified atmospheres to inhibit spoilage. In the presence of 220 ppm sorbate and 280 ppm benzoate, growth of Z. rouxii was completely inhibited if the product atmosphere contained 80% carbon dioxide and 20% nitrogen. Use of high hydrostatic pressure has been shown to kill spoilage organisms (Karatzas et al., 2001; San Martin et al., 2002). For example, Palou et al. (1998) demonstrated that Z. bailii was killed when pressures reached 517 MPa. High-intensity pulsed electrical fields (HIPEF) represent another non-chemical means for preserving foods and this method has effectively inhibited spoilage of orange juice by Saccharomyces cerevisiae (Elez-Martínez et al., 2004).

10.9 Future trends

10.9.1 Identification of spoilage yeasts
At present, many food microbiology laboratories still identify spoilage yeasts using phenotypic-based methods. However, such methods can prove laborious, as well as time consuming, and results can therefore be delayed as well as uncertain. In contrast, molecular methods offer a more reliable and accurate means for identification and are well-suited to recognize new spoilage species, e.g. Z. lentus (Steels et al., 1999). At present, the most reliable molecular method is sequencing of diagnostic nuclear- and mitochondrial-encoded genes (e.g. ACT-1, COX2, D1/D2 rDNA) and non-coding regions (e.g. ITS rDNA), although a number of rapid, molecular-based identification systems are just now beginning to appear. These include diagnostic probes and chip-based systems developed from species-specific gene sequences. Furthermore, through the use of PCR-based typing techniques, such as microsatellite PCR fingerprinting (van der Vossen and Hofstra, 1996), AFLP typing (Vos et al., 1995) and RAPD analysis (Williams et al., 1990), the opportunity now exists
for differentiating between individual spoilage strains, either of the same or different species, and identifying their sources of origin (Baleiras Couto et al., 1996). Some of these diagnostic and typing methods, as well as those being developed for quantitation of spoilage organisms, are discussed in more detail in Chapter 2.

**10.9.2 New methods for preservation**

One of the greatest challenges facing food producers is development of more effective preservation methods. Some of these new methods were presented in Section 10.8, and others have been discussed by Gould (1996) and Raso and Barbosa-Canovas (2003). Multi-parameter modeling represents a new approach for development of effective preservation methods. Reyns et al. (2000) presented a kinetic analysis that combined both high-pressure and temperature inactivation of *Z. bailii*. Other studies utilizing multiple parameters have been reported by Braun and Sutherland (2004), Evans et al. (2004) and Quintas et al. (2005). Brul et al. (2002) discussed a genomics approach in which the effect of preservation processes on food spoilage yeasts can be followed by measuring gene expression using DNA micro-array technology. This latter approach will not only provide further insights into the different resistance mechanisms used by spoilage yeasts to protect themselves against the effects of preservatives (e.g. sorbic acid), but will also, no doubt, lead to the development of more effective preservation strategies and hence greater product safety.

**10.10 Sources of further information and advice**

The following references are especially noted and provide extensive information on yeasts causing food spoilage, methods for their isolation and information on identification and taxonomy.

Yeasts as spoilage organisms (Walker and Ayres, 1970)

Fungi and Food Spoilage, 2nd edition (Pitt and Hocking, 1997)

Yeasts in Food, Beneficial and Detrimental Aspects (Boekhout and Robert, 2003)


Yeasts: Characteristics and Identification (Barnett et al., 2000)

**10.11 References**


BOEKHOUT T and ROBERT V (2003), Yeasts in Food, Beneficial and Detrimental Aspects, Hamburg, Behr’s Verlag.


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SHEHATA E T A M (1960), ‘Yeasts isolated from sugar-cane and its juice during the production of aguardente de cana’, Appl Microbiol, 8, 73–75.


Zygosaccharomyces and related genera


VAN ESC F (1987), ‘Yeasts in soft drinks and fruit juice concentrates’, De Ware (n) Chemicus, 17, 20–31 (English reprint; Brygemeteren 4 July/August 1992, 9–20).


11

Saccharomyces and related genera

g. h. fleet, the university of new south wales, australia

11.1 Introduction

Yeasts within the genus Saccharomyces are best known for their positive contributions to food and beverage production – especially through the roles of Saccharomyces cerevisiae and Saccharomyces bayanus in the fermentation of alcoholic beverages, and the roles of S. cerevisiae and Saccharomyces exigus in the fermentation of bread and other bakery products (Rose and Harrison, 1970, 1993). Nevertheless, there are circumstances when these same species can spoil the very commodity they produce. Within S. cerevisiae, for example, there is significant metabolic diversity. Some strains produce high amounts of hydrogen sulphide and acetic acid, resulting in wines and beers with unacceptable quality. In these cases, the fermentation process has failed and the initial raw material is spoiled and wasted. Re-growth of S. cerevisiae or S. bayanus in finished beer or wine causes unacceptable turbidity, sediment, gassiness and off-flavours. The product is spoiled and vast quantities of beer and wine are lost because of such activities. In addition to S. cerevisiae, S. bayanus and S. exigus, there are other lesser-known species within the genus which, from time to time, are also linked to food and beverage spoilage. This chapter considers the contribution of Saccharomyces species to food and beverage spoilage. It describes the taxonomic status of the genus, the diversity of foods and beverages in which the species are found, factors affecting their survival and growth in these ecosystems, their metabolic impact on product quality, and methods for their analysis and control. Oda and Ouchi (2000) and Viljoen and Heard (2000) have given earlier overviews of this topic.
11.2 Taxonomy of *Saccharomyces*

The genus was first described in 1838 by Meyen and later defined by Reess in 1870, with *S. cerevisiae* as the type species (Barnett, 1992; Oda and Ouchi, 2000). Species of *Saccharomyces* are characterised as round, oval or cylindrical cells that reproduce asexually by multilateral budding. Septate hyphae is not formed but pseudohyphae may be produced. The vegetative cells are predominantly diploid (or higher ploidy) that undergo sexual reproduction by sporulation to form an ascus containing one to four round or slightly oval shaped ascospores, with smooth walls. The ascus has a persistent integrity, but eventually the haploid ascospores germinate and conjugate to re-form a diploid cell. Vigorous fermentation of hexose sugars is a trait of the genus, but the profile of sugars fermented or assimilated varies with the species. Throughout the past 100–150 years, many species within the genus have been described on the basis of various combinations of morphological, physiological and biochemical properties. As information has evolved, species have been established, dis-established, re-established or transferred to other genera, causing much confusion to researchers and industry professionals. Thus, wine yeasts well known as *Saccharomyces ellipsoideus* or *Saccharomyces oviformis* in the old literature are now described as *S. cerevisiae* or *S. bayanus*. Well-known brewing yeasts such as *Saccharomyces carlsbergensis* and *Saccharomyces uvarum* have been re-named as *S. cerevisiae* or *Saccharomyces pastorianus*. The historical development of these changes and underlying motivations have been comprehensively reviewed by Barnett (1992) and Vaughan-Martini and Martini (1995), where informative tables showing changes to species names over time have been published. A similar table is given in Deák and Beuchat (1996).

Molecular approaches to yeast taxonomy are now being used to classify *Saccharomyces* yeasts on phylogenetic principles, but this process is still in a state of development. Initially, DNA hybridisation homologies were used to redefine the genus (Vaughan-Martini and Martini, 1995, 1998) and, to a significant extent, this was supported by electrophoretic karyotyping data (Vaughan-Martini *et al*., 1993; Cardinali and Martini, 1994). More recently, sequence data of ribosomal DNA and other genes are being applied (Oda *et al*., 1997; Kurtzman and Robnett, 1998, 2003; Daniel and Meyer, 2003). Species recognised in the last taxonomic description of the genus by Vaughan-Martini and Martini (1998) and Barnett *et al.* (2000) are listed in Table 11.1 along with their differentiating properties. Since then, several new species have been described, based on combinations of phenotypic and genetic criteria. These species include *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae* (Naumov *et al*., 2000a), *Saccharomyces naganishii*, *Saccharomyces humaticus* and *Saccharomyces yakushimaensis* (Mikata *et al*., 2001) and *Saccharomyces turicensis* (Wyder *et al*., 1999) (Table 11.1). The probiotic yeast *Saccharomyces boulardii* is currently recognised as a variety of *S. cerevisiae* (Vaughan-Martini and Martini, 1998; van der Aa Kuhle and Jespersen, 2003), but can be distinguished from
<table>
<thead>
<tr>
<th>Species</th>
<th>Fermentation</th>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Resistance to cycloheximide</th>
<th>Growth temperature</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maltose</td>
<td>Sucrose</td>
<td>Trehalose</td>
<td>Melibiose</td>
<td>Raffinose</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>v</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. bayanus</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>v</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. paradoxus</td>
<td>v</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>S. pastorianus</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>S. cariocanus</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>S. kudriavzevii</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>?</td>
<td>+</td>
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<tr>
<td>S. mikatae</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>S. barnetti</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>S. castellii</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>v</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>S. dairenensis</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. exigus</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. kunashirensis</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. martiniae</td>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. rosinii</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. servazzii</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. spencerorum</td>
<td>–</td>
<td>+</td>
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<td>S. transvaalensis</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>S. unisporus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. kluyveri</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. naganishii</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. humaticus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. yakushinaensis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. turicensis</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Reactions: +, positive; –, negative; v, variable.
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*S. cerevisiae* by many molecular criteria including chromosome karyotyping, sequencing and various PCR-based (polymerase chain reaction) methods (Mitterdorfer *et al.*, 2002; Posteraro *et al.*, 2005). Nevertheless, it is very closely related to *S. cerevisiae*.

Over the years, *Saccharomyces* species have been divided into two main groups – the *Saccharomyces sensu stricto* group and the *Saccharomyces sensu lato* group, largely based on DNA hybridisation homology, but also supported by karyotyping data and ecological associations. The *sensu stricto* group comprises all those species phylogenetically related to *S. cerevisiae*, and generally associated with the production of alcoholic beverages. These are *S. cerevisiae*, *Saccharomyces paradoxus*, *S. pastorianus* and *S. bayanus* and, more recently, *S. cariocanus*, *S. kudriavzevii* and *S. mikatae*. There is increasing genetic and DNA sequencing evidence to suggest that *S. pastorianus* is a hybrid of *S. cerevisiae* and *S. bayanus* and that, generally, there has been significant interspecific hybridisation within species of *Saccharomyces sensu stricto* (Masneuf *et al.*, 1998; Naumov *et al.*, 2000b; de Barros Lopes *et al.*, 2002; Kurtzman and Robnett, 2003). The *sensu lato* group comprises most other species in the genus except *Saccharomyces kluyveri*, which has very little DNA homology with other species in this group. Based on sequence data of multiple genes, *Saccharomyces sensu lato* forms a clade distinct from *Saccharomyces sensu stricto*, providing strong arguments to relocate some of these species to other genera (Kurtzman and Robnett, 2003). According to Vaughan-Martini and Martini (personal communication), the next taxonomic revision of the genus *Saccharomyces* will recognise the following species only: *S. bayanus*, *S. cerevisiae*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus* and *S. pastorianus*, and possibly *S. uvarum*. This restructure is supported by the phylogenetic analysis of Kurtzman (2003) who proposes taxonomic reorganisation of the genus as shown in Table 11.2, where only species within the *sensu stricto* group are retained as *Saccharomyces*, and other species are re-located to genera of *Kazachstania*, *Naumovia* and *Lachancea*.

### 11.3 Association with food and beverage spoilage

The species of *Saccharomyces* show considerable diversity in habitats, with some species being intimately associated with food and beverage fermentations (Phaff and Starmer, 1987). In most cases, these are controlled, deliberate fermentation processes with positive commercial objectives (e.g. production of bread and alcoholic beverages). Nevertheless, there are many occasions where the presence and growth of *Saccharomyces* yeasts are not wanted, and the outcome is product spoilage. Interestingly, the type or representative strains of many species within the genus were isolated from foods or beverages (e.g. *S. barnetti*, *S. castellii*, *S. cerevisiae*, *S. dairenensis*, *S. exigus*, *S. pastorianus*, *S. turicensis*, *S. unisporus*) (Vaughan-Martini and Martini, 1998). However, the other species in the genus (Table 11.1) have soil, plant or
insect origins. There has been much debate about the natural origin of the so-called ‘domesticated’ or ‘industrialised’ species of *S. cerevisiae* and *S. bayanus*, which are intimately associated with winery and brewing environments (Mortimer and Polsinelli, 1999; Torok *et al*., 1996). There is a view that such species may not occur in natural habitats, but have evolved as ‘industrial’ species through selective adaptation from *S. paradoxus*, which is the species naturally associated with plant environments (Vaughan-Martini and Martini, 1995; Querol *et al*., 2003). Numerous researchers have not been able to isolate *S. cerevisiae* or *S. bayanus* from the surfaces of healthy, freshly harvested fruits, including grapes, leading to the conclusion that they are not naturally associated with such habitats (Martini *et al*., 1996). Nevertheless, with appropriate enrichment methods, it is possible to isolate *S. cerevisiae* from the surfaces of fruits (Fleet *et al*., 2002; Fleet, 2003a) and other plant ecosystems, along with *S. paradoxus* (Sniegowski *et al*., 2002; Sweeney *et al*., 2004). Moreover, *S. paradoxus*, not normally associated with industrial fermentations, was recently found as an indigenous species in wine fermentations (Redzepovic *et al*., 2002). Thus, knowledge and understanding about the ecology of *Saccharomyces* yeasts are still evolving. Interestingly, DNA sequences homologous with *S. cerevisiae* were detected in residues of a wine jar recovered from an ancient Egyptian tomb dated at about 3000 BC (Cavalieri *et al*., 2003).

| *S. cerevisiae* | *Saccharomyces* |
| *S. paradoxus* | | |
| *S. mikatae* | | |
| *S. cariocanus* | | |
| *S. kudriavzevii* | | |
| *S. pastorianus* | | |
| *S. bayanus* | | |
| *S. servazzii* | | |
| *S. unisporus* | | |
| *S. transvaalensis* | | |
| *S. martiniae* | | |
| *S. spencerorum* | | |
| *S. rostii* | | |
| *S. kunashirensis* | | |
| *S. exigus* | | |
| *S. turicensis* | | |
| *S. barnetti* | | |
| *S. castellii* | | |
| *S. dairenensis* | | |
| *S. kluyveri* | | |
| *Lachancea* | | |

Table 11.2 Proposed reorganisation of the taxonomic classification of species within the genus *Saccharomyces* (Kurtzman, 2003)
The spoilage of foods and beverages by yeasts, generally, has been the topic of many comprehensive reviews (Walker and Ayres, 1970; Fleet, 1990a, 1992; Deák, 1991; Tudor and Board, 1993; Thomas, 1993; Deák and Beuchat, 1996; Loureiro and Querol, 1999; Loureiro, 2000). In particular, the reader is referred to the extensive and informative tables given in Deák (1991), Tudor and Board (1993) and Deák and Beuchat (1996) that summarise individual species, the foods and beverages spoiled by these species and the relevant literature references. These tables include good coverage of the *Saccharomyces* species. According to Deák and Beuchat (1996), *S. cerevisiae* is one of the most frequently reported yeasts, isolated from foods and beverages. Table 11.3 gives an overview of the species of *Saccharomyces* associated with food and beverage spoilage.

### 11.3.1 Alcoholic beverages

*Saccharomyces cerevisiae* and *S. bayanus* are frequently implicated in the spoilage of alcoholic beverages such as beer, wine and cider (Thomas, 1993; du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003). Inappropriate strains of these species may grow during the alcoholic fermentation stage of these processes, leading to final product with grossly unacceptable flavours (e.g. excessive esters, diacetyl, sulphur volatiles, acetic acid) (Dufour et al., 2003; Lambrechts and Pretorius, 2000; Fleet, 2001). To avoid this outcome, most beer brewers and many winemakers now use selected strains of *S. cerevisiae* or *S. bayanus* as starter cultures to conduct their fermentations (Degre, 1993; Henschke, 1997). The grape juice used for wine fermentations, however, is not sterile and contains numerous indigenous species of yeasts, including *Saccharomyces*. These species may grow in conjunction with any added starter culture, and positively or negatively affect the quality of the final wine (Lambrechts and Pretorius, 2000; Fleet, 2003b). A similar situation occurs in traditional cider fermentations (Morrissey et al., 2004).

### Table 11.3 *Saccharomyces* species associated with the spoilage of various food and beverage commodities

<table>
<thead>
<tr>
<th>Commodity group</th>
<th><em>Saccharomyces</em> species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits, fruit concentrates, fruit juices, drinks</td>
<td><em>S. cerevisiae, S. bayanus, S. pastorianus,</em></td>
</tr>
<tr>
<td>Vegetable salads</td>
<td><em>S. exiguus, S. dairenensis, S. bayanus, S. unisporus</em></td>
</tr>
<tr>
<td>Dairy products</td>
<td><em>S. cerevisiae, S. dairenensis, S. exiguus, S. kluyveri</em></td>
</tr>
<tr>
<td>Meat products</td>
<td><em>S. cerevisiae, S. exiguus</em></td>
</tr>
<tr>
<td>Bakery products</td>
<td><em>S. cerevisiae, S. exiguus, S. unisporus, S. bayanus,</em></td>
</tr>
<tr>
<td>High-sugar products</td>
<td><em>S. cerevisiae</em></td>
</tr>
<tr>
<td>Alcoholic beverages</td>
<td><em>S. cerevisiae, S. bayanus, S. pastorianus</em></td>
</tr>
</tbody>
</table>
Many beer and wine products after packaging contain residual, fermentable sugars as part of their sensory profile and requirements for consumer appeal. Refermentation of these products at this stage by *Saccharomyces* species is not an uncommon problem and leads to significant product spoilage (swollen, exploding package; cloudy, gassy product with off-flavours). (Thomas, 1993; Louriero and Malfeito-Ferreira, 2003). *Saccharomyces cerevisiae var diastaticus* can be particularly troublesome to the brewing industry because it also secretes a glucoamylase which enables it to ferment residual starch dextrins in packaged beer. Moreover, this strain can lead to phenolic off-flavours because of its ability to decarboxylate phenolic acids into flavour active phenols such as 4-vinyl guaiacol and styrene (Dufour et al., 2003). *Saccharomyces cerevisiae* occurs in African sorghum beers where in addition to its positive role in production, it may also cause spoilage (van der Aa Kuhle et al., 2001; Jespersen, 2003). The tolerance of *S. cerevisiae* and *S. bayanus* to high concentrations of ethanol (e.g. >15% v/v) is an underlying factor that contributes to their spoilage of alcoholic beverages (Chi and Arneborg, 2000; Bisson and Block, 2002).

### 11.3.2 Fruit products and carbonated beverages

Fleet (2003a) has reviewed the association of yeasts with fruit and fruit products. As mentioned already, *Saccharomyces* species are infrequently isolated from the surfaces of fresh, undamaged fruits, unless specific enrichment methods are used. Generally, species of *Cryptococcus*, *Rhodotorula*, *Metschnikowia*, *Aureobasidium* and *Candida* are prevalent at such habitats. In contrast, fruit juice concentrates, fruit pulps, packaged fruit juices and drinks and soft drinks are particularly prone to fermentative spoilage with *S. cerevisiae*, *S. bayanus* and to a lesser extent, *S. pastorianus* (Torok and King, 1991a; Deák and Beuchat, 1993a,b; Thomas, 1993; Sancho et al., 2000; Stratford et al., 2000; Arias et al., 2002; Heras-Vazquez et al., 2003). The ability to exhibit good growth at low pH (e.g. pH 3.0), high sugar concentration (50% w/v glucose), and low water activity (0.87–0.90) and to resist inactivation by heat processing are properties that favour yeasts in these products (Put and de Jong, 1980; Stratford et al., 2000). Occasionally *Saccharomyces kluyveri* (Deák and Beuchat (1996), *S. unisporus* (Heras-Vazquez et al., 2003), and *S. exigus* (Stratford and James, 2003) are also isolated from these products at the stage of spoilage.

### 11.3.3 Vegetable-salad products

*Saccharomyces* yeasts are rarely isolated from fresh vegetables or vegetable-type products, but more detailed and systematic research is needed. Generally, basidiomycetous yeasts are found in these habitats (Fleet 1990a, 1992; Deák and Beuchat, 1996). However, vegetable products that have been fermented, salted or acid preserved present altered ecosystems where, among other
yeasts, *Saccharomyces* species are notable for causing spoilage. *Saccharomyces exiguus* and *S. dairenensis* have been prominent in the spoilage of salad vegetables containing mayonnaise (Dennis and Buhagiar, 1980; Brocklehurst et al., 1983; Brocklehurst and Lund, 1984; Tornai-Lehoczki et al., 2003) while *S. unisporus* and *S. bayanus* have caused the spoilage of salads fermented with lactic acid bacteria (Bonestroo et al., 1993; Savard et al., 2002). Factors contributing to the specificity of this ecology are considered to be tolerance of the yeasts to lactic and acetic acids, and capacity to grow at the low temperatures (5–10°C) used to store these products. Vegetable silages, have also been reported to contain *S. exiguus* and *S. dairenensis* (Middelhoven and Franzen, 1986). Pectolytic strains of *S. cerevisiae* and *S. kluyveri* have been implicated in the softening and gaseous spoilage of fermented olives (Vaughn et al., 1972).

11.3.4 Dairy products

*Saccharomyces cerevisiae* gives very good growth in pasteurised milk but is rarely involved in spoilage of this product because of competition from faster growing bacteria (Fleet, 1990b; Roostita and Fleet, 1996a). *Saccharomyces cerevisiae*, *S. dairenensis*, *S. kluyveri* and *S. exiguus* have been sporadically isolated from a diversity of fermented dairy products but their occurrence and significance are generally secondary to other species such as *Debaryomyces hansenii, Yarrowia lipolytica* and *Kluyveromyces marxianus*, which are better suited to the high-salt, high-fat, high-protein and lactose-enriched environments that these products may present (Fleet and Mian, 1987; Rohm et al., 1992; Roostita and Fleet, 1996b; Suzzi et al., 2000; Vasdinyei and Deák, 2003; Frohlich-Wyder, 2003). They have been isolated from yoghurts that have undergone fermentative spoilage, and they have undefined positive and negative roles as part of the indigenous, maturation flora of many cheeses and other fermented dairy products such as kefir (Fleet, 1990b; Hansen and Jakobsen, 2001; Beshkova et al., 2002; Addis et al., 2003; Frohlich-Wyder, 2003; Narhvus and Gadaga, 2003).

11.3.5 Meat products

The occurrence and significance of yeasts in fresh and processed meat, poultry and seafood products have been well reviewed (Fung and Liang, 1990; Dillon and Board, 1991; Tudor and Board, 1993; Samelis and Sofos, 2003). Apart from the very occasional isolation of *S. cerevisiae* and *S. exiguus*, *Saccharomyces* species are not significant in these products. Recent surveys of yeasts in poultry meat products and processed red meats (Viljoen et al., 1998; Ismail et al., 2000; Hinton et al., 2002) confirm these conclusions. Meat products present ecosystems that have little fermentable sugars, are high in protein and fat content, and may contain significant salt. These conditions are not conducive to the growth of *Saccharomyces* yeasts.
11.3.6 Bakery products
The positive roles of *S. cerevisiae* (baker’s yeast) in the fermentation of conventional type breads, and combined *S. cerevisiae* and *S. exigus* in the production of sourdough and other traditional bakery products are well documented (Pulvirenti *et al.*, 2004; Vernocchi *et al.*, 2004; Foschino *et al.*, 2004). These yeasts are prevalent in the baking environment and, inevitably, will also contaminate the final products. If such contamination is excessive and the products are stored for lengthy periods under inappropriate conditions, these species can grow causing chalky, white spot discoloration of the bread and distinct fermentative off-flavours (Seiler, 1980; Legan and Voysey, 1991). *Saccharomyces unisporus*, *S. bayanus* and *S. pastorianus* may also be involved in the fermentation of some traditional bakery products but further research is needed to determine whether their presence has positive or negative outcomes (Hammes *et al.*, 2005).

11.3.7 High-sugar products
Foods containing particularly high concentrations of sugars (e.g. sugar syrups, jams, molasses, fruit concentrates, confectionery) are selective habitats for fermentative yeast spoilage. While osmo- or xerotolerant yeasts such as *Zygosaccharomyces rouxii*, *Schizosaccharomyces* spp. and some *Candida* and *Pichia* spp. usually predominate in these spoilage outbreaks, it is not unusual to isolate *S. cerevisiae* from these products (Tilbury, 1980; Tokuoka, 1993).

11.4 Public health significance of *Saccharomyces*
Although not seen as spoilage in the context of sensory acceptability, foods are also considered unacceptable, and rejected, if they pose a risk to public health. Yeasts are rarely, if at all, associated with outbreaks of foodborne infections or intoxications (Fleet, 1992). Nevertheless, many yeast species are considered as opportunistic pathogens, capable of causing a range of mucocutaneous, cutaneous, respiratory, central nervous and systemic infections, occasionally with fatal consequences (Hazen and Howell, 2003; Georgiev, 2003). It is pertinent to note here that *S. cerevisiae* has pathogenic potential and, over the years, has been linked to a number of infections in humans (Murphy and Kavanagh, 1999; Wheeler *et al.*, 2003; Llanos *et al.*, 2004). Its sub-species *S. cerevisiae* var *boulardii*, now used as a biotherapeutic and probiotic agent, has also been implicated in several cases of human infections, principally fungaemia (Cassone *et al.*, 2003; van der Aa Kuhle *et al.*, 2005).
11.5 Factors affecting survival and growth

Control of commodity spoilage by *Saccharomyces* species depends on the application and maintenance of processing and storage conditions that either inactivate (kill) the cells, or prevent their growth. Many environmental factors (e.g. temperature, pH, water activity, chemical preservatives, physical matrix) determine the physical and chemical limits of tolerance and growth of these yeasts, but precise descriptions of such limits have become a challenging task, because the impact of one factor is often moderated by the influence of other factors (Fleet, 1992; Deák and Beuchat, 1996). For example, the minimum temperature for growth varies, depending on the pH of the medium or the concentrations of any sugar or salt that may be present. The effect of combined environmental stresses on the survival and growth of yeasts is often difficult to predict because it may be additive or synergistic (interactive), where the outcome is significantly greater (or less) than adding the responses to single stresses (Praphailong and Fleet, 1997; Betts et al., 2000; Battey et al., 2002).

Two other concepts are also important in determining the yeast survival or growth response, namely, the physiological status of the cell and any adaptive behaviour. Stationary phase cells are, generally, more tolerant of physical and chemical stresses than exponential phase cells, and cells exposed to sublethal stresses may initiate adaptive reactions to enhance their survival and growth responses. Most food and beverage commodities present ecosystems where yeast cells are simultaneously exposed to multiple stresses and, because of their long shelf-life, there is good opportunity for adaptive responses to occur (Fleet, 1999; Querol et al., 2003). The reader is referred to Walker (1977), Rose (1987), Fleet (1992, 1999), Deák (1991) and Deák and Beuchat (1996) for more comprehensive accounts of factors affecting the survival and growth of yeasts, in general. Ingram (1958) gives a good account of the early literature on this topic. Unfortunately, few data are known about factors that affect the growth and survival of *Saccharomyces* species other than *S. cerevisiae*.

11.5.1 Temperature

*Saccharomyces* species generally exhibit strong growth in the range 15–30 °C. Growth rates are substantially decreased and lag phases are extended as the temperature decreases below 10 °C. Growth can occur at temperatures below 5 °C, and these yeasts have been reported to spoil fruit products stored at temperatures as low as –5 °C to –10 °C. Grape juice stored at –2 °C to –5 °C exhibited fermentative spoilage after 4–8 weeks, and a *Saccharomyces* strain isolated from frozen berries exhibited growth on agar media at –9 °C after 3 months (Pederson et al., 1961; Walker, 1977). The lowest temperature at which growth ceases for these yeasts is not well described, but there are good data showing growth of *S. cerevisiae*, *S. bayanus*, *S. unisporus*, *S. dairiensis* and *S. exigus* at 4–7 °C generally within a 1–3 week period (Recca and Mrak, 1952; Brocklehurst et al., 1983; Jermini and Schmidt-
Lorenz, 1987; Bonestroo et al., 1993; Savard et al., 2002). *Saccharomyces bayanus* is reported to be more cryotolerant than *S. cerevisiae*, growing faster at lower temperatures (Sipiczki, 2002). As mentioned already, the minimum growth temperature is moderated by other factors such as pH, sugar concentration and salt concentration (Fleet, 1992; Betts et al., 1999). Thus, the presence of 50% (w/v) glucose shifts the minimum temperature for growth of *S. cerevisiae* from values below 5 °C to values near 10 °C (Jermini and Schmidt-Lorenz, 1987). Cells (10⁹ cfu/ml) of *S. cerevisiae*, washed free of nutrients and stored in weak phosphate buffer (pH not indicated) lost approximately 95% of their viability after 2 years at 3–5 °C, and 99% of their viability after 1 year at −40 °C (Tanguay and Bogert, 1974).

Most *Saccharomyces* yeasts do not grow above 37 °C, but a few strains of *S. cerevisiae* have been reported to grow at 41–43 °C (Barnett et al., 2000). At higher temperatures (e.g. 60–65 °C), they are rapidly inactivated. Numerous heat inactivation studies have been conducted with *S. cerevisiae* because of its significance in the spoilage of heat pasteurised fruit juices and carbonated beverages (Fleet, 1992; Thomas, 1993; Stratford and James, 2003). Decimal reduction values (*D*) at 55–60 °C for *S. cerevisiae* are in the range 1–20 minutes, depending on the strain and food matrix, and make it one of the most heat-resistant yeasts that has been examined. Corresponding *z* values range from 4 to 6.5 °C (Put et al., 1976; Put and de Jong, 1982; Torok and King, 1991b; Shearer et al., 2002). However, its thermal tolerance is significantly affected by other factors, and is increased by the presence of sugars (Beuchat, 1983; Torreggiani and Toledo, 1986) and decreased by conditions such as lower pH and presence of preservatives (Fleet, 1992; Shearer et al., 2002). Moreover, the ascospores of *S. cerevisiae* are about 100-fold more heat resistant than the vegetative cells, as determined by comparing their *D*₆₀ values (Put and de Jong, 1980, 1982; Fleet, 1992). Heat processing (e.g. 70–75 °C for 10–15 minutes; 95 °C for 3–5 seconds) is one of the hurdles used to control yeast spoilage of fruit juices, fruit drinks and soft drinks (Put and de Jong, 1980; Stratford et al., 2000).

### 11.5.2 Sugar and salt concentration; water activity (*a*ₜ)

The *Saccharomyces* species do not grow at *a*ₜ values less than 0.85–0.88, and this distinguishes them from the more osmo- or xero-tolerant yeasts in genera such as *Zygosaccharomyces*, *Pichia*, *Debaryomyces* and *Schizosaccharomyces* (Tokuoka, 1993; Lagos et al., 1999). In addition to concentration, the type of solute determines the minimum *a*ₜ for growth. For one strain of *S. cerevisiae*, isolated from candied apple, the minimum *a*ₜ for growth was 0.89 (glucose), 0.91 (fructose), 0.89 (sucrose) and 0.92 (NaCl) (Tokuoka et al., 1985). Under optimal conditions, *Saccharomyces* spp. do not grow in environments where the sugar (glucose) concentration exceeds 50% (w/v) or the salt (NaCl) concentration exceeds 10–12% (w/v) (Table 11.1). However, there is significant species and strain variation in response
to these conditions (Vaughan-Martini and Martini, 1998; Betts et al., 1999; Hansen and Jakobsen, 2001) and, moreover, the upper limits for growth are significantly decreased in the presence of other stresses such as low pH, low temperature and the presence of other antimicrobials (Tilbury, 1980). For example, *S. cerevisiae* may grow in the presence of 7.5% (w/v) NaCl at pH 5.0–7.0, but not at pH 3.0, where its salt tolerance was decreased to 5% (w/v) (Praphailong and Fleet, 1997).

### 11.5.3 Acids and pH

There are few, systematic, comparative studies on the growth responses of *Saccharomyces* species to pH, and the weak organic acids (propionic, acetic, sorbic, benzoic) and inorganic acids (sulphurous, carbonic) that are commonly used as food preservatives. Like most yeasts, they give strong growth under acid conditions (pH 3.0–7.0), with limiting values being around pH 1.5–2.5 and pH 8.0–8.5, at least for *S. cerevisiae* (Praphailong and Fleet, 1997; Betts et al., 1999). Inhibition by the weak organic and inorganic acids is strongest when they occur in their undissociated form (e.g. at pH 3.5–4.0). Some minimum concentrations reported to inhibit *S. cerevisiae* at pH 3.5 are: sulphurous acid (sulphur dioxide, 75–100 mg/l; benzoic acid 100–600 mg/l; sorbic acid 200–600 mg/l; acetic acid 10 g/l; and propionic acid, 6 g/l (Fleet, 1992; Praphailong and Fleet, 1997).

Maimer and Busse (1992) investigated the combined effects of Brix and sorbic acid concentrations on growth and gas production by *S. cerevisiae* in strawberry homogenates at pH 4.0. Up to 600 ppm of sorbic acid was needed to inhibit the yeast, but this depended on the Brix value, with less acid being needed as the Brix increased. No yeast growth and gas production were observed in homogenates at 45°Brix with 200 ppm of sorbic acid.

Savard et al. (2002) have reported the effects of acetic and propionic acids on the growth of *S. bayanus* and *S. unisporus* but the experiments were conducted in a fermented vegetable juice medium, pH 3.74, that also contained lactic acid. Consequently, it is not possible to compare these data with those published for *S. cerevisiae*. However, *S. unisporus* was substantially more resistant than *S. bayanus* to the inhibitory effects of propionic and acetic acids.

### 11.5.4 Ethanol and carbon dioxide (CO₂)

Ethanol and carbon dioxide are the main end-products of fermentative spoilage by *Saccharomyces* yeasts and, consequently, become significant for their impact on the continued growth and survival of the organisms. Bisson and Block (2002) have reviewed the ethanol tolerance of *S. cerevisiae* and *S. bayanus* and note the importance of distinguishing between the properties of ethanol production and ethanol tolerance. Most strains of *S. cerevisiae* and *S. bayanus* can survive exposure to ethanol concentrations as high as 20%
(v/v), but may not produce such high amounts during fermentation. The maximum amount produced during fermentation varies with the strain, with the majority having the ability to produce 10–15% (v/v). However, many factors affect ethanol tolerance and production by yeasts, and these include temperature, dissolved oxygen concentration, pH and presence of organic acids, and culture conditions (Bisson and Block, 2002; Pina et al., 2004). There appears to be no literature on the comparative ethanol tolerance of species of Saccharomyces other than S. cerevisiae and S. bayanus.

Although Saccharomyces species are quite tolerant of CO₂ at normal, atmospheric pressure, their growth is inhibited at approximately 1 × 10⁵ – 4 × 10⁵ Pa (2 atmospheres), and the cells are inactivated at higher pressures (e.g. 30 atmospheres) (Jones and Greenfield, 1982; Ison and Gutteridge, 1987; Slaughter, 1989; Fleet, 1992).

11.5.5 New processing technologies
In recent years, a diversity of alternative technologies has been researched and developed for processing foods and beverages. These new approaches are being driven by consumer demands for less intensively processed products that have a more natural, healthier appeal, but still retain the functional qualities of good shelf-life and stability, safety and sensory appeal (Gould, 2000; Food and Drug Administration Report, 2001).

Plant extracts have been examined for their potential to serve as novel antimicrobials that could be used to minimise or replace the use of traditional preservatives such as benzoic acid or sorbic acid. Essential oils extracted from various herbs and species and other plants inhibit a broad spectrum of food spoilage yeasts, including S. cerevisiae (Conner and Beuchat, 1984; Araujo et al., 2003). The essential oils of garlic and onion are particularly effective, with minimum inhibitory concentrations being around 20 ppm for S. cerevisiae. Moreover, these extracts exhibit their anti-yeast effect across a wide pH range (e.g. pH 3.0–8.0) (Kim et al., 2004). Vanillin (4-hydroxy-3-methoxy benzaldehyde), which is extracted from vanilla beans, is inhibitory to S. cerevisiae and other yeasts at concentrations of 20 mM, but lower concentrations can be effective when combined with other hurdles such as decreased temperature and lower pH (Cerrutti and Alzamora, 1996; Fitzgerald et al., 2004). Many plant extracts are powerful flavourants, so the challenge becomes one of developing applications where yeast inhibition is effective but the extract does not compromise sensory appeal of the product (Araujo et al., 2003; Fitzgerald et al., 2004).

Exponential phase cells of S. cerevisiae are inactivated by exposure to low-intensity electric fields and high-intensity pulsed electric fields, although somewhat more slowly by the former process (Qin et al., 1996; Aronsson and Ronner, 2001; Guillou and El Murr, 2002; Ranalli et al., 2002; Geveke and Brunkhorst, 2003; Elez-Martinez et al., 2004). These concepts are being developed as non-thermal strategies for food processing. A 5-log reduction
in the viability of *S. cerevisiae* in orange juice was achieved after exposure to a high-intensity, pulsed electric field for 1000 µs (35 kV/cm, bipolar pulses with 4 µs width, 200 Hz, 32°C). A similar log-reduction by thermal processing required exposure of the juice to 90°C for 1 minute (Elez-Martinez *et al*., 2004). Many variables such as electric field strength, time of exposure, temperature, pH and water activity affect the efficacy of yeast inactivation by electric fields which appear to disrupt membrane function within the cells (Ranalli *et al*., 2002; Elez-Martinez *et al*., 2004). The inactivation kinetics of other *Saccharomyces* species by electric fields are yet to be reported.

Exposure to high hydrostatic pressure is another non-thermal process that is being used in food and beverage production to inactivate microorganisms, including yeasts (Smelt, 1998). Exponential phase cells of *S. cerevisiae* are quickly inactivated at pressures above 300 MPa. At this pressure, *D* values are around 1 minute or less at 25–30°C (Pandya *et al*., 1995; Chen and Tseng, 1997; Zook *et al*., 1999; Basak *et al*., 2002), but stationary phase cells are slightly more resistant (Brul *et al*., 2000; Donsi *et al*., 2003). Under similar conditions, *D* values for ascospores of *S. cerevisiae* are about 7–10 minutes, being much more resistant to the process (Parish, 1998; Zook *et al*., 1999).

11.5.6 Stress and adaptation

Food and beverage ecosystems are rarely static with respect to their intrinsic and extrinsic properties. Moreover, products that are stored for any lengthy period present a long time-frame relative to the yeast cell cycle (Fleet, 1999). Thus, opportunities exist for the adaptation and selection of yeast strains with properties to survive and grow in the new environment. The wine industry, for example, has relied on the use of sulphur dioxide as an antioxidant and antimicrobial for hundreds of years, so it is not surprising to find wine strains of *S. cerevisiae* with enhanced ability to resist this substance (Querol *et al*., 2003). Similarly, it would not be unusual to find more osmotolerant strains of *Saccharomyces* in the fruit juice concentrate industry or more benzoate or sorbate-tolerant strains associated with the carbonated beverage industry. Erasmus *et al.* (2003) have discussed the metabolic adaptation of *S. cerevisiae* to high-sugar environments.

Yeasts react quickly to change in the external environment by shifting their metabolic and physiological behaviour to tolerate the new conditions. For example, an array of protective, heat shock proteins is produced in response to temperature stresses, intracellular glycerol is produced as an osmoprotectant against increases in external sugar or salt concentrations, and proton and anion pumping mechanisms are activated to accommodate acid and pH stresses. These responses are well coordinated and regulated at the genetic level, and substantial research has been done to understand the associated molecular mechanisms. Discussion of these mechanisms is outside
the scope of this chapter and the reader is referred to detailed reviews by Bauer and Pretorius (2000), Piper et al. (2001) and the book by Hohmann and Mager (2003).

11.6 Biochemical basis of spoilage

Fermentation of sugars (glucose, fructose, sucrose, maltose) is the principal spoilage reaction of *Saccharomyces* species. The main products of the reaction are carbon dioxide and ethanol. Carbon dioxide gives the product a gassy, frothy appearance and causes packaged products to swell and explode. In addition, the products develop a distinctive alcoholic, fermentative smell and taste. A vast array of secondary metabolites (e.g. organic acids, higher alcohols, esters, aldehydes, ketones, sulphur volatiles) are also produced and contribute to changes in the sensory properties of the product. The production of these secondary metabolites, their sensory thresholds and impact, and the biochemical mechanisms of their formation are well known for *S. cerevisiae* and *S. bayanus* because of their importance in contribution to the flavour of alcoholic beverages (Fleet, 1992; Lema et al., 1996; Berry, 1997; Heard, 1999; Lambrechts and Pretorius, 2000; Wang et al., 2003). The profile of secondary products generated can vary significantly, depending on yeast strain and environmental conditions, but are not well described for *Saccharomyces* species other than *S. cerevisiae* and *S. bayanus* (Pérez-Coello et al., 1999; Plata et al., 2003). Some strains of *S. cerevisiae* and *S. bayanus* can produce overpowering, spoilage concentrations of acetic acid and hydrogen sulphide (H₂S). Generally, these concentrations are greater than 1–1.5 g/l for acetic acid (Radler, 1993; Henschke, 1997) and greater than 75–100 µg/l for H₂S (Rauhut, 1993; Spiropoulos et al., 2000; Mendes-Ferreira et al., 2002).

*Saccharomyces* yeasts are not known for their production of extracellular hydrolytic enzymes such as proteases, lipases, amylases, pectinases and cellulases (Vaughan-Martini and Martini, 1998; Barnett et al., 2000). Consequently, they are unlikely to degrade the macromolecules of food structure. However, more detailed, systematic analyses of *Saccharomyces* species for these enzymes are needed (Charoenchai et al., 1997; Buzzini and Martini, 2002). Some strains of *S. cerevisiae* and *S. bayanus* are reported to be pectolytic (McKay, 1990; Gainvors et al., 1994; Blanco et al., 1994; van Rensburg and Pretorius, 2000; Gognies et al., 2001; Radoi et al., 2005) and there are conflicting reports on extracellular protease production by these yeasts (Fleet, 1992; van Rensburg and Pretorius, 2000; Hansen and Jakobsen, 2001). Intracellular lipases, proteases and nucleases will be released when the yeast cells autolyse, and these enzymes can be expected to affect food and beverage properties (Charpentier and Feuillat, 1993; Connew, 1998; Zhao and Fleet, 2003).
11.7 Isolation, enumeration, identification

The isolation, enumeration and identification of *Saccharomyces* yeasts from foods and beverages follow the same principles and strategies for yeasts, in general. These involve the sequential operations of: rinsing or maceration of the sample; dilution of the suspension; enumeration of the yeast cells in suspension by agar plating, most probable number, membrane filtration or microscopic methods; purification of isolates, and identification of isolates to either genus, species or strain level. Details of these procedures have been reviewed by Fleet (1992), Beuchat (1993), Deák and Beuchat (1996), Kurtzman *et al.* (2003) and Deák (2003).

General media for their isolation and cultivation include malt extract agar, glucose–yeast extract–peptone agar, tryptone–glucose–yeast extract agar, WL–nutrient agar, and dichloran–rose bengal–chloramphenicol agar (Beuchat, 1993; Kurtzman *et al.*, 2003). Various antibiotics (e.g. oxytetracycline, gentamicin, chloramphenicol, penicillin) may be added to suppress the growth of bacteria, and supplementation with either propionic acid, biphenyl or dichloran is used to restrict the growth of filamentous fungi (Beuchat, 1993; Deák and Beuchat, 1996). Ethanol–sulphite–yeast extract agar has been used as a medium for the selective culture and isolation of *Saccharomyces* yeasts and exploits their tolerance to ethanol and sulphur dioxide (Kish *et al.*, 1983), but it is not a convenient medium to prepare, and other ethanol-tolerant yeasts may grow on this medium (Heard and Fleet, 1986). Lysine agar is a very useful medium to enumerate non-*Saccharomyces* yeasts when they are present in foods or beverages along with *Saccharomyces* species. It exploits the fact that most *Saccharomyces* species cannot utilise lysine as a nitrogen source and will not form colonies on this medium. However, some *Saccharomyces* (e.g. *S. unisporus*, *S. kluyveri*, Table 11.1), occasionally found in food ecosystems, can utilise lysine and grow on this agar (Heard and Fleet, 1986). Generally, agar plating media are incubated at 25–30°C for 2–7 days, after which colonies are examined.

Selection of any medium and cultivation conditions for the isolation and enumeration of *Saccharomyces* species should not overlook the fact that most foods and beverages are processed to varying extents and are likely to harbour a proportion of sublethally injured cells that may require resuscitation before analysis. Injured cells might not form colonies on acidified media or media containing increased concentrations of salt (NaCl) or sugar, thereby giving an underestimation of the true viable population (Beuchat, 1984; Deák and Beuchat, 1996; Fleet and Mian, 1998). Fleet and Mian (1998) examined the induction of sublethal injury in cells of *S. cerevisiae* after heating at 50°C for 5–15 minutes, or freezing at –196°C for 60 minutes. Approximately 20–25% of the cells were injured by the heat treatment as evidenced by their inability to grow on acidified malt extract agar (pH 3.5) or malt extract agar with 5% NaCl. Freezing the cells induced 55–65% injury as determined by culture on these two media. The injured cells could be resuscitated by incubation in malt extract broth (2%) at 25°C for 3 hours.
A useful list of media for the cultivation of yeasts is given in Kurtzman et al. (2003).

PCR-denaturing gradient gel electrophoresis (DGGE) is now being applied as a culture-independent molecular approach for the detection of yeast species in foods and beverages (Giraffa, 2004; Prakitchaiwattana et al., 2004), and several studies have reported the detection of *S. cerevisiae* by this method in wine fermentations (Mills et al. 2002), sourdough fermentation (Meroth et al., 2003) and coffee bean processing (Masoud et al., 2004). This approach should detect injured or non-culturable cells, since it depends on the analysis of extracted DNA, but numerous operations are involved and their conditions need to be properly optimised (Prakitchaiwattana et al., 2004).

The standard, conventional procedure for identifying *Saccharomyces* species is based on determination of a vast array of phenotypic tests: cell and sporulation morphology, fermentation of sugars, assimilation of various carbon and nitrogen substrates, growth at different temperatures and growth in the presence of various concentrations of glucose and salt (NaCl) (Vaughan-Martini and Martini, 1998) (see Table 11.1). To decrease the workload, time and tedium of these tests, various diagnostic kits for yeast identification have been developed. Generally, these kits assay the growth response of yeasts on a few, carefully selected substrates and have computerised systems for processing and comparing the data with reference databases. Such kits include the API 20C, ID 32C, SIM, RapID yeast Plus and Yeast Ident-Food systems and have been evaluated by various researchers over the years (Rohm et al., 1990; Torok and King, 1991a; Deák and Beuchat, 1993b, 1996; Velázquez et al., 2001; Arias et al., 2002; Robert, 2003). The BIOLOG system embraces similar principles, but uses a greater diversity and number of substrates, and has a more extensive database (Praphailong et al., 1997). Various *Saccharomyces* species (but not all) are included in the databases of these systems that generally give good but not unequivocal data. These kits provide a ‘first stage’, rapid diagnosis, that should be followed up with more definitive phenotypic or molecular tests.

Fatty acid profiling has been used as a rapid method to identify and differentiate *Saccharomyces* species from other yeasts (Malfeyto-Ferreira et al., 1997; Loureiro, 2000) and to differentiate *Saccharomyces* species within the *sensu stricto* and *sensu lato* groups (Botha and Kock, 1993). Electrophoretic isoenzyme profiles based on the esterase, acid phosphatase and glucose-6-phosphate dehydrogenase combination of enzymes, can differentiate *S. cerevisiae*, *S. bayanus*, *S. pastorianus* and *S. paradoxus* (Duarte et al., 1999).

Molecular methods have now been developed to the point that they can be routinely applied to yeast identification. They are faster and more convenient than cultural methods, and are considered to give definitive, more reliable data. In addition to genus and species identification, they also permit strain typing and differentiation. Application of these methods to food and beverage yeasts has been reviewed by Loureiro and Querol (1999), Kurtzman et al.
The various species of *Saccharomyces* can be distinguished and identified by sequencing of the ribosomal DNA genes (either 18S, 26S or ITS regions) (Naumov et al., 2000a; Mikata et al., 2001; Kurtzman and Robnett, 2003) and by electrophoretic karyotyping (Vaughan-Martini et al., 1993; Cardinali and Martini, 1994). A faster, simpler method for species identification is based on PCR-RFLP (restriction fragment length polymorphism) analysis of the ITS and other regions of ribosomal DNA and comparison of the fragment patterns, but digestion of the DNA with a combination of three to four different endonucleases (e.g. Hae III, Hpa II, Scr F1 and Taq I) may be needed to discriminate between all species, especially those in the *Saccharomyces sensu stricto* group (Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Naumova et al., 2003). PCR-RFLP analyses have received widespread application for the identification of *Saccharomyces* species, as well as other yeasts, in foods and beverages (e.g. Redzepovic et al., 2002; Naumova et al., 2003; Vasdinyei and Deák, 2003; Heras-Vazquez et al., 2003; Loureiro and Malfeito-Ferreira, 2003; Posteraro et al., 2005).

Simpler, faster methods are based on PCR profiles generated with oligonucleotide primers that target intron splice sites and other repetitive regions in DNA (de Barros Lopes et al., 1998; Hierro et al., 2004), but these approaches require more rigorous evaluation. Manzano et al. (2004) have described a rapid PCR-DGGE procedure that differentiates species within the *Saccharomyces sensu stricto* group. Kosse et al. (1997) outlined a strategy for designing oligonucleotide probes for the rapid identification of yeasts associated with the spoilage of yogurts. The probes targeted specific regions of the 18S rRNA, and included analysis for *S. cerevisiae*, *S. kluyveri* and *S. servazzii*.

As mentioned already, there is significant diversity in the metabolic profiles of *Saccharomyces* strains. Within the one species, there can be beneficial and detrimental strains. The ability to distinguish between strains is an important commercial need within this genus, especially for *S. cerevisiae* and *S. bayanus*. A broad range of molecular ‘fingerprinting’ methods have been examined for this purpose and are reviewed in Giudici and Pulvirenti (2002), van der Vossen et al. (2003) and Loureiro and Malfeito-Ferreira (2003). These methods include various applications of random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), RFLP and PCR-microsatellite procedures (Baleiras Couto et al., 1996; Hennequin et al., 2001; Mitterdorfer et al., 2002; Howell et al., 2004; Cocolin et al., 2004; Posteraro et al., 2005). A simpler, faster approach is based on RFLP analysis of mitochondrial DNA, where no PCR amplification of DNA is required (Querol et al., 1992; Lopez et al., 2001). This method has been widely used to differentiate wine strains of *S. cerevisiae* and *S. bayanus* (Loureiro and Malfeito-Ferreira, 2003; Schuller et al., 2004).
11.8 Prevention of spoilage

Because *Saccharomyces* species are widely distributed ecologically, and are widely used industrially, it is inevitable that they will contaminate many foods and beverages. The application of ‘total chain’ Hazard Analysis Critical Control Point (HACCP) and other quality management programmes is essential to prevent or minimise contamination at both the pre-harvest and post-harvest stages of production. Insects such as bees, wasps and fruit flies are major vectors of yeasts, and their control requires special consideration (Lachance et al., 1995). Poor cleaning and sanitation of process equipment provide another risk factor that contributes significantly to outbreaks of food and beverage spoilage by yeasts, and good effective practices are essential to prevent or minimise such occurrences. For heat-processed products, such as fruit juices, fruit pulps, fruit concentrates, sugar syrups and carbonated beverages, it is essential that time–temperature combinations be sufficient to cause yeast inactivation, and take into consideration the potential presence of any ascospores, and the protective effect of high-sugar concentrations within the product. Many *Saccharomyces* are psychrotrophic and can grow well at refrigeration temperatures. Refrigeration, therefore, will not necessarily prevent product spoilage. The lower the temperature, the longer the shelf-life, so good control of temperature is a critical requirement. Weak acid preservatives are used to control yeasts in many heat-processed and refrigerated products. For maximum efficiency of these agents, proper management of their concentration and product pH is essential, but preservative resistant strains can compromise this initiative. Finally, routine monitoring of raw materials and end-products should be conducted to ensure that they meet appropriate specifications (e.g. yeasts not detected in 1–10 g or 100–200 ml of product) (Loureiro and Querol, 1999; Stratford et al., 2000; Loureiro and Malfeito-Ferreira, 2003).

11.9 Future trends

The taxonomy of the *Saccharomyces* genus is still uncertain and remains in a state of change and development. Recent molecular analyses suggest that several of the species currently recognised in the genus are phylogenetically unrelated. Taxonomic revision and reconstruction of the genus are needed, and a likely outcome is that some species, especially those in the *sensu lato* group, will be transferred to other genera as proposed in Table 11.2. More research, using combinations of cultural and molecular, culture-independent methods is required to better define the ecological habitats of species within the genus. For many species, this ecology is poorly described, and for others (e.g. *S. cerevisiae, S. bayanus, S. paradoxus*) controversy still exists. Knowledge about the physical and chemical limits of growth and survival for these yeasts is very scant and more focused, systematic determination is required.
In particular, more information is needed about the impact of the newer processing technologies. Fundamental studies to understand the mechanisms by which these yeasts tolerate and respond to environmental stresses may yield novel strategies for controlling their growth and potential to spoil foods and beverages. The capacity to quickly identify and differentiate species and strains within the genus has been greatly facilitated by molecular techniques. Many molecular options are now available to perform these tasks, but they require evaluation and standardisation with respect to accuracy, precision, reproducibility, cost and convenience.

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12

Candida and related genera

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12.1 Introduction

To start, the terms ‘Candida’ and ‘related genera’ must be defined. Neither can be simply interpreted. Candida is the taxonomic name of an asexual (imperfect, anamorphic) yeast genus whose definition and circumscription need more detailed discussion and will be given below. It is the nomenclatural type genus of the family Candidaceae, and, in this sense, related genera means other taxons belonging to the family, such as Brettanomyces, Geotrichum, Kloeckera and some ten more imperfect genera (Table 12.1). The genus Candida, on the other hand, comprises a number of yeast species that have sexual (perfect, teleomorphic) counterparts belonging to several genera, among others Citeromyces, Debaryomyces, Issatchenkia, Metschnikowia, Yarrowia and more, which can also be considered related genera. It is the Editor’s intention that this second interpretation is to be followed in this chapter; its content, however, is to be restricted to certain teleomorphic genera to which Candida species are related, whereas several others (e.g. Saccharomyces, Kluyveromyces, Pichia) are the subjects of other chapters.

12.2 Brief history of the genus Candida

Viljoen and Kock (1989) gave a detailed account of the taxonomic adventures of the genus, to which the reader is referred for more information and further references. The history of the yeasts currently belonging to the genus goes back more than 150 years, though the name Candida was introduced only in
1923. Among the first yeasts discovered was one named *Torula* by Turpin in 1838. This name was, however, given earlier to a filamentous fungus by Person in 1796, hence Berlese changed the yeast taxon for *Torulopsis* in 1895. Nevertheless, the name *Torula* remained in use for a long time; moreover it was extended to a number of non-sporulating yeasts also not producing pseudohyphae. Others, with various kinds of pseudohyphae and also developing pellicle on liquids, were called *Mycoderma*, *Mycotorula*, *Asporomyces* and others. At the same time, in the medical field, *Monilia*, *Oidium*, *Blastomyces* and other names were in use, despite the fact that these were applied earlier to mycelial fungi producing conidia. In an effort to clear the confused situation, Berkhout, in 1923, introduced the name *Candida* for all those asexual yeasts that produced true or pseudohyphae, with the type species *Candida vulgaris* (now a synonym of *C. tropicalis*).

The nomenclatural and taxonomical adventures of this group of yeasts have not ceased. Lodder in 1934 excluded the red colored yeasts from the genus *Torulopsis*, among them the type species *T. pulcherrima*, rendering the taxonomic position of the genus uncertain. In correction, a new type species, *T. colliculosa*, was denoted by Lodder and Kreger-van Rij in 1952, which, however, turned out to be the anamorphic form of *Saccharomyces fermentati* (now *Torulaspora delbrueckii*). Van Uden and Buckley, in the 2nd edition of the taxonomic treatise of the yeasts in 1970, considered that the traditional criterion for separating *Candida* and *Torulopsis*, i.e. the presence or absence of hyphae, was uncertain and arbitrary. The two genera were formally merged by Yarrow and Meyer in 1978, under the name *Candida*, which was conserved contrary to the nomenclatural priority of *Torulopsis*. By this act, both the number and the heterogeneity of species in the genus increased tremendously. Unlike the majority, several species showed characteristics (such as cell wall composition and urease reaction) related to basidiomycetous yeasts, and many of these became reclassified in different genera as *Rhodotorula*,

<table>
<thead>
<tr>
<th>Genera belonging to the family:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aciculoonidium</em></td>
</tr>
<tr>
<td><em>Arxula</em></td>
</tr>
<tr>
<td><em>Blastobotrys</em></td>
</tr>
<tr>
<td><em>Botryozyma</em></td>
</tr>
<tr>
<td><em>Brettanomyces</em></td>
</tr>
<tr>
<td><em>Candida</em></td>
</tr>
<tr>
<td><em>Geotrichum</em></td>
</tr>
<tr>
<td><em>Kloeckera</em></td>
</tr>
<tr>
<td><em>Myxozyina</em></td>
</tr>
<tr>
<td><em>Schizoblastosporion</em></td>
</tr>
<tr>
<td><em>Sympodimyces</em></td>
</tr>
<tr>
<td><em>Trigonopsis</em></td>
</tr>
</tbody>
</table>

*According to Kurtzman and Fell (1998).
Cryptococcus, Apiotrichum and Vanrija. Finally, Van der Walt in 1987 proposed that the genus Candida and the whole family of Candidaceae should be reserved for yeasts of ascomyceteous affinity. The genus has been described in this sense by Meyer et al. (1998) in the latest edition of the taxonomic treatise. Still, a few anamorphic species with basidiomycetous teleomorphs bear the name Candida (e.g. C. scottii and C. japonica corresponding to Leucosporidium scottii and Filobasidium capsuligenum, respectively). The large number of species currently recognized as Candida remain heterogeneous in physiological and biochemical properties and show different anamorphic–teleomorphic (am./tm.) relationships. The treatment of the genus obviously needs further resolution.

12.3 Characteristics

Despite significant emendations, the genus Candida can be defined only as a broad group of asexual acomycetous yeasts, which, in common with several anamorphic genera, is characterized by a typical two-layered cell wall not containing xylose, rhamnose or fucose, and not showing positive Diazonium blue B or urease reactions. The genus Candida is distinguished from similar related genera mostly in negative properties, such as:

- not producing acetic acid (genus Brettanomyces);
- not budding bipolarly (Kloeckera, Schizoblastosporion);
- not having triangular cells (Trigonopsis);
- not producing blastoconidia on denticles, branches or conidiophore (Blastobotrys, Botryozyma, Sympodiomyces, Aciculoconidium);
- not producing arthroconidia (Arxula, Geotrichum);
- not producing extracellular slime and mucoid colonies (Myxozyma).

The genera listed in brackets are those belonging to the family Candidaceae. When all those possessing some peculiar traits are excluded, the genus Candida remains just a depository of the remaining anamorphic species whose cells may be of spherical, ellipsoidal or cylindrical shape, budding multilaterally, and may form true or pseudohyphae. Several of the genera listed above contain only a single species, some comprise 5 to 11 species. In contrast, 163 species of Candida were listed in the fourth edition of The Yeasts (Kurtzman and Fell, 1998); and since then dozens of new species have been described yearly (Table 12.2). In the preparation of the new edition of The Yeasts, at least 230 Candida species will be considered (MA Lachance, personal communication).

12.4 Teleomorphic connections

A number of Candida species have teleomorphic counterparts. Some species
were originally isolated in asexual form and later found to sporulate; these are considered for teleomorphic ascomycetous species (e.g. \textit{C. kefyr} = \textit{Kluyveromyces marxianus}). Other species have mating types and are heterothallic, thus when one of the mating types is isolated, it appears as a non-sporulating organism; when, however, both mating types are mixed, sexual spores are easily formed (e.g. \textit{C. lipolytica} = \textit{Yarrowia lipolytica}). In several cases, the anamorph–teleomorph relationships have been determined by molecular methods (DNA–DNA hybridization, rDNA sequencing; e.g. \textit{C. krusei} with \textit{Issatchenkia orientalis}). Currently, at least a dozen ascomycetous genera have \textit{Candida} anamorphs; the most frequent and food-related species are listed in Table 12.3. The anamorph of the most widely known species, \textit{Saccharomyces cerevisiae}, has been described as \textit{C. robusta}; this name is not in use because sporulating strains are common as the diploid cells of \textit{S. cerevisiae} can directly transform into asci with up to four ascospores.

Teleomorphic connections are also found in other anamorphic genera of the family Candidaceae. Most well known are \textit{Kloeckera} with \textit{Hanseniaspora}, and \textit{Brettanomyces} with \textit{Dekkera}. Species of \textit{Geotrichum} may have telemorphs in both \textit{Dipodascus} and \textit{Galactomyces}; in turn, the ascosporogenous genus \textit{Stephanoascus} has anamorphs in four different genera (\textit{Arxula}, \textit{Candida}, \textit{Blastobotrys} and \textit{Sympodiomyces}).

In the following treatment, the anamorph/teleomorph connections will be mentioned; however, species normally found in the ascosporogenous state are referred to in other chapters (e.g. \textit{Saccharomyces}, \textit{Kluyveromyces}, \textit{Pichia}, \textit{Torulaspora}).

### 12.5 Classification

In its present concept, the genus \textit{Candida} is extremely heterogeneous. Recent progress in the phylogenetic grouping of ascomycetous yeasts has also touched anamorphic species. Kurtzman and Robnett (1998) analyzed the phylogenetic
relationships among approximately 500 species of ascomycetous yeasts including about 190 members of Candida and other anamorphic genera, based on the extent of divergence in the variable D1/D2 domain of 26S rDNA. The polyphyly of Candida and Pichia was apparent, species of each genera belonged to several main clades. The Saccharomyces clade included C. glabrata and C. milleri, in addition to the anamorphic Candida states of Saccharomyces, Kluyveromyces and Torulaspora. The Debaryomyces clade, with C. famata, the anamorph of D. hansenii, also included the Pichia species with Q9 ubiquinone, e.g. P. guilliermondii (am. C. guilliermondii), and many Candida species (C. zeylanoides, C. diddensiae, C. tenuis); whereas C. albicans and several clinically important yeasts (C. tropicalis, C. parapsilosis) clustered with Lodderomyces elongisporus. More distantly related were Pichia (Hyphopichia) burtonii (am. C. variabilis) and C. catenulata. Other clades formed were the Pichia–Williopsis (with P. anomala, am. C. pelliculosa, and P. jadinii, am. C. utilis), the Pichia–Citeromyces (with the latter’s anamorph, C. globosa), and the Pichia–Issatchenka–Dekkera clade, with the type species, P. membranifaciens (am. C. valida), P. fermentans (am. C. lambica), P. nakasei (am. C. citrea), as well as I. orientalis (am. C. krusei) and other Candida species without known teleomorphs (C. vini, C. inconspicua, C. diversa). The Metschnikowia clade also contained Yarrowia lipolytica and Clavispora lusitaniae, with the respective anamorphs (C. pulcherrima, C. reukauffii, C. lipolytica, C. lusitaniae) as well as several other Candida

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**Table 12.3** Anamorph Candida species belonging to various teleomorph genera

<table>
<thead>
<tr>
<th>Anamorph</th>
<th>Teleomorph</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. colliculosa</td>
<td>Torulaspora delbrueckii</td>
</tr>
<tr>
<td>C. domercqiae</td>
<td>Wickerhamiella domercqiae</td>
</tr>
<tr>
<td>C. edax</td>
<td>Stephanoascus smithiae</td>
</tr>
<tr>
<td>C. famata</td>
<td>Debaryomyces hansenii</td>
</tr>
<tr>
<td>C. globosa</td>
<td>Citeromyces matritensis</td>
</tr>
<tr>
<td>C. guillermondii</td>
<td>Pichia guillermondii</td>
</tr>
<tr>
<td>C. helenica</td>
<td>Zygoascus helenicus</td>
</tr>
<tr>
<td>C. holmii</td>
<td>Saccharomyces exiguus</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>Kluyveromyces marxianus</td>
</tr>
<tr>
<td>C. krusei</td>
<td>Issatchenka orientalis</td>
</tr>
<tr>
<td>C. lambica</td>
<td>Pichia fermentans</td>
</tr>
<tr>
<td>C. lipolytica</td>
<td>Yarrowia lipolytica</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>Clavispora lusitaniae</td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td>Pichia anomala</td>
</tr>
<tr>
<td>C. pulcherrima</td>
<td>Metschnikowia pulcherrima</td>
</tr>
<tr>
<td>C. sorbosa</td>
<td>Issatchenka occidentalis</td>
</tr>
<tr>
<td>C. sphaerica</td>
<td>Kluyveromyces lactis</td>
</tr>
<tr>
<td>C. utilis</td>
<td>Pichia jadinii</td>
</tr>
<tr>
<td>C. valida</td>
<td>Pichia membranifaciens</td>
</tr>
<tr>
<td>C. variabilis</td>
<td>Pichia burtonii</td>
</tr>
<tr>
<td>C. vini</td>
<td>Pichia fluxuum</td>
</tr>
</tbody>
</table>
species (C. etchellsii, C. stellata, C. lactis-condensi, C. magnoliae, C. rugosa). The Stephanoascus clade comprised divergent ascosporogenous genera with Candida anamorphs (Wickerhamiella, Zygoascus) and morphologically varied anamorphic genera (Arxula, Blastobotrys, Sympodiomyces, Trigonopsis), as well as related Candida species such as C. versatilis, C. cantarellii. Recently, Kurtzman (2003) proposed new genera in the classification of the members of Saccharomycetaceae; the formal phylogenetic arrangement of the rest of ascomycetous yeasts has still remained to be done.

A most thorough approach for the phylogenetic grouping of Candida has been made by Suzuki and Nakase (1998, 1999, 2002) and Suzuki et al. (1999) who examined the cell wall composition, ubiquinone type, and 18S rDNA sequences for almost all species. The Japanese authors arrived at conclusions largely similar to those made by Kurtzman and Robnett (1998). They established four phylogenetically distinct clusters according to the presence or absence of galactose in cell wall (I, galactose absent; II and III, galactose predominant; and IV, galactose present); and within these clusters subgroups were formed based on the types of ubiquinone. In this way, group IA with Q6 included, among others, C. holmii, C. glabrata, C. colliculosa; IB with Q7 (C. utilis, C. pelliculosa, C. boidinii, C. valida, C. krusei, C. lambica); IC with Q8 (C. lusitaniae, C. globosa); ID with Q9 (C. albicans, C. tropicalis, C. parapsilosis). Cluster IIA contained species with Q8 and galactose in cell wall (C. magnoliae, C. etchellsii, C. lactis-condensi) but also without galactose (C. stellata); IIB with Q9 and galactose (C. versatilis, Wickerhamiella) and without galactose (C. apicola); IIC: Dipodascus ingens (galactose +), Pichia pastoris (galactose −); and IID with Q9 (Dipodascus, Galactomyces, Yarrowia) and Q8 (C. incommunis). To cluster III belonged members of Stephanoascus, Zygoascus and Arxula, whereas cluster IV included lipomycetous genera (Lipomyces, Dipodascopsis, Myxozyma) as well as some Candida with Q9 (C. cantarellii, C. vinaria). The four clusters are tentatively marked with names of order rank (I, ‘Saccharomycetales’; II, ‘Dipodasccales’; III, ‘Stephanoascales’; IV, ‘Lipomycetales’); however, no formal proposal has been yet made (M. Suzuki, personal communication).

12.6 Growth and survival

As the Candida species make up about one-quarter of all yeasts known, the ecological factors affecting them and their stress responses and resistance toward those are by and large the same as those that are relevant for the whole group of yeasts. These have been amply discussed in recent treatments (Deák and Beuchat, 1996; Spencer and Spencer, 1997; Boekhout and Robert, 2003), and only specific examples and particulars related to certain Candida species will be described here.

The temperature limits and range fell between 0 and 40 °C for most Candida species. The majority of them are mesophilic and grow best at 25 to 35 °C;
the maximum limiting temperature may be less than 37 °C for some species (e.g. *C. zeylanoides*, *C. vini*), whereas for others, in particular those associated with warm-blooded animals, it can be over 45 °C (e.g. *C. albicans*, *C. glabrata*). The lower limit of growth may fall a few degrees below zero if the solutes are not yet frozen, as in the case of many food systems. Under these conditions, *C. famata*, *C. valida*, *C. lipolytica* can often be isolated from chilled foods at −1 to 4 °C. The growth rate at these temperatures is much slower than at optimum condition; the generation time may be as long as several days. A few species can be considered as true psychrophilic for not growing at temperatures higher than 20 °C, however, most yeasts capable of growth at low temperatures also grow better at 20–25 °C, being psychrotrophic or psychrotolerant rather than psychrophilic. Some *Candida* species show a remarkably wide range of growth temperature, e.g. for *C. valida* it extends from 5 to 45 °C, whereas others have a much narrower range, e.g. between 28 to 42 °C for *C. sloofii* (tm: *Arxiozyma telluris*).

The other main environmental factor affecting growth is the availability of free water; in food relations often expressed in terms of water activity ($a_w$). Most yeast grows well at $a_w$ values in the range of 0.90 to 0.95, but tolerates lower values around 0.80 because of increased solute concentrations. In food microbiology, a particular group of yeasts called osmophilic has gained special attention, being able to grow at high sugar concentrations corresponding to $a_w$ as low as 0.61. This value cited from earlier literature was not confirmed by recent reinvestigations (Jermimi and Schmidt-Lorenz, 1987; Tokouka and Ishitani, 1991); nevertheless $a_w$ values of 0.71–0.75 brought about at sugar concentrations of 55–65% permit the growth only of certain yeasts, mainly the species of Zygosaccharomyces. Many other yeasts tolerate $a_w$ values lower than 0.80 adjusted by either sugars or salt; these accordingly may be called osmophilic or halophilic, but a more appropriate general term would be xerotolerant, all the more that in most cases there is no absolute requirement of high sugar or salt for growth. Among the *Candida* (or the corresponding teleomorphs), *C. famata*, *C. versatilis*, *C. halonitratophila* are notable halotolerant, and *C. lactis-condensi*, *C. colliculosa* and *C. domercqiae* grow well at high sugar concentrations. The $a_w$ tolerance is affected by the kind of solute, temperature, pH and other growth conditions (Tokouka, 1993).

Acidity and pH also are growth limiting environmental factors. Yeasts, in general, prefer a slightly acidic habitat and have optimum growth at pH values between 4.5 and 5.5. However, the growth-limiting pH can be much lower, and several species, e.g. *C. krusei* and *C. valida*, are known to grow even at pH 1.5. Tolerance to pH depends on the type of the acidulant, organic acids being more inhibitory than inorganic acids. Yeasts are considerably more resistant to acidic than alkaline conditions. Many of them stop growing at pH 8.0, and those that grow at pH 10.0–10.5 can be considered alkali tolerant; among the latter are several *Candida*, e.g. *C. famata*, *C. colliculosa*, *C. lipolytica*, *C. parapsilosis* and *C. pulcherrima*. 
Week organic acids used as preservatives, such as sorbic acid and benzoic acid, can effectively inhibit the growth of yeasts when applied in appropriate concentrations and at pH low enough to retard the dissociation of weak acids (in general, both preservatives inhibit yeast growth in concentrations 300–800 mg L\(^{-1}\) below pH 4.5). Hence, combination of these preservatives with a weak organic acid enhances their effectivity, e.g. acetic acid itself is inhibitory and moreover decreases the pH. *Zygosaccharomyces bailii* is regarded as the most resistant yeast to preservatives; however, several *Candida* species, such as *C. krusei* and *C. valida*, possess and develop tolerance to them. Moreover, some of them may be able to degrade sorbic acid into a product (pentadien) producing off-odor.

Ethanol tolerance of yeasts, with the exception of specific wine yeast strains of *Saccharomyces cerevisiae*, is generally less than 10% (v/v). It is well known that after the fermentation of must starts, most of the indigenous (‘wild’) yeasts from grapes die out with increasing concentrations of ethanol. Nevertheless, some yeast strains of *C. stellata* and *C. pulcherrima* may survive for longer in the fermenting wine. If non-*Saccharomyces* yeasts survive at high populations, it could be a potential cause of wine spoilage; on the other hand some may contribute to the development of wine flavour (Lema *et al.*, 1996).

A variety of physical and chemical agents can be used to inactivate yeasts. High temperature is most effective. Yeasts are usually killed within a few minutes at temperatures over 55 °C, the decimal reduction (\(D\)) values are generally less than 1 minute in the temperature range of 60–65 °C. The death rate increases tenfold when the temperature rises by 4–5 °C, and also increases with lowering pH, but decreases with lowering \(a_w\). Unlike bacterial endospores the sexual spores of yeasts are not significantly more resistant than vegetative cells. Data in the literature are, however, conflicting on the heat resistance of yeasts (Török and King, 1991; Fleet 1992; Engel *et al.* 1994; cf. Tchango *et al.*, 1997).

Freezing is detrimental to yeast cells and the loss of viability is strongly influenced by the freezing rate. Cells in the exponential phase of growth are more susceptible to freezing than stationary phase cells (Park *et al.*, 1997); spores being arrested in cell cycle may show higher resistance. Freezing does not cause immediate death of the total cell population, but the ratio of survivors decreases with time in the frozen state. A similar effect is exerted by dehydration. These conditions cause sublethal injury of cells, which can be repaired under favorable circumstances (Fleet and Mian, 1998). Injured cells become more susceptible to preservative factors and can be inactivated by a combination of milder treatments (so-called hurdle technology). These aspects and the possible future application of novel, alternative preservative technologies have been recently discussed by Deák (2004) and will not be further elaborated here.
12.7 Occurrence in foods

Several recent compilations appeared on the occurrence of yeasts in foods, mostly cataloguing the various species and focusing on spoilage aspects (Fleet, 1990, 1992; Tudor and Board, 1993; Thomas, 1993). Deák (1991) has made a thorough survey of yeasts in specific types of foods, extended and updated by Deák and Beuchat (1996); and in these surveys the distribution and frequencies of yeast species were also assessed. Table 12.4 shows the selected data of most frequent Candida species in foods overall and in certain main food types. C. famata, C. pelliculosa, C. valida, C. colliculosa, C. kefyr and C. krusei appear among the most widespread and frequent species (each of them may occur in sporulating form), but their distribution varies with different types of foods. C. famata can be predominant in meat products, C. pelliculosa and C. krusei in acidic foods, C. kefyr in dairy products, C. colliculosa in low a\textsubscript{w} foods, and C. valida in beverages; these species, however, may be accompanied and even surpassed by other yeasts in specific cases.

Loureiro and Querol (1999) gave an overview on the types of spoilage caused by yeasts in different kind of foods. Frequently visible physical alterations appear in gas production, turbidity, sediments and surface films, sometimes in texture changes and discoloration, whereas sensory changes resulting in off-flavors and off-odors may be less evident. These symptoms of deterioration may be produced by spoilage yeasts in general, and the group of Candida yeasts cannot be noted for any specific signs. Their fermentative vigour is usually less than that of Saccharomyces and related species; on the other hand, several species of Candida produce abundant pseudohyphae, leading to the development of surface films. The various spoilage defects are determined by the nature of food commodity, the type of yeast and the size of yeast population; spoilage symptoms start to appear when yeast counts reach 10\textsuperscript{5} cells/g or higher.

The main species on fruits are Hanseniaspora and their Kloeckera anamorphs, besides C. pulcherrima, C. membranifaciens, C. stellata, C. famata are among the normal residents on many fruits. Basidiomycetous species (Rhodotorula, Sporobolomyces) dominate the yeast population on raw vegetables, and the ascomycetous yeasts are represented mostly by weakly or non-fermenting species, such as C. krusei, C. valida, C. pelliculosa, C. lambica and Geotrichum candidum. Fruit juices and soft drinks are produced and preserved in many different ways, hence the yeasts capable of causing spoilage are also different. The most notorious is Z. bailii; besides C. stellata, C. sake and a variety of other species may cause spoilage, which is usually due to a single species in bottled drinks. Fermented, salted and acid preserved foods are composed of a range of vegetables and may contain other ingredients, as in several indigenous foods. These are characterized by a rich microbiota in which yeasts are always associated with lactic acid bacteria. Candida species frequently participate in the fermentation or occur as oxidative organisms in the film developing on the surface. C. versatilis, C. etchellsii,
### Table 12.4 Frequencies of common *Candida* species in foods

<table>
<thead>
<tr>
<th>Species</th>
<th>All foods</th>
<th>Fruits, beverages</th>
<th>Meat, dairy products</th>
<th>Low $a_w$ products</th>
<th>Low pH products</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>0.54</td>
<td>0.37</td>
<td>1.40</td>
<td>–</td>
<td>0.53</td>
</tr>
<tr>
<td><em>C. apicola</em></td>
<td>0.52</td>
<td>0.65</td>
<td>0.56</td>
<td>1.41</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. boidinii</em></td>
<td>0.55</td>
<td>0.43</td>
<td>0.56</td>
<td>–</td>
<td>1.05</td>
</tr>
<tr>
<td><em>C. cantarellii</em></td>
<td>0.15</td>
<td>0.23</td>
<td>0.14</td>
<td>0.35</td>
<td>–</td>
</tr>
<tr>
<td><em>C. catenulata</em></td>
<td>0.45</td>
<td>0.28</td>
<td>0.52</td>
<td>–</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. colliculosa</em></td>
<td>3.64</td>
<td>4.68</td>
<td>1.95</td>
<td>7.53</td>
<td>2.10</td>
</tr>
<tr>
<td><em>C. dattila</em></td>
<td>0.66</td>
<td>0.98</td>
<td>0.28</td>
<td>1.41</td>
<td>–</td>
</tr>
<tr>
<td><em>C. diddensiae</em></td>
<td>0.20</td>
<td>0.03</td>
<td>0.42</td>
<td>–</td>
<td>0.26</td>
</tr>
<tr>
<td><em>C. etchellsii</em></td>
<td>0.45</td>
<td>0.48</td>
<td>0.35</td>
<td>1.06</td>
<td>1.58</td>
</tr>
<tr>
<td><em>C. famata</em></td>
<td>6.72</td>
<td>4.61</td>
<td>8.65</td>
<td>5.64</td>
<td>4.73</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>0.72</td>
<td>0.86</td>
<td>0.35</td>
<td>0.70</td>
<td>1.05</td>
</tr>
<tr>
<td><em>C. globosa</em></td>
<td>0.26</td>
<td>0.14</td>
<td>0.42</td>
<td>0.70</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. guillermondii</em></td>
<td>2.52</td>
<td>2.40</td>
<td>2.23</td>
<td>1.41</td>
<td>2.37</td>
</tr>
<tr>
<td><em>C. holmii</em></td>
<td>1.76</td>
<td>1.11</td>
<td>2.42</td>
<td>1.88</td>
<td>2.63</td>
</tr>
<tr>
<td><em>C. inconspicua</em></td>
<td>0.58</td>
<td>0.41</td>
<td>0.73</td>
<td>–</td>
<td>0.79</td>
</tr>
<tr>
<td><em>C. intermedia</em></td>
<td>0.84</td>
<td>0.74</td>
<td>0.84</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>3.36</td>
<td>2.21</td>
<td>3.91</td>
<td>4.11</td>
<td>0.79</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>3.20</td>
<td>3.23</td>
<td>2.56</td>
<td>1.76</td>
<td>3.95</td>
</tr>
<tr>
<td><em>C. lactis-condensii</em></td>
<td>0.15</td>
<td>0.49</td>
<td>0.14</td>
<td>0.94</td>
<td>0.53</td>
</tr>
<tr>
<td><em>C. lambica</em></td>
<td>1.60</td>
<td>1.60</td>
<td>1.30</td>
<td>1.88</td>
<td>1.05</td>
</tr>
<tr>
<td><em>C. lipoLYtica</em></td>
<td>1.14</td>
<td>0.37</td>
<td>2.09</td>
<td>–</td>
<td>1.58</td>
</tr>
<tr>
<td><em>C. lusitaniae</em></td>
<td>0.22</td>
<td>0.28</td>
<td>0.14</td>
<td>0.70</td>
<td>–</td>
</tr>
<tr>
<td><em>C. magnoliae</em></td>
<td>0.66</td>
<td>0.46</td>
<td>0.84</td>
<td>0.70</td>
<td>1.18</td>
</tr>
<tr>
<td><em>C. norvegica</em></td>
<td>0.45</td>
<td>0.46</td>
<td>0.37</td>
<td>–</td>
<td>0.53</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>2.80</td>
<td>1.38</td>
<td>4.42</td>
<td>1.12</td>
<td>5.26</td>
</tr>
<tr>
<td><em>C. pelliculosa</em></td>
<td>4.56</td>
<td>4.25</td>
<td>4.19</td>
<td>3.53</td>
<td>5.52</td>
</tr>
<tr>
<td><em>C. pulcherrima</em></td>
<td>1.06</td>
<td>2.46</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. rugosa</em></td>
<td>0.60</td>
<td>0.18</td>
<td>1.26</td>
<td>–</td>
<td>1.18</td>
</tr>
<tr>
<td><em>C. sake</em></td>
<td>1.32</td>
<td>1.72</td>
<td>0.74</td>
<td>0.47</td>
<td>2.10</td>
</tr>
<tr>
<td><em>C. sorbosa</em></td>
<td>0.15</td>
<td>0.28</td>
<td>–</td>
<td>–</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. sphaerica</em></td>
<td>0.60</td>
<td>0.28</td>
<td>0.98</td>
<td>0.47</td>
<td>0.53</td>
</tr>
<tr>
<td><em>C. stellata</em></td>
<td>0.95</td>
<td>1.66</td>
<td>0.21</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>2.08</td>
<td>1.85</td>
<td>2.05</td>
<td>1.41</td>
<td>1.58</td>
</tr>
<tr>
<td><em>C. utlis</em></td>
<td>0.50</td>
<td>0.74</td>
<td>0.19</td>
<td>0.47</td>
<td>0.53</td>
</tr>
<tr>
<td><em>C. valida</em></td>
<td>4.32</td>
<td>4.43</td>
<td>3.35</td>
<td>3.53</td>
<td>3.95</td>
</tr>
<tr>
<td><em>C. variabilis</em></td>
<td>1.78</td>
<td>0.55</td>
<td>3.63</td>
<td>2.11</td>
<td>2.16</td>
</tr>
<tr>
<td><em>C. versatilis</em></td>
<td>0.95</td>
<td>0.92</td>
<td>0.84</td>
<td>0.35</td>
<td>0.79</td>
</tr>
<tr>
<td><em>C. vini</em></td>
<td>0.84</td>
<td>1.11</td>
<td>0.42</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td><em>C. zeylanoides</em></td>
<td>1.14</td>
<td>0.37</td>
<td>2.42</td>
<td>0.47</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*Species marked with asterisk has teleomorphic state, see Table 12.3.

Frequencies are calculated separately for all species considered in each column (56–99 species) based on the number of occurrences, the types of food, and the isolates found.

Data are from a broad literature survey, partly published by Deák and Beuchat (1996).

*C. famata, C. pelliculosa, C. krusei* and *C. holmii* can be isolated frequently in such kind of foods. In foods of low water activity (high-sugar products, concentrates, dried foods, pasta, bakery products, etc.) xerotolerant yeasts
can occur, typically *Zygosaccharomyces rouxii* and related species, as well as *C. colliculosa*, *C. lactis-condensi* and *C. versatilis*. Baking inactivates most microorganisms, but in the leavening of rye bread and sour dough bread, some specific yeast, such as *C. holmii*, may contribute in addition to the baker’s yeast strains of *S. cerevisiae*.

Yeast in dairy products are as diverse as the products are varied, and among the most frequently isolated species *C. kefyr*, *C. sphaerica*, *C. famata*, *C. zeylanoides*, *C. lipolytica* and *Geotrichum candidum* can be found. The first two species contribute in the making of fermented dairy products, and together with the others play a role in the ripening of cheeses, sometimes bringing about their spoilage as well. Yeasts form a small but permanent component of the microbiota of fresh meat and poultry and their products. Species capable of growth at low temperature and also with proteolytic and lipolytic activity are characteristic in these foods; such as *C. famata*, *C. lipolytica*, *C. zeylanoides*, *C. catenulata* and *C. intermedia*. Certain yeasts that are considered as pathogenic or opportunistic agents, such as *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, have been reported from meat, poultry, fish and shellfish; however, there are no indications for the direct implication of these sources in human yeast infections.

Disregarding the potential safety hazard posed by the vigorous gas production by some yeasts leading to the explosion of packages, there are a few rare cases reported, which raised growing concern from a public health point of view (Loureiro and Querol, 1999). In recent years an increasing number of food-associated yeasts have been isolated from clinical cases, though no food has been directly implicated in transmitting opportunistic pathogenic species (Hazen, 1995; de Hoog, 1996). Direct symptoms may be attributed to the formation of allergic metabolic compounds, and it is also suspected that degradation of preservative factors by yeast may open the way for pathogenic bacteria. In this respect, the significance of foodborne yeasts should be properly appreciated.

### 12.8 Control measures

Yeasts are natural contaminants of raw materials and ingredients used in product formulations. They may occur in the air of the production plant and get into the product via the hands of worker. High numbers of contaminants can frequently build up on processing equipment, and this environment selects the most fitting spoilage types. Hence, due attention should be given to understanding yeast contamination throughout the complete chain of processing from raw material to the final product in order to avoid its introduction, and exerting control over the potential spoilage species during the manufacturing process.

Several examples give evidence of the risk posed by the potential of yeasts. Spoilage of mayonnaise was caused by *I. orientalis* (am. *C. krusei*)
capable of growing in the presence of 1.5% acetic acid and 0.01% sorbate, and the source of contamination was found to be the plastic containers, which were insufficiently cleaned. Various raw ingredients of delicatessen salads proved to be the primary sources of spoilage yeasts, \textit{P. membranifaciens}, \textit{I. orientalis}, \textit{S. exiguus} and \textit{Z. bailii} (Deák, unpublished observations). Secondary contaminants on bread came from the bakery environment, in particular the slicing machines and packaging materials; spoilage yeasts are introduced into many bakery and confectionery products through the fillings and coatings after baking (Legan and Voysey 1991). \textit{Z. mellis} may contaminate crystalline sugar by means of bees and wasps; moreover, if a xerophilic mold, \textit{Wallemia sebi}, is present, it may stimulate the growth of the yeast by inverting sucrose into monosaccharides, eventually increasing the spoilage risk of high-sugar products such as marzipan and cakes (Vindeløv and Arneborg 2001). Contamination of processing equipment can usually be attributed to poor hygienic practices; however, Laubscher and Viljoen (1999) reported resistance of dominant dairy yeasts to regularly used commercial sanitizers and cleaning compounds.

In order to prevent and eliminate quality deterioration and spoilage of food caused by yeasts, the first line of intervention is the conscientious application of effective technology by making use of wholesome raw materials and good quality ingredients under continuous care of hygiene (good manufacturing practice (GMP) and good hygiene practice (GHP). Adherence to this principle calls for control measures and monitoring based on a HACCP (Hazard Analysis Critical Control Point) system for quality and safety assurance. Potential critical control points (CCPs) should be defined, specifications and critical limits for monitoring should be established, and standardized analytical procedures for monitoring should be applied. Regarding yeasts, however, a sound scientific basis is lacking and there are insufficient details on ecology to be of use in assuring adequate quality and safety management. Lack of knowledge also prevents the development of predictive models. If a product formulation is based on data obtained from predictive modeling, it would be possible to prevent outgrow of spoilage contaminants in such a product. Predictive microbiology has been focused on foodborne pathogenic bacteria, and models for spoilage yeasts are less developed (Kalathenos et al. 1995; Sorensen and Jacobsen, 1997; Betts et al., 2000; Battey et al. 2002). The ComBase program (www.combase.cc) provides a freely downloadable growth predictor mainly for pathogenic bacteria but also for \textit{Saccharomyces cerevisiae} (Baranyi and Tamplin, 2004).

Microbiological testing is traditionally based on culture methods. Their inherent slowness, however, precludes immediate intervention. Hence, rapid, instrumental and automated techniques are being introduced. Moreover, end-product testing and the meaningless total counts such as ‘yeasts and molds’ are being replaced by more indicative methods. The use of acidified media, and more recently of media supplemented with antibacterial antibiotics, comes from the view of attaching primary role to spoilage bacteria as compared
Food spoilage microorganisms

with the rest: yeasts and molds. However, these groups of fungi are not only widely different from each other but also highly varied in their physiological and metabolic attributes which determine their ecological significance. If the ecological factors such as low pH, low $a_w$, less preservative and others permit, yeasts can easily overgrow bacteria and become the primary cause of spoilage. With the advancement in the microbial ecology of foodborne yeasts the critical control points in the entire production line can be determined and effective quality management systems such as HACCP can be introduced. These measures also allow food producers to apply new food preservation strategies and meet changes in a consumer’s expectation. Based on the understanding of yeast ecology not only can the risk of spoilage be predicted but appropriate measures can also be taken to prevent or reduce it.

12.9 Specific detection, enumeration, identification

The great diversity and large number of species in Candida and related genera make it impossible to apply any method specific for the whole group. There are two possibilities: either to choose a method applicable for all yeasts, or to use one that is specific for a single species or a limited group of closely related species.

Methods for the detection, enumeration and isolation of yeasts have been recently reviewed by Deák (2003a) who also refers to numerous earlier compilations on the subject. For general purposes, dichloran rose bengal chloramphenicol (DRBC) agar or tryptone glucose yeast extract chloramphenicol (TGYC) agar can be recommended; both allow the recovery of all kinds of yeasts while inhibiting bacterial growth and the former also reduces fungal spreading. Acidified media (an appropriate basal medium adjusted by tartaric acid to pH 3.5) can be suitable for recovering yeasts from certain low pH types of foods, e.g. pickles, cheeses, fruit juices. By the same token, a low pH medium containing 0.5% acetic acid (or 0.3% acetate and 0.1% potassium sorbate) is appropriate for detecting preservative and acid-resistant yeasts, such as C. valida, C. krusei, C. parapsilosis, besides Zygosaccharomyces bailii. For the enumeration of yeasts from high-sugar foods, malt extract agar with 30 to 50% glucose can be used, however, to recover osmophilic yeasts, the diluents should also be osmotically balanced. The dichloran 18% glycerol agar (DG18) is satisfactory for the enumeration of a wider range of xerotolerant yeasts, such as C. famata, C. versatilis, C. etchellsii, C. lactis-condensi. A variety of specific, differential and selective media have been developed for the detection of wild yeasts in breweries and wineries. Their working principle relies on the inclusion of inhibitors, differential dyes or selected carbon- and/or nitrogen sources. A recent reference source is Van der Kuhle and Jespersen (1998). Similar strategies have been applied in developing media for the detection of specific foodborne yeasts, and to this end, significant progress has been made in recent years. Species
targeted were *S. cerevisiae*, *Z. bailii*, *T. delbrueckii*, *D. hansenii*, *K. marxianus*, *Y. lipolytica*, *I. orientalis* and their respective *Candida* anamorphs (Loureiro and Malfeito-Ferreira, 2003; Siloniz *et al.*, 2000).

The selective detection of *C. albicans* is of particular importance in clinical mycology. Hence, a number of media have been developed for the direct detection and presumptive identification of this and other opportunistically pathogenic yeasts (Baumgartner *et al.*, 1996). Of these, reference to the CHROMagar *Candida* is relevant here for it allows the discrimination by colony color of a number of yeast species, hence it can be a differential medium in the investigation of foodborne yeasts (Tornai-Lehoczki *et al.*, 2003).

Identification of yeasts traditionally relies on phenotypic characters (morphology, as well as physiological and biochemical tests (Yarrow, 1998; Barnett *et al.*, 2000)); however, in recent years, molecular methods have served not only for the phylogenetic classification but also for fast and reliable identification of yeasts (Kurtzman *et al.*, 2003). Neither elaborated method could be routinely used in food industrial laboratories. For the detection of yeasts without previous cultivation a promising molecular method would be the fluorescent *in situ* hybridization (FISH) of whole cells with rRNA-targeted oligonucleotide probes (Stender *et al.*, 2001). By the same token, specific oligonucleotide probes could be placed on DNA chips and used for detection and identification (Perez-Ortin *et al.*, 2002). In industrial practice, miniaturized and simplified identification methods would suffice in most cases; commercially available kits have, however, been developed for the purposes of clinical diagnostics and their database is restricted (Deák and Beuchat, 1993, 1996; Velasquez *et al.*, 2001). Detailed treatment of this subject is beyond the scope of this chapter; interested readers may turn to references above and further sources therein.

Reference should also be made to the various novel, non-conventional techniques developed in recent decades for the rapid, often instrumental and automated detection, enumeration and identification of yeasts. These have been amply discussed by Deák (1994, 1995, 2003b), van der Vossen *et al.* (2003) and Loureiro and Malfeito-Ferrera (2004), and a few examples only will be referred to here. Impedance or conductance changes can be correlated with the yeast counts, and commercially available instruments allow the rapid detection and monitoring of yeasts (Deák and Beuchat, 1995). Loureiro (2000) advocates the concept of zymological indicators based on the determination of fatty acid profiles. Flow cytometry provides a rapid and sensitive method for cell counting which can also discriminate between live and dead yeast cells (Malacrino *et al.*, 2001).

### 12.10 Future trends

*Candida* species represent about one-quarter of all yeasts known, and the size of the genus will extend with the description of new species. Subdivision
of the genus will inevitably follow as the internal relations are better understood. More detailed classification will facilitate identification, and contribute to deeper exploration of yeast biodiversity in foods and natural habitats. According to the ecological concept, though artificial, foods can be considered as ecosystems that provide specific niches and habitats for yeasts and other microorganisms. Yeasts are usually minor components of the microbiota, and cannot compete with bacteria in most food ecosystems. If, however, the ecological factors such as low pH, low $a_w$ or others permit, yeasts can easily overgrow bacteria and become the primary cause of spoilage. In the light of the ecological approach, the once neglected and underestimated role of yeasts in food spoilage will be given due concern.

Recent progress in the ecological diversity of yeasts in foods and beverages indicates the following main directions of future research: (1) improving detection and enumeration by rapid, specific and differential methods; (2) increasing use of molecular and biochemical techniques for identification and typing; (3) better understanding of yeast responses to preservative stresses; (4) elucidation of emerging technologies on growth, survival and spoilage potential; (5) investigation of yeast interaction with bacteria and molds.

By gaining more knowledge about the ecology and biodiversity of yeasts in food, their beneficial function can be better exploited and the harmful activities can be controlled.

12.11 Sources of further information


12.12 References

Candida and related genera


352 Food spoilage microorganisms


Candida and related genera


13

*Dekkera/Brettanomyces* spp.

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13.1 Introduction

This chapter describes yeast species of the genera *Dekkera/Brettanomyces* that are, presently, being given particular attention owing to their ability to produce off-flavors and off-tastes in premium quality wines. The main focus will be put on their occurrence, spoilage activities and control. Claussen first described the yeasts of the genus *Brettanomyces* in 1904 (Gilliland, 1961). Afterwards several strains were mentioned using different names such as *Mycotorula intermedia*, *Monilia vini* and *Oospora vini* (Licker *et al.*, 1998). This nomenclature was revised and the first taxonomic study of this genus was made by Custers, in 1940, who described the species *B. bruxellensis*, *B. lambicus*, *B. clausenii* and *B. anomalus*, all reproducing by budding (van der Walt and van der Kerken, 1958). The production of ascospores, observed by van der Walt and van der Kerken (1960), led to the introduction of the genus *Dekkera* by van der Walt (1964). The taxonomy of these genera suffered several rearrangements and, presently, *D. anomala* and *D. bruxellensis* are the two accepted species in the genus *Dekkera* (Kurtzman and Fell, 1998; Barnett *et al.*, 2000; Boekhout *et al.*, 2002). Their anamorphs are *B. anomalus* and *B. bruxellensis*, respectively. The other three species, *B. naardenensis*, *B. custersianus* and *B. nanus*, are only mentioned as imperfect forms (Table 13.1). The most recent manuals of classical identification (Kurtzman and Fell, 1998; Barnett *et al.*, 2000; Boekhout *et al.*, 2002) describe the morphological, biochemical and physiological characteristics of these species. Their distinction is made on the basis of five key characters (Table 13.2). Some of them have variable results that make identification troublesome, mainly between *D. bruxellensis* and *D. anomalala*, which are distinguished only by the presence of non-septate filaments in the latter species. According
<table>
<thead>
<tr>
<th>Species</th>
<th>Synonyms</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. anomala/B. anomalus</td>
<td>Monilia vini, Oospora vini, Mycotorula clausenii, B. anomalus, B. clausenii, D. clausenii, Torulopsis cylindrica, B. cidri, B. dublinensis, C. beijingensis</td>
<td>Spoiled soft drink (type strain), beer, stout beer, cider, sherry vat, apple, kefyr</td>
</tr>
<tr>
<td>D. bruxellensis/B. bruxellensis</td>
<td>B. bruxellensis, B. lambicus, Mycotorula intermedia, B. intermedia, B. intermedius, B. custersii, B. patavins, B. vini, B. schanderlii, D. intermedia, B. abstinens, D. abstinens, D. lambica</td>
<td>Belgian stout beer (type strain), lambic beer, grape must, sour wine, sparkling wine, tea-beer, brewery equipment, dry ginger ale, cola, wine, secondary fermentation, sherry</td>
</tr>
<tr>
<td>B. custersianus</td>
<td>D. custersiana</td>
<td>Equipment at a Bantu beer brewery (type strain), olives</td>
</tr>
<tr>
<td>B. naardenensis</td>
<td>D. naardenensis</td>
<td>Lemonade (type strain), soda water, carbonated lemonade, carbonated tonic water, carbonated soft drink</td>
</tr>
<tr>
<td>B. nanus</td>
<td>Eeniella nana</td>
<td>Bottled beer (type strain)</td>
</tr>
</tbody>
</table>
Table 13.2  Key characters of Dekkera/Brettanomyces spp. (Smith, 1998) (V, variable result)

<table>
<thead>
<tr>
<th>Species</th>
<th>Lactose fermentation</th>
<th>Galactose assimilation</th>
<th>Glucitol assimilation</th>
<th>Succinate assimilation</th>
<th>Non-septate filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. anomala</td>
<td>V</td>
<td>+</td>
<td>–</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. custersianus</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>B. naardenensis</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

To Boekhout et al. (2002) the classification scheme for Dekkera/Brettanomyces spp. is as follows:

Fungi (Kingdom)
  Eumycota (Division)
    Ascomycotina (Subdivision)
      Hemiascomycetes (Class)
        Saccharomycetales (Order)
          Saccharomycetaceae (Family)
            Dekkera (Genus)
        Candidaceae (Family, mitosporic members of Saccharomycetales)
          Brettanomyces (Genus)

13.2  Occurrence

13.2.1  Preference for fermented products

The yeasts of the genus Brettanomyces were first described as being responsible for a slow stock beer fermentation after the completion of the primary fermentation, with the production of typical strong flavors (Gilliland, 1961). Concerning wines, the first mention is a strain of Mycotorula intermedia isolated from French grape must by Krumbholz and Tauschanoff, in 1933 (van der Walt and van der Kerken, 1958). The first strains described in taxonomical manuals were isolated from these two industries (brewing and wine) and were identified as B. bruxellensis and B. anomalus (Table 13.1). These are the most common species of the genus (Deak and Beuchat, 1996).

Table 13.3 lists the main references reporting the isolation of Dekkera/Brettanomyces spp. from different sources. These yeasts are mostly associated with fermented products, particularly with the post-fermentation or aging period of alcoholic beverages, like wine, beer, cider, kombucha (a tea-based beverage (typically black tea) of Asian origin, fermented by mixed cultures of bacteria and yeasts forming a surface pellicle called the ‘tea fungus’; Steels et al., 2002) and tequila. Some reports describe their isolation from cheeses or fermented milks. Besides food industries, Dekkera/Brettanomyces
Table 13.3  Occurrence and spoilage activities of *Dekkera/Brettanomyces* spp. in foods and beverages (see Table 13.1 for synonyms)

<table>
<thead>
<tr>
<th>Species</th>
<th>Product</th>
<th>Prevalence</th>
<th>Spoilage effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wine industries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. schanderlii</em></td>
<td>Bottled claret wine</td>
<td>3 out of 207 wine contamination strains</td>
<td>–</td>
<td>Domercq (1956)</td>
</tr>
<tr>
<td><em>B. vini</em></td>
<td>Red wines</td>
<td>4 out of 1070 wine contamination strains</td>
<td>–</td>
<td>Peynaud (1958)</td>
</tr>
<tr>
<td><em>Brettanomyces</em> spp.</td>
<td>Winery equipment and walls</td>
<td>17 out of 132 contamination strains</td>
<td>–</td>
<td>Peynaud (1959)</td>
</tr>
<tr>
<td><em>B. intermedius,</em></td>
<td>Bottled dry or semi-sweet</td>
<td>50% of total contaminant yeasts</td>
<td>Turbidity</td>
<td>Van der Walt and</td>
</tr>
<tr>
<td><em>B. schanderlii</em></td>
<td>white wines, dry sherry</td>
<td>recovered from 60 samples</td>
<td></td>
<td>Van Kerken (1958, 1959)</td>
</tr>
<tr>
<td></td>
<td>Red wines, medium-sweet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sparkling wines, newly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>fermented wines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. intermedius,</em></td>
<td>White musts before fermentation,</td>
<td>Absence from grapes in vineyard and</td>
<td>–</td>
<td>Van der Walt and</td>
</tr>
<tr>
<td><em>B. schanderlii</em></td>
<td>winery material, equipment and</td>
<td>transport vehicles, and from husks and</td>
<td></td>
<td>Van Kerken (1961)</td>
</tr>
<tr>
<td></td>
<td>floor, breeding ground of</td>
<td>pomace</td>
<td></td>
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</tr>
<tr>
<td></td>
<td><em>Drosophila</em> spp.</td>
<td>Only one must out of ten showed contamination</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. intermedius</em></td>
<td>White and red wines and still-</td>
<td>Variable frequency (0–100% of samples)</td>
<td>–</td>
<td>Wright and Parle (1974)</td>
</tr>
<tr>
<td></td>
<td>wash fermentations</td>
<td>and level (0–92% of total flora)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absence from winery floors, walls or</td>
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<tr>
<td></td>
<td></td>
<td>equipment</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. intermedius</em></td>
<td>Fermenting red grape juices</td>
<td>One strain among more than 800 strains</td>
<td>–</td>
<td>Poulard (1978)</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Red and white must fermentation</td>
<td>Early, middle and end of fermentation,</td>
<td>–</td>
<td>Urbina and Beltran (1984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>present in 6 out of 666 samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. schanderlii</em></td>
<td>Red must fermentation</td>
<td>Middle fermentation, present in one out of</td>
<td>–</td>
<td>Urbina and Beltran (1984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>666 samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. intermedius,</em></td>
<td>Red and white wine</td>
<td>Mixed contamination with bacteria</td>
<td>Mousiness</td>
<td>Heresztyi (1986a)</td>
</tr>
<tr>
<td><em>B. lambicus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. intermedius</em></td>
<td>Barrelled red wine</td>
<td>Sole contaminant</td>
<td>Unpleasant taints</td>
<td>Gaia (1987)</td>
</tr>
<tr>
<td>Species</td>
<td>Product</td>
<td>Prevalence</td>
<td>Spoilage effect</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
<tr>
<td><em>Brettanomyces</em></td>
<td>Healthy and sour rotten grapes</td>
<td>Present in one plant over 4 or 5 plants of 2 plant cultivars, out of 5 analyzed Average of 0.41 log_{10} cfu/g in healthy grapes, and of 1.37 log_{10} cfu/g in damaged grapes</td>
<td>–</td>
<td>Guerzoni and Marchetti (1987)</td>
</tr>
<tr>
<td><em>B. intermedius</em></td>
<td>Bulk white wine</td>
<td>50% of isolates</td>
<td>Turbidity</td>
<td>Ciolfi <em>et al.</em> (1988)</td>
</tr>
<tr>
<td><em>D. intermedia, B. lambicus</em></td>
<td>Bulk wines</td>
<td>Sole contaminant</td>
<td>Phenolic taint</td>
<td>Froudière and Larue (1989)</td>
</tr>
<tr>
<td><em>B. custersianus</em></td>
<td>Red must fermentation</td>
<td>12.5% of total isolates in 3rd day of fermentation</td>
<td>–</td>
<td>Querol <em>et al.</em> (1990)</td>
</tr>
<tr>
<td><em>D. bruxellensis</em></td>
<td>Red must fermentation</td>
<td>12.5% of total isolates in 3rd day of fermentation</td>
<td>–</td>
<td>Querol <em>et al.</em> (1990)</td>
</tr>
<tr>
<td><em>B. intermedius</em></td>
<td>Bulk wines</td>
<td>Up to 96% of isolates</td>
<td>Turbidity</td>
<td>Ciolfi (1991)</td>
</tr>
<tr>
<td><em>D. intermedia, B. lambicus</em></td>
<td>Bulk or barrelled wines</td>
<td>Absence from grapes, musts or bottled wines</td>
<td>Acetic and other acids production</td>
<td>Longo <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><em>D. intermedia</em></td>
<td>Non-fermented musts</td>
<td>Low frequency</td>
<td>–</td>
<td>Chatonnet <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>B. intermedius, B. lambicus</em></td>
<td>Bulk, bottled and barrelled wines</td>
<td>–</td>
<td>Phenolic taint</td>
<td>Salvadores <em>et al.</em> (1993)</td>
</tr>
<tr>
<td><em>B. lambicus</em></td>
<td>Bulk red wines</td>
<td>Present in 2 out of 4 wineries in proportions up to 7.4% of total isolates</td>
<td>–</td>
<td>Ibeas <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Barrelled sherry wines and respective surface films</td>
<td>Sole cycloheximide resistant yeast isolated</td>
<td>Acetic acid production</td>
<td>Ibeas <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Cabernet Sauvignon wines</td>
<td>Up to an average of 700 cfu/ml</td>
<td>–</td>
<td>Mitrakul <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><strong>B. bruxellensis</strong></td>
<td>Sparkling white wines</td>
<td>Sole contaminant</td>
<td>Turbidity</td>
<td>Malfeito-Ferreira et al. (1997)</td>
</tr>
<tr>
<td>---------------------</td>
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</tr>
<tr>
<td>Brettanomyces spp.</td>
<td>Sparkling wines</td>
<td>27.5% of strains isolated from 15 samples</td>
<td>No spoilage was detected</td>
<td>Ciani and Ferraro (1997)</td>
</tr>
<tr>
<td>Brettanomyces spp.</td>
<td>Pinot noir wines</td>
<td>25% of bottled wines, 50% of bulk wines, up to $10^3$ cfu/ml</td>
<td>Phenolic taint</td>
<td>Gerbaux et al. (2002)</td>
</tr>
<tr>
<td>Dekkera spp.</td>
<td>Early stages of wine fermentation</td>
<td>17% of the non-Saccharomyces yeast flora</td>
<td>–</td>
<td>Esteve-Zarzoso et al. (2000)</td>
</tr>
<tr>
<td>D. anomala</td>
<td>Must fermentation</td>
<td>2.4% of species recovered during the first stage of fermentation</td>
<td>–</td>
<td>Esteve-Zarzoso et al. (2001)</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>Sherry biological aging</td>
<td>Up to 66.6% of species isolated from sherry pellicles during biological aging</td>
<td>–</td>
<td>Esteve-Zarzoso et al. (2001)</td>
</tr>
<tr>
<td>B. bruxellensis</td>
<td>Bulk red wines</td>
<td>57% of 74 samples, up to $2.5 \times 10^3$ MPN/ml, less than 1% of total flora</td>
<td>Phenolic taint</td>
<td>Rodrigues et al. (2001)</td>
</tr>
<tr>
<td>B. bruxellensis</td>
<td>Air from crush, tank, barrel and bottling rooms</td>
<td>3 to 100 cfu/250 l of air</td>
<td>Phenolic taint</td>
<td>Connell et al. (2002)</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>Bottled red wine</td>
<td>Up to an average of $12.7 \times 10^3$ cfu/ml</td>
<td>Phenolic taint</td>
<td>Phister and Mills (2003)</td>
</tr>
<tr>
<td>Dekkera spp.</td>
<td>Early and late must fermentation</td>
<td>Up to 26.3% of isolates in late fermentation</td>
<td>–</td>
<td>Ganga and Martínez (2004)</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>Red wines</td>
<td>$&lt;10$ to $1.5 \times 10^4$ cfu/ml</td>
<td>Phenolic taint</td>
<td>Cocolin et al. (2004)</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>Red must in first days of fermentation</td>
<td>4 out of 40 isolated colonies</td>
<td>–</td>
<td>Hierro et al. (2004)</td>
</tr>
<tr>
<td><strong>Beer industries</strong></td>
<td><strong>B. bruxellensis, B. lambicus</strong></td>
<td>Fermentation and maturation of lambic beer</td>
<td>About $10^3$ to $10^5$ cfu/ml, predominant species from 8 to 24 month storage,</td>
<td>–</td>
</tr>
<tr>
<td>Brettanomyces spp.</td>
<td>Lambic beer after one year of maturation</td>
<td>Only yeast species present, together with acetic and lactic bacteria</td>
<td>–</td>
<td>Kumara and Verachtert (1991)</td>
</tr>
<tr>
<td>B. lambicus</td>
<td>Lambic beer overattenuated</td>
<td>–</td>
<td>–</td>
<td>Kumara et al. (1993)</td>
</tr>
<tr>
<td>Species</td>
<td>Product</td>
<td>Prevalence</td>
<td>Spoilage effect</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------</td>
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</tr>
<tr>
<td><em>Brettanomyces</em></td>
<td><strong>Lambic beer fermentation</strong></td>
<td>Common contaminants</td>
<td>–</td>
<td>Nedervelde and Debourg (1995)</td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brettanomyces</em></td>
<td><strong>Fermentation and maturation of</strong></td>
<td>Predominant yeast during maturation</td>
<td>–</td>
<td>Martens <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>spp.</td>
<td><strong>acidic ales of Roeselare</strong></td>
<td>after 20–24 months of fermentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cider industries</strong></td>
<td><em>Brettanomyces</em></td>
<td>Cider industries, water from the</td>
<td>–</td>
<td>Davenport (1976)</td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td>apple washing and conveying channels</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. anomalus</em></td>
<td><strong>stored apples</strong></td>
<td>among other 11 different species</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Kombucha production</strong></td>
<td><em>Brettanomyces</em></td>
<td>Cellulosic pellicle</td>
<td>56% of analyzed species</td>
<td>Mayser <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Cellulosic pellicle</td>
<td>–</td>
<td>–</td>
<td>Liu <em>et al.</em> (1996)</td>
</tr>
<tr>
<td></td>
<td>fermenting liquor</td>
<td>fermentations of different origin</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Up to $10^6$ cfu/ml, representing 90%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>of flora after 22 days of fermentation;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>up to $&gt;3 \times 10^3$ cfu/ml, dominant</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>contaminant during maturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tequila industry</strong></td>
<td><em>B. bruxellensis</em></td>
<td>Tequila fermentation (all stages)</td>
<td>Low proportion</td>
<td>Lachance (1995)</td>
</tr>
<tr>
<td><em>B. anomalus</em></td>
<td>Agave rots, cooked agave, cooked agave,</td>
<td>Low proportion</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>agave extract, early and intermediate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>fermentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dairy industries</strong></td>
<td><em>D. anomalala</em></td>
<td>Harzer cheese (yellow cheese type)</td>
<td>One of the 4 predominating species</td>
<td>Engel and Rosch (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in acid curd quarg</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. anomalus</strong></td>
<td>Airag fermented milk</td>
<td>44% of isolates</td>
<td>–</td>
<td>Naersong et al. (1996)</td>
</tr>
<tr>
<td><strong>D. bruxellensis</strong></td>
<td>Yoghurt</td>
<td>One species among other 11 isolated species</td>
<td>–</td>
<td>Kosse et al. (1997)</td>
</tr>
<tr>
<td><strong>B. anomalus</strong></td>
<td>Kefir</td>
<td>One species among other 5 isolated species</td>
<td>–</td>
<td>Wyder et al. (1997)</td>
</tr>
<tr>
<td><strong>D. custersoniana</strong></td>
<td>Cheddar cheese</td>
<td>One species present among other 6 species. Present in raw milk, hands, cheese air, equipment and cheese curd</td>
<td>–</td>
<td>Laubsher and Viljoen (1999)</td>
</tr>
<tr>
<td><strong>D. naardenensis</strong></td>
<td>Cheddar cheese</td>
<td>One strain out of 77 isolates from other species. Present in cheese ripening, absent from processing line and plant</td>
<td>–</td>
<td>Welthagen and Viljoen (1999)</td>
</tr>
<tr>
<td><strong>D. bruxellensis</strong>, <strong>D. anomala</strong></td>
<td>Amasi fermented milk</td>
<td>5 <em>D. bruxellensis</em> and 1 <em>D. anomala</em> out of 80 isolates</td>
<td>–</td>
<td>Gadaga et al. (2002)</td>
</tr>
<tr>
<td><strong>D. anomala</strong>, <strong>D. bruxellensis</strong></td>
<td>Ewe’s cheese</td>
<td>16 of <em>D. anomala</em> and 3 of <em>D. bruxellensis</em> out of 261 isolates</td>
<td>–</td>
<td>Cosentino et al. (2001)</td>
</tr>
<tr>
<td><strong>D. anomala</strong>, <strong>D. bruxellensis</strong></td>
<td>Feta cheese</td>
<td>28.6% of samples contaminated with <em>D. anomala</em>, 19% with <em>D. bruxellensis</em> (Dairy A), 18.6% with <em>D. anomala</em> (Dairy B)</td>
<td><em>D. anomala</em> (&gt;10⁶ cfu/g) was the probable cause of swelled samples by gas production</td>
<td>Fadda et al. (2001)</td>
</tr>
<tr>
<td><strong>B. anomalus</strong>, <strong>B. bruxellensis</strong></td>
<td>Blue veined cheese</td>
<td>Presence in floors, walls and equipment, absence from processing or maturation</td>
<td>–</td>
<td>Viljoen et al. (2003)</td>
</tr>
<tr>
<td><strong>Bioethanol industries</strong></td>
<td><strong>B. intermedius</strong></td>
<td>Alcoholic fermentation for distillation</td>
<td>Up to 70 × 10⁶ cells/ml</td>
<td>Acetic acid production</td>
</tr>
<tr>
<td><strong>B. bruxellensis</strong></td>
<td>Alcoholic fermentation for distillation</td>
<td>–</td>
<td>–</td>
<td>Uscanga et al. (2000)</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td><strong>D. bruxellensis</strong></td>
<td>Greek-style black olives</td>
<td>3 strains out of 125 isolated strains from other species</td>
<td>–</td>
</tr>
<tr>
<td>Species</td>
<td>Product</td>
<td>Prevalence</td>
<td>Spoilage effect</td>
<td>Reference</td>
</tr>
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</tr>
<tr>
<td>B. clausenii</td>
<td>Cassava flour</td>
<td>One species among other 9 isolated species</td>
<td>–</td>
<td>Okagbue (1990)</td>
</tr>
<tr>
<td>D. bruxellensis</td>
<td>Sourdough fermentation</td>
<td>One isolate in one out of 4 fermentation batches, one isolate among 17 from rye flour</td>
<td>–</td>
<td>Meroth <em>et al.</em> (2003)</td>
</tr>
</tbody>
</table>
spp. have also been reported in industrial ethanol fermentations. Outside these types of environment, the yeasts are scarcely mentioned. Occasional reports include isolation from olives, flours and carbonated beverages (Table 13.3), bees and air at ground level in fruit orchards (see references cited in Licker et al., 1998), honey and tree exudates (van der Walt, 1970).

Despite the preference for fermented products, Dekkera/Brettanomyces spp. are not common contaminants of such commodities. Table 13.3 lists the data reported for their prevalence among analyzed samples. These yeasts are usually not dominant and are reported in a low percentage of analyzed samples. Furthermore, in many other ecological surveys, these yeasts are not referred to (see reviews of Deák and Beuchat, 1996, and Loureiro and Malfeito-Ferreira, 2003). Thus, they may be regarded as infrequent contamination yeasts that appear in high numbers mainly when other microorganisms have been inhibited. The physiological and metabolic explanations for this observation are not fully understood (see below). The contamination of products by pure cultures of Dekkera/Brettanomyces spp. is rare but has been described in sparkling wines for D. bruxellensis (Ciani and Ferraro, 1997; Malfeito-Ferreira et al., 1997) and for D. naardenensis in spoilt non-juice soft drinks (van Esch, 1992; Deák and Beuchat, 1995). The increase in their predominance appears to be the result of exceptional resistance to minimal nutrient conditions, which is seen as determinant in the survival during production and storage steps and, then, in their development when the environment becomes favourable (Uscanga et al., 2000). For instance, in lambic beer Brettanomyces is commonly isolated after one year of maturation when Saccharomyces are no longer found (Kumara and Verachtert, 1991).

13.2.2 The relevance of D./B. bruxellensis in the genera
The abundance of the five species of Dekkera/Brettanomyces is not uniform. D. bruxellensis is the best represented species among the sources listed in Table 13.3, mainly due to its isolation from wine-related environments and to its dissemination by all types of industries. Recent studies using molecular identification approaches have revealed that D. bruxellensis is the only species isolated from wines (Phister and Mills, 2003; Dias et al., 2003b; Bellon et al., 2003; Cocolin et al., 2004; Martorell et al., 2006). The species B. custersonianus and D. anomala have been reported, only once each, in must fermentation (Querol et al., 1990; Esteve-Zarzoso et al., 2001) but not in wines. The reference not to the species but to the genus Brettanomyces, in many of the isolates from wines referred to in Table 13.3, is probably related with the above-mentioned difficulty to differentiate the species D. anomala from the D. bruxellensis by conventional methodologies. Thus, in wines it is common to see the reference to Dekkera/Brettanomyces spp. without species distinction (see review of Fugelsang, 1997). Moreover, despite the fact that earlier isolates of D. anomala were not associated with wine (see Table 13.1) it has become usual to look at this species as a common contaminant of wine
(see review of Deák and Beuchat, 1996). The fact that some recent research on D./B. bruxellensis continues to include culture collection strains of D. anomala for comparative purposes (Delfini et al., 2002; Silva et al., 2004; Cocolin et al., 2004) or mention it as relevant to wines (Gerós et al., 2000), contributes to the perpetuation of this unjustified view. However, as far as we are aware, D./B. bruxellensis is the sole species isolated from wines.

Dekkera anomala is mainly linked to traditional beer fermentations (see Table 13.1) but it has not been isolated in brewing industries according to the most recent reports (see Table 13.3). Although some of the references to the genus Brettanomyces spp. could hypothetically be B. anomalous, the fact is that dairy industries are presently the most frequent environment related with this species (see Table 13.3). The fermentation or assimilation of lactose (see Table 13.2) could justify the apparent higher relative frequency of D. anomala when compared with D. bruxellensis, but the number of analyzed samples is too low to support this statement unequivocally. In the cider industry, B. anomalus and B. bruxellensis were detected by Morrisey et al. (2004) but their proportions were not reported. The work of Lachance (1995), concerning tequila fermentation, mentions the recovery of B. anomalus only from agave rots in the field and of B. bruxellensis during fermentation. A possible explanation for the apparent higher frequency of D. anomala in fermented products with an ethanol content lower than wine, may be related to an ethanol tolerance lower than that of D. bruxellensis.

The isolation of B. custersianus, B. naardenensis and, in particular, B. nanus is seldom described and is practically restricted to the initial works of taxonomical characterization that provided the isolates mentioned in Table 13.1 (van der Walt, 1961; Kolfschoten and Yarrow, 1970; Smith et al., 1981). Other occasional references are listed in Table 13.3. In a technical report, van Esch (1992) states that B. naardenensis is the typical contaminant of preserved non-juice soft drinks (tonic water, lemon-lime and cola), with a moderate requirement for oxygen. This author considers this species as the main spoiler (by turbidity) in such products, which have low nitrogen content, high carbon dioxide concentrations, very low pH and are regarded as the least susceptible to yeast spoilage. In juice containing soft drinks or in fruit juices these species are not prevalent (van Esch, 1992).

13.2.3 Dissemination
As stated before, most works are on D. bruxellensis, and so dissemination studies are mainly concerned with this species in the wine industry. However, its dissemination in wine-related environments has not been properly established. From early reports (see review of Licker et al., 1998) and from Table 13.3 it emerges as a common, although mostly not predominant, contaminant in wines and grape juice. Van der Walt and van Kerken (1961) and Alguacil et al. (1998) mentioned the isolation from cellar equipment. In contrast, Wright and Parle (1974) did not recover it from winery floors, walls
or equipment. The most surprising observation is that it has not been consistently recovered from grapes in the vineyard, although it is relatively frequent in grape juice fermentation (Table 13.3). Despite occasional technical and Internet references, only one scientific report mentioned their isolation from grapes, particularly from sour rot damaged grapes (Guerzoni and Marchetti, 1987). This report is in agreement with our empirical knowledge that the incidence of *D./B. bruxellensis* in wines is higher in poor quality vintages characterized by higher proportions of rotten grapes. Recent broad studies have not found their presence on sound and damaged grapes (Jolly *et al*., 2003; Prakitchaiwattana *et al*., 2004) or must fermentation (Clemente-Jimenez *et al*., 2005). Davenport (1974) did not find *Brettanomyces* spp. in the vineyard after analyzing soil, rootlets, leaves, bark, grapes in different stages of maturity and dormant buds of vines. Van der Walt and van Kerken (1961) did not recover *Brettanomyces* spp. from husks and pomaces but others have isolated them from these sources (Alguacil *et al*., 1998). Only Alguacil *et al*. (1998) refer to its isolation from the flies *Drosophila* spp. in the winery plant. Similarly, it has only been mentioned once in winery air samples (Connell *et al*., 2002).

Therefore, according to present knowledge *D./B. bruxellensis* may be regarded as a winery contaminant but its primary source and routes of contamination should be investigated more deeply. Particularly, the transmission from the vineyard, especially from damaged grapes, to the winery and the persistence in both vineyard and winery environments deserve more attention. These dissemination studies should also include molecular techniques of infraspecific typing (Bellon *et al*., 2003; Martorell *et al*., 2006) to follow routes of contamination.

In other food and beverage industries the primary sources of *Dekkera/Brettanomyces* spp. are also not well established. *B. anomalus* and *B. bruxellensis* were found in stored apples for cider making, but apples in the orchard were not analyzed (Morrissey *et al*., 2004). The work of Lachance (1995), concerning tequila fermentation, mentioned the recovery of *B. anomalus* from agave rots in the field, while *B. bruxellensis* was only detected during tequila fermentation. Acetate esters have been shown to attract *Drosophila melanogaster* flies (Zhu *et al*., 2003) and Lachance (1995) hypothesized that fruit flies attracted by acetic acid smell carry it into the processing winery. In the dairy industry, *B. anomalus* and *B. bruxellensis* were recovered from floors, walls and equipment of cheese factories but not from air, brine or cheeses (Viljoen *et al*., 2003). *D. custersiana* and *D. naardenensis* were also isolated from several origins in cheddar cheese factories (Laubsher and Viljoen, 1999; Welthagen and Viljoen, 1999). We are not aware of published works on the dissemination of these yeasts in brewing, kombucha and ethanol production plants.

### 13.2.4 Old wooden vats: a well-known ecological niche

The traditional wooden vats are used for sherry, red wine, lambic beer or
cider production and Dekkera/Brettanomyces spp. are common contaminants of such industries (Table 13.3). In wines, the onset of problems related with Dekkera/Brettanomyces spp. activity coincided with the worldwide increase in the utilization of oak barrels since 1990. Chatonnet et al. (1990) demonstrated the higher rate of wine spoilage in old used barrels than in new ones, suggesting that this type of vessel is a preferential ecological niche for these species. In addition, according to our empirical knowledge, the wines matured in stainless steel tanks with oak chips are not as affected by D. bruxellensis. This suggests that it is the continuous utilization of barrels that promotes the infection and persistence of these yeasts inside the wood. It is not clear if these yeasts are more easily adapted to colonize wood than other species but the assimilation of cellobiose, resulting from degradation of wood lignin by barrel toasting, should be a stimulating factor. However, the absence of cellobioase activity is frequent among D. bruxellensis strains recovered from lambic beer (Vanderhaegen et al., 2003) and cellobiose assimilation by this species is described as variable by taxonomical manuals (Smith, 1998; Barnett et al., 2000). These yeasts have been also recovered from wine stored in stainless steel or concrete tanks (Chatonnet et al., 1992; Rodrigues et al., 2001) and so, most probably, the survival in wood vats has other reasons. Among these, the diffusion of oxygen is essential either by stimulating yeast growth or by reducing the levels of molecular sulphite active against yeasts. In addition, it is believed that cell immobilization in the wood structure contributes to the protection against preservatives (Swaffield et al. 1997). The ability to form pseudomycelium might as well favor the colonization of the porous structure of the wood and of the spaces between the staves and the grooves.

13.3 Spoilage activities

Microbial spoilage is not easily defined, particularly in fermented foods and beverages, where the metabolites produced contribute to the flavor, aroma and taste of the final products. In fact, for cultural or ethnic reasons, there is little difference between what is perceived as spoilage or beneficial activity (Fleet, 1992). An excellent example of this is the production of 4-ethylphenol by Dekkera/Brettanomyces spp. in fermented beverages, where spoiling depends on the type of product and on the awareness of the technologists for the problem. In fact, until the enlightenment of the origin of 4-ethylphenol in wines by Chatonnet et al. (1992), these species were not regarded as a serious threat for the wine industry but, today, they are the most serious microbial problem of modern enology worldwide. In contrast, they are desirable in several traditional Belgian beers where volatile phenols contribute to flavor characteristics (Vanderhaegen et al., 2003). In other fermentations, such as cider and kombucha, these yeasts are also believed to play a role in aroma characteristics. However, only now the levels of 4-ethylphenol in cider and beer begin to be carefully studied (Picinelli, Vanderhaegen, personal
communications) perhaps influenced by the information provided by research on wines. In these fermented drinks, whether volatile phenols should be regarded as a taint or not is still to be determined. Other exceptions to their spoilage attributes are the possibility of using cellobiose fermenting *B. bruxellensis* in cellulose fermentation for ethanol production (Spindler et al., 1992; Park et al., 1999, 2000) and of using *B. anomalus* in the production of cerebrosides for functional foods to prevent cancer (Tanji et al., 2004). The ability to ferment lactose by *B. anomalus* (Bothast et al., 1986) may also be exploited to convert cheese whey to single-cell protein (Sandhu and Waraich, 1983).

### 13.3.1 Wine spoilage by volatile phenols

The most relevant spoilage effects of *D. bruxellensis* are the off-flavors and off-tastes provoked by volatile phenols, especially in fashionable premium red wines matured in oak casks, where it is responsible for serious economic losses. The origin of volatile phenols is related with the sequential activity of two enzymes, which decarboxylate hydroxycinnamic acids present in grape juices or wines (ferulic, *p*-coumaric and caffeic acids) into hydroxystyrenes (4-vinylguaiacol, 4-vinylphenol, 4-vinylcathecol, respectively), which are then reduced to ethylphenols (4-ethylguaiacol, 4-ethylphenol, 4-ethylcathecol, respectively) (Steinke and Paulson, 1964). The decarboxylation step is present in a large number of bacteria, fungi and yeast species (Degrassi et al., 1995; Edlin et al., 1995; Suezawa, 1995; Suezawa et al., 1998). However, the reduction step is much less frequent and, in yeasts, has been reported as particularly effective in the species *D. bruxellensis*, *D. anomalus*, *Pichia guilliermondii*, *Candida versatilis*, *C. halophila* and *C. mannitofaciens* (Table 13.4). The *Candida* species producing high levels of volatile phenols are associated with soy sauces (Suezawa, 1995). The species *P. guilliermondii* was isolated from grapes, grape juices and winery equipment (Dias et al., 2003b), but, apparently, do not grow and produce 4-ethylphenol in wines with average ethanol levels of 12% (v/v) (unpublished observations).

The knowledge of wine spoilage by volatile phenols produced by *D. bruxellensis* is relatively recent. Tucknott et al. (1981) reported that these yeasts were the only species isolated from wines with mousy and other ill-defined off-odors. Hereszty (1986b) demonstrated the production of volatile phenols by *Brettanomyces* in grape juice, but lactic acid bacteria were thought to be also responsible for their production in wines (Cavin et al., 1993). Hock (1990) described the concern caused by these yeasts in Californian wines, but the problem was then related to obnoxious flavors and odors, not specifically to the production of 4-ethylphenol (Kunkee and Bisson, 1993). Sponholz (1992) mentioned the production of 4-ethylphenol by *Brettanomyces* but did not relate it to phenolic taint. Ibeas et al. (1996) were not yet aware of the production of volatile phenols although mentioned the detection of contaminated sherry wines with disagreeable flavor characteristics. By that
Table 13.4  Production of volatile phenols by yeast species

<table>
<thead>
<tr>
<th>Species</th>
<th>4-vinyl derivative</th>
<th>4-ethyl derivative</th>
<th>Growth medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. bruxellensis</td>
<td>+</td>
<td>+</td>
<td>Synthetic defined medium with <em>p</em>-coumaric acid</td>
<td>Chatonnet <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>C. vini, M. pulcherrima, P. membranifaciens, S. cerevisiae, T. delbrueckii</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. anomalus</td>
<td>+</td>
<td>+</td>
<td>Synthetic defined medium with <em>p</em>-coumaric, caffeic or ferulic acids</td>
<td>Edlin <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>Candida versatilis, C. halophila, C. mannitofaciens</td>
<td>+</td>
<td>+</td>
<td>High salt (15% NaCl) medium with ferulic or <em>p</em>-coumaric acids</td>
<td>Suezawa (1995)</td>
</tr>
<tr>
<td>D. hansenii, P. subpelliculosa, P. anomala, C. famata</td>
<td>+</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. etchellsii, C. nodaensis, C. halonotrophila, P. farinosa, Z. rouxii</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. lambica, R. rubra/glutinis, R. minuta</td>
<td>+</td>
<td>–</td>
<td>Synthetic defined medium with ferulic acid</td>
<td>Donaghy <em>et al.</em> (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>--------</td>
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<td>--------</td>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>B. bruxellensis</strong></td>
<td>+</td>
<td>+</td>
<td>Grape juice with ferulic or p-coumaric acids</td>
<td></td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong>, <strong>Z. bailii</strong>, <strong>S. ludwigi</strong></td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D. bruxellensis</strong>, <strong>D. anomala</strong>, <strong>P. guilliermondii</strong></td>
<td>+</td>
<td>+</td>
<td><strong>Self</strong></td>
<td></td>
</tr>
<tr>
<td><strong>P. guilliermondii</strong>, <strong>C. wickerhamii</strong>, <strong>D. hansenii</strong></td>
<td>+</td>
<td>–</td>
<td>Synthetic defined medium with p-coumaric acid</td>
<td></td>
</tr>
<tr>
<td><strong>C. wickerhamii</strong>, <strong>C. cantarelli</strong>, <strong>D. hansenii</strong>, <strong>K. lactis</strong></td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. naardenensis</strong>, <strong>B. custersianus</strong>, <strong>B. nanus</strong></td>
<td>Not assessed</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shinohara et al. (2000)

Dias et al. (2003b), unpublished data
time, the research of Chatonnet et al. (1992, 1995, 1997) was important to demonstrate that the genera *Dekkera/Brettanomyces* should be regarded as the sole agents of phenolic off-flavors in wines. Thus, the next monographs covering wine microbiology issues described thoroughly, for the first time, the characteristics of *Dekkera/Brettanomyces* spp., including the production of phenolic off-odors described as ‘medicinal’, ‘Band-aid®’, ‘barnyard-like’ or ‘horsey’ (Boulton et al., 1996; Fugelsang, 1997; Ribéreau-Gayon et al., 2000). The discussion on the benefits or defects of phenolic flavors is still lively, supported by many leading wine writers that look at phenolic wines as having a positive ‘Brett character’ (Arvik et al., 2002). Winemakers share the opposite opinion based on the fact that wine fruitiness and complexity is replaced by a dominant phenolic flavour (Arvik et al., 2002; Coulter et al., 2003). In addition to off-flavors, the volatile phenols in high amount also affect wine mouthfeel by increasing bitterness and astringency, described as metallic taste (Coulter et al., 2003).

**Relevance of volatile phenols**

The value of a preference threshold may measure the spoilage effect of molecules with sensorial activity. This is defined as the minimum concentration under which 50% of the tasters, in a 70 person jury, statistically rejected the sample (Chatonnet et al., 1992). For instance, in Bordeaux red wines, the preference threshold for 4-ethylphenol is about 620 µg/l, and for the mixture (10:1) of 4-ethylphenol and 4-ethylguaiacol is 426 µg/l (Chatonnet et al., 1992). Below these concentrations, volatile phenols may contribute favorably to the complexity of wine aroma by imparting aromatic notes of spices, leather, smoke or game, appreciated by most consumers. Above those levels, wines are clearly substandard for some consumers but remain pleasant for others. To increase the difficulty in the definition of spoilage, these thresholds are dependent on grapevine variety and on the style of wine (Gato et al., 2001; Coulter et al., 2003).

Analysis of volatile phenols from different countries showed that more than 25% of the red wines had levels higher than the preference threshold of 4-ethylphenol of 620 µg/l (see review of Loureiro and Malféito-Ferreira, 2003). Although the results are not based on random sampling, they demonstrate that volatile phenols are a worldwide concern. Moreover, as sensory detection of volatile phenols depends on the type of wine (Gato et al., 2001; Coulter et al., 2003), a higher proportion of wines may be badly affected by volatile phenols. 4-Ethylguaiacol is present in about one-tenth of the 4-ethylphenol concentration (Chatonnet et al., 1992) but this rate is not always observed (Rodrigues et al., 2001; Coulter et al., 2003). The other volatile phenol, 4-ethylcathecol, is only now under study because its detection in red wines by gas chromatography requires previous sample derivatization and so it is not detected in the analysis of the other volatile phenols (Hesford and Schneider, 2004). The fact that the precursors of 4-ethylcathecol, caffeic acid and its esters, are present in relatively high concentrations in wines (Lee and Jaworski...
and that the detection threshold for 4-ethylcathecol (described as having a phenolic smell similar to that of 4-ethylphenol) is lower than that of the other volatile phenols (Porret et al., 2004), suggest that its influence in phenolic taint should not be neglected. Occasional discrepancies between sensorial detection and concentration of 4-ethylphenol and 4-ethylguaiacol may be explained by the hidden presence of 4-ethylcathecol.

Volatile phenol precursors in raw materials
The hydroxycinnamic acids in nature are found in free or in esterified forms. Apparently, the free forms are consumed directly by the yeasts but the esters should be previously hydrolyzed (Grando et al., 1993). In wines the bound forms are mainly tartaric or anthocyanin esters (Gil et al., 1995; Romero and Bakker, 2000; Ibern-Gómez et al., 2002) that may be hydrolyzed by fungal enzymes present in commercial pectinase preparations or by grape juice heating (Gerbaux et al., 2002). The concentrations of hydroxycinnamic acids in beers are comparable to those of red and white wines (Montanari et al., 1999; Gorinstein et al., 2000) but the esterified forms have different origins. In brewing, the cell walls of barley and wheat have mainly ferulic acid esterified to arabinoxylans and esterified or etherified to lignin surfaces (Coghe et al., 2004). Feruloyl esterases have been characterized and related proteins have been sequenced in fungi and bacteria (Crepin et al., 2004). However, the hydrolytic or esterasic activity has not been deeply studied regarding yeasts. Grando et al. (1993) stated that S. cerevisiae did not hydrolyze the tartaric esters but Coghe et al. (2004) suggested that brewer’s yeast possesses feruloyl esterasic activity. We are not aware of these esterasic activities in Dekkera/Brettanomyces spp. If S. cerevisiae is able to convert the esterified forms into the free forms and afterwards into vinyl derivatives, then B. bruxellensis may reduce them to 4-ethylphenol in the absence of hydroxycinnamic acids (Dias et al., 2003a).

In large-scale production beers the main concern is 4-vinylguaiacol, reflecting the ferulic acid decarboxylation by some strains of S. cerevisiae, and, to a lesser extent, by temperature during wort boiling (Coghe et al., 2004). The absence of 4-ethylguaiacol in these industrial types of beers may be explained by the absence of contamination by Dekkera/Brettanomyces spp. In contrast, traditional Belgian beers contaminated by these yeasts show high contents of ethylphenols (Vanderhaegen, personal communication) as do ciders (Picinelli, personal communication). The fact that the respective raw materials bear amounts of precursors (Picinelli et al., 1997; Suárez et al., 1998; Montanari et al., 1999; Gorinstein et al., 2000; Alonso-Salces et al., 2004) high enough to produce volatile phenols higher than the respective detection thresholds, provides a sound basis for further research in this field.

Concerning kombucha, there are no data available on volatile phenol contents, but knowing that the raw material (tea) bears esters of caffeic and...
p-coumaric acid (Clifford, 1999) it is likely that those molecules may be produced by the Dekkera spp. contaminants. In traditional ewe’s milk cheeses there is a detrimental descriptor known as ‘stable smell’ very similar to the smell of 4-ethylphenol. In these products the precursor might come from the hydroxycinnamic acids probably present in the extracts of the plant cardoon (Cynara cardunculus) used as milk rennet. Knowing that Dekkera spp. are present in cheese-making plants, the activity of these yeasts should also be studied in these products.

13.3.2 Spoilage by other metabolites
Besides volatile phenols, Dekkera/Brettanomyces spp. are known for producing other metabolites with undesirable properties, such as acetic acid, tetrahydropyridines, isovaleric and other fatty acids. However, they have not the technological relevance and prevalence of volatile phenols. Concerning wines, Schanderl, in 1950, described their influence on bottled wine spoilage by production of acetic acid and haziness (van der Walt and van der Kerken, 1958). Peynaud and Domercq (1956) referred to the production of acetic acid and ‘mousy’ off-odors and off-tastes in grape juice. Heresztyn (1986a) showed that the mousy taints were due to the production of tetrahydropyridines. These molecules are produced in the presence of ethanol and lysine and are mainly noticed by its retronasal odor (Grbin and Henschke, 2001).

Presently other activities are under study in B. bruxellensis. These yeasts have esterasic ability to hydrolyze sugar-bound glycosides but this feature was not proved to affect wine flavor under practical conditions (Mansfield et al., 2002). The production of biogenic amines (Caruso et al., 2002) and of 2,3-butanediol and acetoin (Romano et al., 2003) has also been referred in B. bruxellensis, but their detrimental influence on human health or wine aroma, respectively, has to be ascertained. Isovaleric and other fatty acids producing rancid flavours are also linked with Brettanomyces activity (Licker et al., 1998) but their true influence on wine spoilage is not clear (Coulter et al., 2003). These metabolic features seem to be independent, because the concentrations of volatile phenols are not correlated either with acetic acid (Rodrigues et al., 2001) or isovaleric acid levels (Coulter et al., 2003).

Acetic acid production is a concern mainly for fuel alcohol industries. Miniac (1989) showed a correlation between increase in Brettanomyces spp. population, production of acetic acid and decrease in ethanol yield in molasses fermentation. However, other authors claim that acetic acid produced is insufficient to inhibit S. cerevisiae fermentations (Phowchinda et al., 1997; Abbott et al., 2005). The recent events of Brettanomyces spp. contamination in Canadian plants confirm that the problem is still present (Abbott et al., 2005).
13.4 Metabolism and physiology of *Dekkera/Brettanomyces* spp.

There are few studies on the metabolism and physiology of *Dekkera/Brettanomyces* spp. and the following description will mainly address the issues related with their prevalence as spoilage yeasts. These genera have singular metabolic characteristics among foodborne yeasts. Contrarily to *S. cerevisiae*, fermentation is stimulated by the presence of oxygen, in a mechanism described as negative-Pasteur or Custer’s effect (Wikén *et al.*, 1961; Scheffers, 1966; Wijsman *et al.*, 1984). This effect has also been described, in *B. anomalus*, as negligible CO₂ production, in the presence of glucose, under strictly anaerobic conditions (Gaunt *et al.*, 1988). Their physiology is characterized by relative low growth rates in unstressed environments (Table 13.5) and dependent on carbon sources (Dias *et al.*, 2003a). The requirement for vitamins is also commonly referred to in the studies of these yeasts (Fugelsang, 1997); however there is some controversy regarding the vitamins required, as mentioned in the development of culture media (Section 13.5.1). Furthermore, Madan and Gulati (1980) reported that *B. bruxellensis* did not require thiamine, biotin and several other vitamins, for growth. In addition, Uscanga *et al.* (2000) considered *D. bruxellensis* as a nutritionally low demanding yeast, not dependent on magnesium and phosphate ions and only slightly dependent on ammonium ions for growth. Thus, the fastidious connotation of this species requires deeper investigation.

The behavior of *D. bruxellensis* in fermenting media (molasses, grape juice and synthetic medium) with *S. cerevisiae* is characterized by showing null or slow growth until about the end of fermentation (Miniac, 1989; Dias *et al.*, 2003a; Abbott *et al.*, 2005; Gómez-Rivas *et al.*, 2004). In post-fermentation it may grow attaining levels as high as those observed in *S. cerevisiae* (Dias *et al.*, 2003a; Abbott *et al.*, 2005). In wines, *D. bruxellensis* growth shows a typical bell-shaped curve (Fugelsang and Zoecklein, 2003) (Fig. 13.1). It is frequent to observe a decrease in viability after inoculation in wines (Grbin and Henshke, 2000; Gerbaux *et al.*, 2002) dependent on the stress imposed to cells, as exemplified in Fig. 13.1 for the ethanol effect. After the initial death phase the growth is reinitiated by the surviving cells, possibly due to a physiological mechanism responsible for weak acid resistance similar to that described for *Z. bailii* (Piper *et al.*, 2001; Steels *et al.*, 2002).

Concerning relevant technological properties, *Dekkera/Brettanomyces* spp. are known for producing high amounts of acetic acid, probably because of a block of the oxidation of ethanol occurring at the level of succinate dehydrogenase (Sanfacon *et al.*, 1976). However, this ability depends on many factors and published results seem to be frequently contradictory. Acetic acid production is stimulated, in *D. anomalula*, by sugar concentrations higher than 2% (w/v) (Gerós *et al.*, 2000; Dias *et al.*, 2003a) but, in *D. bruxellensis*, Abbott *et al.* (2005) reported the highest production at intermediate (4% (w/v) glucose) concentrations. The modulation of acetic acid production
Table 13.5  General physiological characteristics of technological significance of several yeast species compared with *D. bruxellensis*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>D. bruxellensis</em></th>
<th><em>Z. bailii</em></th>
<th><em>S. cerevisiae</em></th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate with glucose (h⁻¹)</td>
<td>0.11</td>
<td>–</td>
<td>0.34</td>
<td>Silva <em>et al.</em> (2004)</td>
</tr>
<tr>
<td>Growth rate with glucose (h⁻¹)</td>
<td>0.15</td>
<td>0.19</td>
<td>–</td>
<td>Dias <em>et al.</em> (2003a), Sousa-Dias <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>Maximum concentration for growth (ethanol %v/v, pH 3.5)</td>
<td>15.5</td>
<td>–</td>
<td>17.0</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Maximum concentration for growth (SO₂ mg/l, pH 3.5)</td>
<td>70</td>
<td>–</td>
<td>200</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Maximum concentration for growth (SO₂ mg/l, pH 3.5)</td>
<td>75</td>
<td>500</td>
<td>50</td>
<td>Loureiro (1997)</td>
</tr>
<tr>
<td>Maximum concentration for growth (sorbic acid mg/l, pH 3.5)</td>
<td>950</td>
<td>600</td>
<td>300</td>
<td>Loureiro (1997)</td>
</tr>
<tr>
<td>Maximum concentration for growth (acetic acid mm, pH 3.5)</td>
<td>200</td>
<td>400</td>
<td>50</td>
<td>Loureiro (1997)</td>
</tr>
<tr>
<td>Maximum concentration for growth (NaCl M, pH 3.5)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>Loureiro (1997)</td>
</tr>
<tr>
<td>Maximum concentration for growth (glucose %w/v)</td>
<td>35</td>
<td>60</td>
<td>60</td>
<td>Loureiro (1997)</td>
</tr>
<tr>
<td>Maximum concentration for growth (sucrose %w/v)</td>
<td>45</td>
<td>75</td>
<td>65</td>
<td>Loureiro (1997)</td>
</tr>
<tr>
<td>Maximum concentration for growth (CO₂ dissolved volumes)</td>
<td>4.45</td>
<td>3.34</td>
<td>2.23</td>
<td>Ison and Gutteridge (1987)</td>
</tr>
<tr>
<td>Fermentation inhibiting concentrations of dimethyl dicarbonate (mg/l)</td>
<td>250</td>
<td>400</td>
<td>250</td>
<td>Delfini <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Growth inhibiting concentrations of acetic acid (mm) in 10% (v/v) ethanol</td>
<td>78</td>
<td>261</td>
<td>122</td>
<td>Kalathenos <em>et al.</em> (1995)</td>
</tr>
</tbody>
</table>
Dekkera/Brettanomyces spp. was also shown to be dependent on oxygen (Ciani and Ferraro, 1997; Abbott et al., 2005) and ammonium sulphate (Uscanga et al., 2000). Therefore, it should not be surprising that wines heavily contaminated with Dekkera did not show high levels of acetic acid (Ciani and Ferraro, 1997; Rodrigues et al., 2001) probably because the factors stimulating its production are not effective. Further research to elucidate this issue should take into account that acetic acid production is variable among strains cultivated under the same conditions (Freer, 2002) and that acetic acid may also be assimilated by these yeasts (Dias et al., 2003a).

The ability to produce odor active compounds like volatile phenols or tetrahydropyridines is a feature not common in other yeast species. The physiological justification for this is not clear. In the case of hydroxycinnamic acids decarboxylation, Arvik and Henick-Kling (2002) speculated that some ATP may be obtained by a small electron gradient similar to the conversion of malic into lactic acid by Oenococcus oenii but this mechanism has never been demonstrated in eucaryotic cells (Salema et al., 1996). The other hypothesis is that these properties are related with a detoxification process, particularly beneficial in acidic media (Barthelmebs et al., 2001).

13.4.1 Effect of antimicrobial agents
There is a certain controversy on the tolerance of Dekkera/Brettanomyces spp. to antimicrobial agents that may result from the comparison of data obtained in different conditions. Therefore, Table 13.5 provides data on tolerance measures for three different yeast species obtained with the same methodologies. In this way it is possible to compare the resistance of D. bruxellensis with those of Z. bailii, regarded as the most resistant spoilage species, and of S. cerevisiae, the most common fermenting species. The

![Image of Fig. 13.1] Viability of D. bruxellensis ISA 1791 inoculated in red wine with 8 (●), 12 (■) or 14% (v/v) ethanol (▲), at pH 3.50. Wines were incubated in Erlenmeyer flasks with orbital shaking at 25°C (unpublished results).
values reported in Table 13.5 show that *D. bruxellensis* is the most tolerant species only towards sorbic acid and dissolved CO₂. Given that sorbic acid concentrations are not used in such high levels, the above-mentioned predominance of *Brettanomyces* in carbonated beverages might be explained by its tolerance to dissolved CO₂. However, in other products the possible reasons for their predominance do not seem to be related to particular stress tolerances to antimicrobials, given that both *Z. bailii* and *S. cerevisiae* are more tolerant. Therefore, further studies are required to elucidate the physiological reasons underlying the survival and predominance of *Dekkera/Brettanomyces* spp. during the long maturation periods of alcoholic beverages.

The main preservative in wines is sulphur dioxide and *D. bruxellensis* has been regarded as either resistant or sensitive. This yeast is recovered mainly in wines not protected by sulphite (Heresztyn, 1986b) and some authors refer its sensitivity to sulphite higher than 30mg/l (Gerbaux et al., 2002; Chatonnet et al., 1992, 1993). Others state that it should be regarded as resistant (van der Walt and van Kerken, 1961; Gaia, 1987; Ciolfi, 1991) and growth has been reported under more than 30 mg/l of free sulphite (Froudière and Larue, 1989). To this apparent controversy concurs the absence of studies under comparable situations. In fact, sulphite resistance was studied in low ethanol wines of 9.5% (Gaia, 1987) or 11.3% ethanol (Froudière and Larue, 1989) and our results point out that ethanol level is a major factor determining sulphite resistance (Malfeito-Ferreira et al., 2004). In synthetic media, initial ethanol levels of 13% (v/v) (Dias et al., 2003a) and 11.4% (Medawar et al., 2003) were shown to arrest *D. bruxellensis* growth. These yeasts were detected in wines up to about 13% ethanol (Gaia, 1987; Ciolfi, 1991; van der Walt and van Kerken, 1961; Rodrigues et al., 2001). At 15% ethanol growth was not observed (Froudière and Larue, 1989) but Ibeas et al. (1996) isolated *Brettanomyces* from the pellicles of sherry wines with about 15% ethanol. However, it is known that, in sherry wines with 14.5% (v/v) ethanol, *Dekkera* proliferation is prevented by increasing the ethanol content to above 15% (v/v) (Ibeas et al., 1997). Knowing that sherry aging occurs under oxygen presence and yeasts are predominantly in the pellicle, it is probable that *D. bruxellensis* have better conditions to grow. These apparent discrepancies, beside strain variability, could be understood if the many factors affecting wine susceptibility could be all controlled (Loureiro and Malfeito-Ferreira, 2003). Thus, we believe that, in red table wines, a maximum ethanol tolerance of 14–14.5% is a reasonable guideline for these yeasts. This value means that practically all red table wines are susceptible to colonization by these yeasts because this is the average higher ethanol content. Wines with more than 15% ethanol are, as a rule, fortified wines and do not show phenolic taint (Kunkee, Hogg, personal communications).

A different issue is the colonization of white wines. These yeasts have been isolated from white wines, sparkling wines (see Table 13.3) and grow during white wine fermentation (Dias et al., 2003a). However, Malfeito-Ferreira et al. (2001) reported loss of viability of *D. bruxellensis* after
inoculation in a wide range of commercial white wines, by a pH-dependent process. These authors hypothesized that the observed cell death explains the absence of phenolic taint, due to 4-ethylphenol, in white wines (Loureiro and Malfeito-Ferreira et al., 2003).

The utilization of sorbic acid is not an advisable preventive measure because *D. bruxellensis* is resistant to the maximum legal concentration of 200 mg/l in wines, in accordance to the high tolerance reported in synthetic media (Table 13.5). For benzoic acid, van Esch (1992) reported minimum inhibitory concentrations from 100 ppm (*B. anomalus*) to 200 ppm (*B. intermedius, D. anomala, B. naardenensis*) in preserved non-juice soft drinks such as tonic water, lemon-lime and cola, with moderate requirement for oxygen. This author considers those species as the main spoilers by turbidity formation.

In conjunction with sulphite, dimethyldicarbonate (DMDC) is an effective agent against *Dekkera* growth, depending on ethanol content (Malfeito-Ferreira et al., 2004). It may be used to prevent wine contamination in countries where it is legally authorized. In grape juices, 250 mg/l inhibited fermentation of *B. bruxellensis*, while 400 mg/l were shown to delay fermentation by *B. anomalus* (Delfini et al., 2002). In wines matured in barrels it is an efficient tool to prevent *Brettanomyces* blooms, being used in regular additions up to the maximum permitted level of 200 mg/l. It may be also added just before bottling, claimed to be efficient if yeast counts are less than 500 cfu/ml.

Other substances or processes have also been reported as effective against *Dekkera/Brettanomyces* spp. High pressure (400 or 500 MPa for 5 or 15 min at 4 or 20 °C) reduced microbial counts of *S. cerevisiae* and *B. bruxellensis* by more than 99.99% (Puig et al., 2003). The essential oils from aerial parts of *Melissa officinalis* at 500 µg/ml completely inhibited the growth of *D. anomalala* (Araújo et al., 2003). Chitosan, a natural derivative of chitin, appeared to inhibit *D. bruxellensis* growth in mixed bioethanol fermentations with *S. cerevisiae* (Gómez-Rivas et al., 2004). The exploitation of killer toxins produced by other yeasts has also been reported (Comitini et al., 2004) but the sensitivity of *D. bruxellensis* varies within the species and is reduced at lower pH values, similar to those found in wines (Yap et al., 2000). Therefore, the practical efficiency of such measures remains to be ascertained under real winery conditions.

13.5 Detection and identification of *Dekkera/Brettanomyces* spp.

13.5.1 Detection and enumeration techniques

Most of the works listed in Table 13.3 refer that the relative predominance of *Dekkera/Brettanomyces* spp. among other species is almost always low, which has been explained by their supposed fastidious nutritional requirements and relative low growth rates. In addition, the above-mentioned discrepancies
in their presence, or absence, from the same sources analyzed by different authors, may result from inadequate selection of culture media and incubation time. Therefore, particular attention should be given to the selection of adequate cultivation techniques and incubation periods to elicit the detection of these yeasts.

Early ecological studies reflect the awareness of the difficulty of isolating *Dekkera/Brettanomyces* spp. from the environment. Van der Walt and van Kerken (1960) described the difficulties in their recovery from materials heavily contaminated with other yeasts or molds that prevented the detection in plating media. These authors developed selective culture media by using sorbic acid, cycloheximide, aureomycin and chloromycetin as inhibitors of other yeast species and bacteria, and by using maltose and sucrose as carbon sources with an incubation period of two weeks. Wright and Parle (1974) used a medium acidified to pH 4 with tartaric acid and followed the recommendation to use sucrose but reported that the use of cycloheximide, sorbate or ethanol as antimicrobials was not satisfactory. More recent dissemination works relied on the use of cycloheximide (Froudière and Larue, 1990; Chatonnet *et al*., 1992; Fugelsang, 1997; Alguacil *et al*., 1998; Mitrakul *et al*., 1999; Rodrigues *et al*., 2001), gentamicin and oxytetracycline together with ethanol at 10% (v/v) (Alguacil *et al*., 1998) or sorbic acid (Chatonnet *et al*., 1992) to suppress the growth of other yeast species and bacteria. The use of glycerol (Fugelsang, 1997) or trehalose and sucrose (Chatonnet *et al*., 1992) has also been advised as carbon sources and a pH indicator (bromocresol green) to detect media acidification (Chatonnet *et al*., 1992; Fugelsang, 1997; Rodrigues *et al*., 2001). In order to favor the growth of *Dekkera/Brettanomyces* spp. vitamins (e.g. biotin and thiamine) may be added to the media (van der Walt and van der Kerken, 1961) but this has not been always regarded as necessary (Chatonnet *et al*., 1992; Fugelsang, 1997; Alguacil *et al*., 1998).

Rodrigues *et al*. (2001) developed a selective media to be used by plating or by the Most Probable Number technique (MPN). The use of MPN enabled the recovery of *D. bruxellensis* from a higher number of wine samples and enabled its detection in much less than 1% of total microbial flora. The use of liquid growth medium favors the recovery perhaps by acting as a resuscitation medium and reduces mold growth. The use of ethanol as sole carbon and energy source inhibited most fast growing oxidative species (Rodrigues *et al*., 2001).

The most common yeast detection techniques in food industry are dependent on growth on plate media (for a critical review see Loureiro *et al*., 2004). As mentioned before, the detection of *Dekkera/Brettanomyces* spp. is improved by the use of selective and differential media but there are only two commercial media available: BSM, from Millipore and DBDM from STABVIDA (Loureiro *et al*., 2004). Other media used in wineries or by support service laboratories are based on those reported in literature (Loureiro and Malfeito-Ferreira, 2003). When *D. bruxellensis* is the sole or predominant contaminant, a general purpose medium added of cycloheximide is efficient enough (Coulter *et al*.,
2003) given that long incubation periods (up to 15 days) are used, specially for the recovery of stressed cells (Rodrigues et al., 2001). The usual 48–72 h, at 25–30°C, of incubation in routine microbiological control are not enough regarding Dekkera/Brettanomyces spp. Long incubation periods are not totally disadvantageous when these yeasts predominate in the absence of molds, because their colonies appear as pinpoints on plate media, contrasting with larger colonies originating from fast-growing species.

Given the slow growth of these yeasts, early detection depends on the use of direct techniques. Modern direct techniques rely on the conjunction of a series of formerly independent techniques, to which molecular approaches have shown its potential to detect and simultaneously identify contaminant yeasts (see critical review of Loureiro et al., 2004). Microscopy-based techniques are only suitable when contamination is high, owing to operator’s fatigue, and when particular morphologies (e.g. bud scars, boat-shaped and ogival cells) of Dekkera/Bruxellensis spp. are obvious. The utilization of fluorescence microscopy after hybridization with species-specific PNA probes (Dias et al., 2003b) directly on pellets of centrifuged wine is an efficient way to confirm the presence of D. bruxellensis in suspected wines. Positive cells appear as bright green fluorescent, under detection thresholds dependent on the volume of centrifuged (or membrane filtered) samples.

The requirement for high levels of contamination also holds true for the effective utilization of direct molecular techniques, like nested polymerase chain reaction (PCR) (Ibeas et al., 1996) or denaturing gradient gel electrophoresis (DGGE) (Cocolin et al., 2001; Mills et al., 2002). Cocolin et al. (2004) presented several PCR, PCR-RE (restriction endonuclease) and DGGE protocols. These techniques were compared against conventional plate counting and the ability to analyze wine samples directly was shown using the PCR-RE protocol within 8h. The main fault, common to other direct methods, is the high detection limit of about $10^4$ cfu/ml. In fact, with this concentration wines may be already tainted by phenolic off-flavor and Dekkera countings in wines, when reported, are mostly below $10^4$ cfu/ml (Mitrakul et al., 1999; Gerbaux et al., 2002; Rodrigues et al., 2001; Phister and Mills, 2003; Cocolin et al., 2004, footnote (a) of Table 13.6 below). The technique of Ibeas et al. (1996) may be rendered more sensitive if wine is centrifuged and PCR is performed in washed cells, with reported positive results with 10 cells. Real-time PCR assays are claimed to have higher sensitivities. Phister and Mills (2003) reported a test taking only 3 hours and detecting as low as 10 cells/ml of wine. The protocol includes a 10-fold dilution of the wine to avoid PCR inhibitors. Delaherche et al. (2004) also described a real-time PCR protocol but showing a detection limit of $10^4$ cells/ml, which is too high for winery practice, as explained before.

The acceptable levels of D. bruxellensis cell counts should be kept to a minimum, given the high risk associated with its presence (Loureiro and Malfeito-Ferreira, 2003). Then, to monitor these yeasts, techniques should demonstrate adequate high sensitivity. To increase detection threshold in
molecular techniques, authors advise a previous cultivation step (Alguacil et al., 1998; Mills et al., 2002) which, by depending on cell proliferation, overcomes the limitation of absence of viability assessment by the reported molecular techniques. In fact, pre-cultivation seems to be an essential step to recover ascomycetes from natural samples even when direct temperature gradient gel electrophoresis (TGGE) techniques are used (Gadanho et al., 2004). The viable but non-culturable population (Millet and Lonvaud-Funel, 2000) may also be detected after pre-cultivation in recovery media.

### 13.5.2 Identification and typing techniques

Genetic molecular identification is now regarded as most effective in species delineation. Identification molecular techniques rely mostly on sequencing the 600–650 nucleotide D1/D2 domain of large subunit (26S) ribosomal DNA because of its taxonomical significance (see review by Kurtzman et al., 2003). The recent CD-ROM manual of Boekhout et al. (2002) provides, in addition to physiological tests, sequences of 18S, 26S and ITS regions of ribosomal RNA gene for most described species. Regarding Dekkera/Brettanomyces spp. the utilization of molecular biological methods began with the analysis of the mitochondrial genome (Hoeben and Clark-Walker, 1986), of the mitochondrial-encoded cytochrome oxidase subunit gene (COX2) (Hoeben et al., 1993) and of the rRNA genes (Molina et al., 1993). Afterwards, Boekhout et al. (1994), Yamada et al. (1994) and Cai et al. (1996) determined partial sequences of 18S and 26S rRNA genes. These molecular approaches contributed to and are consistent with the present species concept and B. custersianus and B. nanus are regarded as phylogenetically distant species (Smith, 1998). These molecular approaches, either for detection or for identification reveal no distinction between anamorph and teleomorph strains, therefore the separation between Brettanomyces and Dekkera is meaningless.

The above-mentioned molecular sequencing is not a suitable tool for industrial utilization despite the availability of gene sequences databases (see review by Kurtzman et al., 2003). Typing of isolates recovered from microbial control plates using molecular techniques is now common practice using simpler techniques (Loureiro et al., 2004). One of the methodologies is PCR-RFLPs (restriction fragments length polymorphism) of rDNA with the respective database, produced by Querol and co-workers, which is available at the website http://motor.zm.nu. The database includes restriction profiles of the species D. bruxellensis and D. anomalous. Egli and Henick-Kling (2001) also used rDNA RFLPs together with sequencing of ITS regions to identify D. bruxellensis. Discrimination at strain level by molecular typing has been reported in D. bruxellensis by means of random amplification of polymorphic DNA-PCR (RAPD-PCR) (Mitrakul et al., 1999) and of AFLP (Barros-Lopes et al., 1999). Molecular polymorphism is known and type strains do not share the same types of molecular patterns (Hierro et al.,
The analysis of *D. bruxellensis* isolated from a wide variety of wine sources using RAPD-PCR with three different OPAs enabled the detection of 12 different haplotypes, evidencing the heterogeneity of the species (Martorell et al., 2006). The AFLP typing also evidenced a high genetic variation at subspecies level (Bellon et al., 2003). Both methodologies reveal the existence of a dominant strain among the different analyzed sources and that a single winery bears genetically different strains (Bellon et al., 2003; Martorell et al., 2006).

Other methodologies of yeast typing dependent on the analysis of cellular composition (e.g. long-chain fatty acids, enzymes, proteins) are, presently, under less attention than the above-mentioned molecular methods (see review of Loureiro et al., 2004).

### 13.6 Monitoring and control

The monitoring and control of *Dekkera/Brettanomyces* spp. will be discussed regarding the wine industry, because of its greater relevance and threat when compared with other food industries.

#### 13.6.1 Microbial and chemical indicators

The most common and suitable technique to detect and count *D. bruxellensis* is plate counting for most wineries. However, many of them do not have regular microbiological control and most of those having it do not specifically target *D. bruxellensis* (Loureiro and Malfeito-Ferreira, 2003). The small scale of wine enterprises is a serious limitation to the development of a routine microbial control. The costs of equipment from the simplest for plate counting to advanced instruments (e.g. fluorescence microscope, real-time PCR, flow cytometer) and the requirement for skilled labour are still a burden for most small and medium enterprises (see review of Loureiro et al., 2004).

Therefore, the economical losses associated with *D. bruxellensis* activity have moved companies to ask for external support. Wineries requiring the identification of presumptive *D. bruxellensis* strains most commonly rely on the services of external laboratories which use classical plating techniques associated or not with molecular tools for the requested task (Loureiro and Malfeito-Ferreira, 2003). However, we currently apply to the wine industry a simplified detection technique based on growth in liquid medium. Serial dilutions of wine samples are inoculated in test tubes with DBDM broth and incubated at room temperature for up to 15 days. Positive results include observation of haziness and detection of typical phenolic smell. Depending on the positive dilution it is possible to have a semi-quantitative assessment of the contamination by *D. bruxellensis* and take adequate control measures as described below.
An alternative approach to detect the activity of *D. bruxellensis* is to examine wine samples for chemical evidence of past microbial activity. The presence of 4-ethylphenol in high concentrations is an indicator of its activity because, as far as we know, it is the only microorganism growing in wines with that physiological feature. The determination of 4-ethylphenol is done by gas chromatography and provides results faster than microbial plate counts (Rodrigues *et al.*, 2001). It is also usual to have high levels of this phenol and do not detect *D. bruxellensis* indicating that it has been inactivated after the 4-ethylphenol production. Conversely, levels lower than the preference threshold of 4-ethylphenol in the presence of viable *D. bruxellensis* indicate a serious risk for wine stability. Hence, this molecule may be also regarded as a 'spoilage predictor', because its determination even in low concentrations indicates the onset of *D. bruxellensis* activity. Therefore, both 4-ethylphenol and yeast detection should be part of a well-designed monitoring plan.

**Guidelines**

Present scientific knowledge is insufficient to provide, on solid grounds, a risk assessment of wine spoilage by *D. bruxellensis*. Thus, the absence, to the best of our knowledge, of microbiological guidelines for the wine industry is not surprising. The lack of such an important quality control tool does not allow, on the one hand, the adoption of the most adequate technological control measures when these yeasts contaminate the wines. On the other hand, it makes difficult the establishment of commercial contracts that aim to safeguard the occurrence of wine spoilage by these yeasts during retailing. Under these perspectives, the definition of microbiological criteria to adopt in wineries should be based, above all, on 'common sense' and on the technical capacity to control efficiently the levels of wine contamination.

The microbiological criteria to follow should be regarded at two levels: (i) when the wines are stored in bulk, in oak barrels or in tanks; and (ii) when the wine is to be bottled. In the first situation, the main purpose of the oenologist is to avoid the production of volatile phenols by *D. bruxellensis*, in levels high enough to produce off-flavors and off-tastes. Thus, it is not mandatory to eliminate them completely, but to ensure that the level of contamination or activity is low enough to keep wine quality. In the second situation, taking into account that it is not possible to monitor the production of volatile phenols in bottled wine, or to intervene technologically, the main purpose, after bottling, is to have the wine free from these yeasts. Based on these principles, we have established, for many Portuguese wineries, microbiological criteria that have been giving adequate results so far, and are given here as orientation guidelines.

In the first case, for bulk stored wines, it is satisfactory to detect *D. bruxellensis* monthly, bimonthly or even every 3 months (according to the type of wine and of container). The sample volumes are 1, 0.1, 0.01 and 0.001ml, from a blend composed by wine from the interface air/liquid and from different depths of the container. If the result is positive for 1ml, or
less, and the level of 4-ethylphenol is higher than 150 µg/l, an immediate fine filtration is recommended, accompanied by sulfite addition. In the following analysis, after filtration, it is sufficient to monitor the level of 4-ethylphenol, as a rule. For wines before bottling, the criteria are more stringent, and detection should be made on 100, 10 and 1 ml of wine, sampled as described above. In case the result is positive in 1 or 10 ml, a very fine or sterilizing filtration is recommended. If positive detection is only obtained for 100 ml, it is admissible to control viable cells only by addition of preservatives (e.g., 40 mg/l of free sulphite, at pH 3.50). In this case, bottling must be technically correct and dissolved oxygen should be lowered to almost zero. Otherwise, a sterile filtration or, as an alternative, a thermal treatment to destroy viable cells is recommended.

13.6.2 Control measures

The wine technologists have two different attitudes facing the threat of *D. bruxellensis*. One, we can call ‘optimistic’, results from the absence of wines spoiled by these yeasts and the thought that it only happens to the others. The other, ‘pessimistic’, results from traumatic experiences in the past. Naturally, neither one nor the other is the correct attitude. The first runs a high risk that sooner or later will bring a disastrous consequence. The second leads to exaggerated precautions and to high costs, both economically and in wine quality. Then, keeping in mind that it is necessary to learn how to live with these yeasts in the winery, one should look at prevention as the best way of dealing with them. Hence, it is convenient to remember the main factors that favor wine and cellar contamination and subsequent colonization, in order to establish adequate good wine-making practices. Regarding the contamination factors, there is still much lacking in their knowledge that makes the adoption of efficient control measures difficult. However, as mentioned before, it is known that the prevalence of *D. bruxellensis* is higher in vintages of poor sanitary quality grapes. In this case, care should be taken to avoid cross-contaminations that may jeopardize the wines of other vintages. Another favoring factor is the lack of hygiene, both inside and outside the winery. In fact, as mentioned before, *D. bruxellensis* has been recovered from *Drosophila* spp. flies and, particularly, from dirty surfaces of equipment used in wine-making and in wine storage. In this last case, the utilization of used oak barrels is especially relevant, as these constitute the main known risk factor for these yeasts.

Concerning factors promoting wine colonization, special attention should be paid to levels of free sulfite, levels of dissolved oxygen, presence of residual sugars and storage temperature, during all processing but specially just after the end of malolactic fermentation. Careful *D. bruxellensis* monitoring should be performed especially during extended periods between the end of wine fermentation and the onset of malolactic fermentation, when wines are frequently left unprotected by sulfite and kept at higher temperatures.
Hygiene and disinfection

Common disinfectants used in food industry based on alcohol, hydrogen peroxide, chlorine, tenside and quaternary ammonium are effective against many yeast species and also against *D. anomala* (Wirtanen and Salo, 2004). The least effective are those based on persulfate (Wirtanen and Salo, 2004). Laubsher and Viljoen (1999) also reported the effect of several detergents and disinfectants on *D. custersiana*. The most efficient products were alkaline detergents, iodophors and peracetic acid-based sanitizers. The least efficient was a concentrated acid detergent, probably reflecting higher tolerance to acidic media. The production of biofilms on stainless steel surfaces has been shown for *D. anomala* in beer dispensing systems (Storgards, 2000) decreasing the efficiency of sanitation processes (Wirtanen and Salo, 2004). Although there are no data on *D. bruxellensis*, its behavior may be similar to the other *Dekkera* species. Thus, the main concern is how to sanitize, properly, points of complex geometry or difficult access, such as dead ends of filters, valves, gauges or hoses. The winery walls and floors should also be properly cleaned to avoid wine leftovers that may act as growing media or as breeding places for insects carrying contamination yeasts. The permanence of pomaces and husks accumulated outside the winery may also contribute to the dissemination of yeasts carried into the winery by insects.

Less efficient, or virtually impossible, is the sterilization of wooden vats common in traditional fermentation processes and in modern fashionable wine aging. The porous structure of wood protects part of the established populations from the action of chemical or physical agents. The production of off-flavors by chlorine-based sanitizers in contact with wood, and the residues of all the other chemical agents that are difficult to remove, limit their use. Therefore, sanitation with hot water or steam is essential, although not completely efficient. Swaffield et al. (1997) reported differences, although not statistically significant, before and after cleaning of cider wooden vats, with sodium carbonate at 60°C for 20 min. Laureano et al. (2004) reported preliminary results showing that the lowest total microbial counts were obtained after barrel steaming (overpressure 0.5 kgf/cm² for 10 min). However, even after steaming, *D. bruxellensis* was recovered from wood layers up to 4–6 mm below the surface.

Whatever the treatment adopted in wineries, one should bear in mind that, in barrels, the critical factor is the inability of the disinfecting agent (e.g. hot water, steam, ozone) to reach the deeper layers of the wood. Therefore, most care should be paid to avoid the contamination by infected wine in barrels used for the first time (see below). In addition, the recovery of infected barrels implies dismantling and removal of the wood soaked with wine. Recovery procedures where barrel dismantling is avoided do not guarantee the removal of infected layers located between staves and in groove junctions, which are the spots most difficult to sanitize. The last option is the disposal of infected barrels.
Prevention of cross-contamination and of D. bruxellensis growth

The main concern when a contaminated sample appears is to avoid any cross-contamination. This is especially important when processing products from external sources like purchased wines of unknown origin. The best solution, at first, is to detect the presence of *D. bruxellensis* to determine the measures to be taken. In case wines are contaminated, the most efficient measure would be to filter sterilize. Even knowing the difficulty of sterilizing by filtration young wines and the controversy of such measures, our opinion is that it should be considered, particularly when wines are to be matured in costly oak barrels.

Other cross-contamination to avoid is that due to the utilization of sampling or topping devices that may spread the yeasts to the entire set of barrels. Owing to barrel variability it is common to find contaminated and sound barrels and so, within the same plant, attention should be paid to wines used in toppings and to contamination among barrels. It should be kept in mind that *Brettanomyces* growth is identical in wines stored in new or used barrels (Mahaney *et al*., 1998).

Oxygen is essential to wine aging but it also stimulates growth and production of volatile phenols (Malfeito-Ferreira *et al*., 2001). Even under the practical absence of oxygen *D. bruxellensis* grows and produces 4-ethylphenol at lower rates but high enough to affect wine quality (Malfeito-Ferreira *et al*., 2001). Therefore all operations contributing to oxygen diffusion must be minimized, or carefully monitored, like rackings, pumpings, toppings, bottling and, particularly, micro-oxygenation – a fashionable process to accelerate red wine aging. Oxygen also contributes to loss of sulfite, which is the most efficient preservative against these yeasts (see below).

Temperature stimulates growth and volatile phenol production and so low cellar temperatures are preferable (Malfeito-Ferreira *et al*., 2001), even knowing that this measure is not very efficient and difficult to implement in most wineries. However, it is very important to keep in mind the temperature effect in the definition of the monitoring periodicity, which should be more frequent in summer than winter.

Another aspect to keep in mind is the susceptibility of young wines to colonization by these yeasts. Even not knowing the causes of such susceptibility, it is likely to be related with their ability to grow under a very low sugar content (less than 2 g/l reducing sugar) and so ‘dry’ wines are not safe (Coulter *et al*., 2003). Then, residual sugars after fermentation must be as low as possible to minimize yeast growth and volatile phenol production (Coulter *et al*., 2003). In addition, supplementation of slow fermentations with excessive amounts of diammonium phosphate may also increase the probability of wine spoilage (Coulter *et al*., 2003).

Preservatives

The above-mentioned measures are not enough to avoid the risk of contamination and so the proper use of preservatives is essential. Despite the
controversy regarding preservative resistance (see Section 13.4), presently the only effective antimicrobials are sulfite and DMDC. Their judicious utilization, since grape crushing, together with all the above-mentioned measures, is the key to prevent the building up of \textit{D. bruxellensis} contamination. The type of application is different for both substances. Sulfite is in common use in wineries and it is preferable to add an effective killing dose at one time than to add small successive amounts. As mentioned before, additions of 40 mg/l at pH 3.5 seem to be enough to control \textit{D. bruxellensis}, not forgetting that effectiveness depends on the type of wine and that it is essential to guarantee adequate homogenization. The preservative DMDC, where legally authorized, is normally used in regular additions up to the maximum level of 200 mg/l. The efficiency of the treatment is higher when used together with sulfite and when the addition is done using a dosing apparatus that ensures the even distribution of the preservative in the total wine volume. As with sulfite, the different wine susceptibilities determine that the efficiency of the operation must be monitored by \textit{D. bruxellensis} detection after preservative addition.

\textit{Curative measures}

When the wine is off-tainted, there are not effective curative measures to recover it. In this situation, oenologists always weigh the possibility of blending tainted wine with ‘clean’ wine. Although this measure may attenuate the defect of the tainted wine by dilution it can not be seen as a curative measure. In fact, mixtures of wines with null or low levels of 4-ethylphenol are effective only for small proportions of tainted wines because large volumes of ‘clean’ wine must be used to obtain a blend with 4-ethylphenol levels lower than the preference threshold. Then, effective curative measures depend either on the (i) reduction or elimination of the sensorial effect or on the (ii) extraction of odor active molecules from the wine. These strategies have not yet been effectively tested in practical conditions. Guilloux-Benatier \textit{et al.} (2001) hypothesized that yeast lees have the property to adsorb volatile phenols, which was later shown, at least partially, in laboratory conditions, by Chassagne \textit{et al.} (2005). Commonly, when adsorbents are added to wine, favorable aroma compounds are also removed and a balance must be drawn between benefits and losses of wine attributes.

13.7 \textit{Future trends}

The knowledge on \textit{Dekkera/Brettanomyces} spp. has many gaps, as described before. The issues related to their dissemination, survival and spoilage activities are far from being fully understood, demanding much more research effort. We consider of major importance the ecological study of determining the primary sources of contamination and dissemination vehicles. It is also necessary to clarify the reason why white wines do not have significant amounts of 4-ethylphenol, given that it may contribute to the solution of the
Table 13.6  Incidence of *D. bruxellensis* and volatile phenols (4-ethylphenol, 4-EP; 4-ethylguaiacol, 4-EG) in wine samples analyzed by support service laboratories

<table>
<thead>
<tr>
<th>Country (year)</th>
<th>Number of samples for <em>D. bruxellensis</em> detection</th>
<th>Percentage of samples with <em>D. bruxellensis</em> volatile phenol determination</th>
<th>Number of samples for detection determination of 4-EP + 4-EG</th>
<th>Percentage of samples with more than 425 µg/l of 4-EP + 4-EG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia (pre-2001)</td>
<td>–</td>
<td>–</td>
<td>177</td>
<td>74%</td>
</tr>
<tr>
<td>Australia (2001)</td>
<td>–</td>
<td>–</td>
<td>51</td>
<td>45%</td>
</tr>
<tr>
<td>Canada (2004)</td>
<td>7</td>
<td>29%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Portugal 1 (2003–2004)</td>
<td>166&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38%</td>
<td>216</td>
<td>54%</td>
</tr>
<tr>
<td>Portugal 2 (2003)</td>
<td>62</td>
<td>24%</td>
<td>62</td>
<td>12%</td>
</tr>
<tr>
<td>Portugal 2 (2004)</td>
<td>174</td>
<td>20%</td>
<td>80</td>
<td>6%</td>
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<tr>
<td>Spain (2003)</td>
<td>88</td>
<td>26%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spain (2004)</td>
<td>74</td>
<td>35%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>USA, California (2004)</td>
<td>1858&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28%</td>
<td>464</td>
<td>14%</td>
</tr>
</tbody>
</table>

<sup>a</sup> For 91 samples submitted to quantification, the following frequencies of contamination range were obtained: 73% (0–10 cfu/ml), 8% (10–10<sup>2</sup> cfu/ml; 9% (10<sup>2</sup>–10<sup>3</sup> cfu/ml, 9% (10<sup>3</sup>–10<sup>4</sup> cfu/ml), 2% (10<sup>4</sup>–10<sup>5</sup> cfu/ml).

<sup>b</sup> About 10–12 samples of white and sparkling wines contained *D. bruxellensis*. 
problem in red wines. The controversy on many issues related to *D. bruxellensis* behavior demands additional efforts on the design of experiments in order to obtain significant results.

We do not know if the volatile phenol problem is increasing worldwide because we are not aware of incidence studies using random wine samples. Table 13.6 gathers information obtained from support service laboratories, which are not comparable because sampling is not standardized. The overall incidences are probably overestimated because winemakers usually send samples to laboratories when problems are suspected. Results from Australia seem to indicate a decreasing incidence with time resulting from the increasing awareness of winemakers for the problem but this hypothesis has to be confirmed by much more extensive sampling programs (Coulter et al., 2003). The tendency to use oak barrels is still continuing and so it is likely that more wines will become affected in spite of the present knowledge and information already available.

The problems associated with *Dekkera* spp. contamination should also be present in other industries, although they have not been so studied as in wines. In particular, the technologists and scientists involved in beer and cider industries should reflect on the need to increase the research in their fields, given that the volatile phenol precursors are common constituents of plant raw materials and *Dekkera* spp. are likely to occur in related environments.

### 13.8 Sources of further information and advice

The sources for additional information on *Dekkera/Brettanomyces* spp. are scarce. Readers are advised to be particularly cautious with information arising from Internet sources and general wine press that is not scientifically supported (Henschke, personal communication). There are several research laboratories all over the world working on the subject and most reliable information is regularly being published in scientific peer-reviewed journals.

### 13.9 Acknowledgements

The authors wish to thank all colleagues providing personal communications on their research issues and to the laboratories providing the incidence data presented in Table 13.6.

### 13.10 References


390 Food spoilage microorganisms


Dekkera/Brettanomyces spp.


Dekkera/Brettanomyces spp.


Part IV

Spoilage moulds
14

General characteristics of moulds
M. O. Moss, University of Surrey, UK

14.1 Introduction to moulds: representatives of two kingdoms

The term mould is applied to filamentous fungi that, although often visible to the unaided eye as a fluffy growth, sometimes associated with spores, do not produce macroscopic fruit bodies. Mycologists have traditionally studied organisms now known to belong to several Kingdoms of the living world, including the oomycetes, which belong to the Kingdom Chromista (= Stramenopila), as well as the true fungi forming their own kingdom (Alexopolous et al., 1996; Carlile et al., 2001; Dick, 2001), which have in common that they are heterotrophic eukaryotes which generally have a cell wall.

Although one sometimes hears reference to ‘yeasts and fungi’, yeasts are fungi that happen to (usually) be single celled. Because of their distinct morphology and metabolism yeasts were once considered to be a distinct taxonomic group, but it is now recognised that the yeast form has evolved in all the major groups of fungi. There are ascomycete, basidiomycete and zygomycete yeasts, as well as species for which no sexual stage is known, but those most frequently encountered in food spoilage are ascomycetes. A small, but important group of pink yeasts, such as Rhodotorula, are the asexual stages of basidiomycetes.

The walls of most yeasts are predominantly made of mannans, as well as $\beta$-1,3 and $\beta$-1,6 glucans and this would seem to clearly distinguish them from the filamentous fungi, the walls of which are predominantly made of chitin. However, careful analysis of the wall material of yeasts shows the presence of chitin, which is localised at the sites of scars left after budding,
and moulds do contain the same mannans and glucans as minor constituents. As an example of how widespread the yeast habit is across the taxonomic groups of the kingdom Fungi one could consider the well-known yeast *Schizosaccharomyces*. This is an ascomycete that divides by fragmentation rather than budding and is very distantly related to *Saccharomyces*. It is now placed in the Archiascomycetes with *Taphrina*, *Protomyces* and *Pneumocystis* (Alexopolous *et al.*, 1996).

The oomycetes, often known as water moulds, usually reproduce asexually with the production of biflagellate zoospores, in line with their mainly aquatic habitat, which are clearly adapted for dispersal. Sexual reproduction in this group results in the formation of oospores, which are thick-walled structures well adapted for long-term survival rather than dispersal (Fig. 14.1). The walls of these organisms contain cellulose and, apart from the brief period of sexual reproduction, they are essentially diploid. They are not generally associated with food spoilage although the downy mildews, which are plant pathogens, may be responsible for significant losses in agriculture and at least one species, *Saprolegnia parasitica*, is a pathogen of fish, especially when grown in intensive culture such as fish farms.

By contrast, most members of the Kingdom Fungi are haploid, except for a brief period associated with sexual reproduction, although some yeasts such as *Saccharomyces* may have a stable diploid cycle, their walls contain chitin, and, apart from the Chytridiomycota, they have no motile stages during their life cycles. There are four phyla, the Chytridiomycota, Basidiomycota, Ascomycota and Zygomycota, although a fifth, the Glomeromycota, has been established for the group of very specialised vesicular arbuscular mycorhizal fungi previously considered to be zygomycetes (Schüßler *et al.*, 2001). The major phyla of fungi are recognised on the basis of their sexual, perfect or teleomorph stages (Fig. 14.2) and many of these are associated with one or more asexual, imperfect or anamorph stages. However, a significant

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**Fig. 14.1** Growth and reproduction of *Saprolegnia*, an oomycete: (a) zoosporangium; (b) release of zoospores; (c) oogonium.
number of moulds, including many of importance in food spoilage, have no known sexual or perfect stage. They are variously referred to as deuteromycetes, imperfect fungi or mitosporic fungi, and the majority can be shown to have close relationships with ascomycetes and basidiomycetes.

From the perspective of food spoilage, the zygomycetes and ascomycetes with their associated anamorphs are by far the most important fungi, although, among the basidiomycetes, there are species that produce fruit bodies, mistakenly collected as food, which are poisonous (Cooper et al., 2003).

### 14.2 The zygomycetes

Like the oomycetes, sexual reproduction in the zygomycetes results in the production of thick-walled structures, the zygospores, which have considerable survival potential but are not generally adapted for dispersal (Fig. 14.2). Also like the oomycetes the mycelium of the zygomycetes is non-septate, except where septa may separate structures such as chlamydospores, sporangia and zygospores. Indeed, at one time the oomycetes and zygomycetes were considered together as the phycomycetes but this name is no longer acceptable as a taxonomic division.

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**Fig. 14.2** Sexual reproduction in (a) zygomycetes, (b) ascomycetes, (c) basidiomycetes.
Unlike the oomycetes, the zygomycetes are predominantly terrestrial organisms, although some are well adapted for an aquatic existence. One consequence of a terrestrial ecology for a non-motile organism is that dispersal is often by air, although rainsplash, mist and insect dispersal are also important. Two widespread genera of zygomycetes associated with food spoilage are *Mucor* and *Rhizopus*, often referred to as pin-moulds, which produce large numbers of asexual spores in structures known as sporangia on the tips of sporangiophores, which are specialised hyphae growing away from the substrate (Fig. 14.3). As the spores are produced within a closed structure there dispersal clearly requires the breakdown of the sporangial wall and release of the spores. That this process occurs effectively in *Mucor* and *Rhizopus* is demonstrated by the frequency with which these organisms occur as airborne contaminants in the laboratory and on foods. One genus, *Pilobolus*, has evolved to actively fire the whole sporangium a considerable distance away from the animal dung on which it grows.

Despite the success of the sporangium, the zygomycetes includes families of moulds that show a progressive evolution to the production of external propagules, i.e. conidia, for dispersal. Thus *Cunninghamella* produces structures that are functionally conidia over the surface of a swollen sporangiophore tip but each one is structurally a single-spored sporangium and hence has a double wall.

### 14.3 The ascomycetes

The ascomycetes and basidiomycetes have septate hyphae although the septa

![Fig. 14.3 Asexual reproduction of the zygomycetes: (a) *Mucor* and (b) *Rhizopus*.](image-url)
have a pore through which many organelles and cytoplasm can pass so that they are essentially coenocytic rather than cellular.

Sexual reproduction involves the fusion of two compatible haploid nuclei to form a diploid nucleus within a specialised cell destined to become the ascus (or the basidium in the basidiomycetes). Within the ascus the diploid nucleus undergoes meiosis, usually followed by mitosis, to form typically eight haploid nuclei, around each of which a new cell wall is formed, resulting in the generation of eight ascospores in each ascus (Fig. 14.2). Eight is the most common number of ascospores in an ascus but there are examples of 2, 4, 16 or more, and even 1 or 3! Although some ascomycetes do produce individual naked asci, the phylum has evolved an increasingly complex range of structures for packaging numerous asci together. These may be closed (the cleistothecium), flask-shaped with an open neck (the perithecium) or packed together over an extensive cup-shaped structure (the apothecium). Further complexity arises from the packaging of perithecia within the surface of a complex macroscopic structure (e.g. Daldinia and Xylaria frequently seen growing on timber in our woodlands) or from an apothecium being carried on a stalk, becoming extensive and convoluted leading to a macrostructure such as we see in the morel (Morchella).

Ascospores may be important as survival structures, and where this is the dominant role they are packaged in spherical asci, themselves packaged in cleistothecia, structures that are not well adapted for dispersal. Such fungi do not produce macroscopic fruit bodies and their ascospores are frequently relatively heat resistant. They include genera such as Eurotium, capable of active growth at low water activities. Clearly such fungi require a dispersal stage and this is associated with their anamorphs, which in the case of Eurotium is Aspergillus, and there are several important strategies for the dispersal of such spores (see Section 14.4).

Those ascomycetes producing perithecia and apothecia have evolved very effective means for dispersal, the ascus being a device for firing the spores, either all together as a single projectile or one at a time, over a relatively long distance. Once fired away from the ascus the spores may then spread by air dispersal. I recall bending down to photograph a large fruit body of the orange peel fungus, Aleuria aurantia, when I heard a hissing sound and saw a cloud of white smoke rising from the surface of the apothecia as thousands of asci discharged their spores synchronously.

The ascospores are haploid, and usually uninucleate, germinating to give a mycelium of one of the sexually compatible partners. A new round of sexual reproduction requires the fusion of two compatible mycelia and it is easy to see why an asexual process for dispersal may be important when widespread air dispersal of ascospores occurs and initially the compatible mycelia may be separated from each other. Indeed, it is possible to conceive that one haploid strain may become isolated from its compatible partner for so long that it loses the ability to undergo sexual reproduction and so becomes obligately dependent on asexual reproduction and thus a permanent member
of the mitosporic fungi. Sexual reproduction is the obvious mechanism by
which genetic recombination can occur but in the mitosporic fungi some
species have evolved an alternative mechanism, the parasexual cycle, the
result of which is a recombination of characters (Caten, 1981).

It is worth noting that many ascomycetes, and a few basidiomycetes, have
become obligately dependent on an algal and/or cyanobacterial partner to
form the group of organisms known as the lichens. Although some of these,
such as *Umbilicaria esculenta*, may be used as foods they play no role in
food spoilage (Purvis, 2000).

### 14.4 The mitosporic fungi

As discussed above many ascomycetes (and quite a few basidiomycetes)
have one or more anamorphic (imperfect) states. In the past these were
isolated, studied and named as individual species and, even when the connection
to a perfect stage is made, the anamorphic states continue to retain their own
names. There are many mitosporic fungi, including some of considerable
importance as food spoilage organisms, for which teleomorphic (perfect)
stages are unknown and it is clearly important that these be described using
internationally acceptable names. Some, known loosely as coelomycetes,
have their spore-producing structures protected within a larger structure such
as a pycnidium (superficially resembling a perithecium) or acervulus. Many
of these are associated with plants and have been extensively described by
Sutton (1980).

The developmental process of converting actively growing vegetative
mycelium into spores, suitable for dispersal, has evolved in several different
ways, leading to a diversity of spore types for which the usefully descriptive
terms arthrospores, blastospores, porospores, anelosporas, aereirosspores and
phialospores may be used (Fig. 14.4). Many genera producing spores with
dark pigmented walls, referred to as dematiaceous hyphomycetes, have been
described by Ellis (1971, 1976) and many individual genera have their own
monographs such as those for *Penicillium* (Pitt, 1979), *Aspergillus* (Thom &
Raper, 1945) and *Fusarium* (Booth, 1971; Gerlach & Nirenberg, 1982). There
are also excellent texts dealing more specifically with foodborne and
food spoilage fungi (Samson *et al.*, 1995; Pitt & Hocking, 1997).

There are two major mechanisms for air dispersal in this group. Those
such as *Aspergillus* and *Penicillium* produce very large numbers of small
(*ca.* 2–5 \( \mu \)m), unwettable, thick-walled, pigmented spores which are released
into the air by mechanical disturbance and are well adapted for widespread
dispersal and long-term survival in the air. Intimate exposure to oxygen,
sunlight and desiccation makes the air a hostile environment for a small
propagule with a large surface area to volume ratio. A penalty for such a
strategy of widespread air dispersal is the problem of not settling on, or
being impacted onto, an appropriate substrate for germination and growth. If
a propagule is too large it will settle out too quickly; if too small the chance of impaction on a surface is very low. It is interesting to note that many moulds have evolved spore sizes (ca. 15–20 \( \mu \text{m} \)) that are a compromise between the requirement for dispersal and impaction (Gregory, 1973).

Alternatively, moulds such as *Fusarium* produce thin-walled colourless wettable spores which are well adapted for rain splash and mist dispersal. Impaction on a new substrate will be a function of the size of the water droplet rather than the propagule it is carrying. Such spores do not survive for long in the air but are adapted for dispersal from an infected host to a healthy plant nearby.

The mitosporic fungi thus include a wonderfully diverse range of strategies for dispersal which has undoubtedly contributed to their biological success.

### 14.5 The mycelial habit

Although the oomycetes are predominantly aquatic, some have evolved to an increasingly terrestrial environment, often associated with plants as important crop pathogens. A good example is *Phytophthora infestans*, the agent of potato blight and late blight of tomatoes, which produces sporangia borne on the tips of aerial sporangiophores and these may act as either functional conidia in the absence of a film of water, or the source of actively swimming zoospores in a film of water.
The zygomycete *Mucor rouxii* looks like any other member of the genus when grown aerobically on the surface of a medium such as malt extract agar. However, when inoculated into a liquid medium, rich in fermentable sugars, and incubated without shaking, it grows as a budding yeast in response to anaerobic conditions and high concentrations of carbon dioxide. If these yeast cells are inoculated back onto solid media they develop as a filamentous mould. It is interesting to note that, whereas the majority of moulds are clearly aerobic and have a metabolism dependent on oxygen, many but not all yeasts have the ability to ferment carbohydrates as a source of energy as well as a competent oxidative pathway. This makes it possible for them to grow in anaerobic conditions in liquid environments. From the point of view of the efficiency of biomass yield, fermentation is a wasteful process but the products, such as ethanol, are a valuable part of a large segment of the food industry!

In the ascomycetes and basidiomycetes there is an increased specialisation to either a yeast or mycelial growth form. Despite their simple morphology yeasts are not primitive fungi but have often adapted to specialised, usually liquid, environments rich in nutrients such as plant exudates and the body fluids of insects and other animals. They may also have complex nutritional requirements and do not seem to produce and secrete secondary metabolites such as mycotoxins.

The mycelial form, on the other hand, is well adapted for growing over and through solid substrates, and the generation of airborne spores, as well as being associated with a diverse range of secondary metabolism including the production of mycotoxins, an important aspect of the microbiological spoilage of foods (Sinha & Bhatnagar, 1998; DeVries et al., 2002). The fungal hypha grows actively at its tip although it is also capable of extensive branching. When two genetically compatible hyphal tips meet, the actively extending wall is still sufficiently plastic that they may fuse to form a single mycelium, a process known as anastomosis. This adaptation to growth on and through a solid substrate requires the ability to secrete enzymes for degrading complex macromolecules, such as polysaccharides and proteins, to smaller molecules that can be absorbed into the cytoplasm and metabolised to support growth. Indeed, among filamentous fungi we find both the capability to degrade recalcitrant macromolecules, such as lignocellulose, as well as the ability to actively grow at low water activities.

Filamentous fungi often grow on substrates and in environments where there is considerable competition and the secretion of secondary metabolites with antibiotic and toxic properties must be a factor in their success. The biosynthesis of a metabolite, such as aflatoxin, involves a large number of genes, some of which are control genes, and moulds often have mechanisms conferring resistance to their own antimicrobial metabolites. This implies that secondary metabolite biosynthesis and secretion is not a trivial phenomenon in the biology of moulds.
Among the prokaryotes we see in the Streptomycetes a group of bacteria that have evolved similar strategies to the moulds with the formation of a branching filamentous structure, ability to grow on solid substrates, the production of airborne spores on aerial structures, and the production of antibiotics.

### 14.6 Spoilage organisms

Members of the fungal kingdom are responsible for the degradation and recycling of large quantities of organic material and are important agents in the major nutrient cycles of the biosphere. These activities are generally referred to as biodegradation but, when the organic materials are important for the well-being of humans, such as foods, animal feeds and the raw materials used in their production, then the process is considered to be spoilage.

The four most important factors influencing the growth of moulds on foods are the available nutrients, pH, temperature and water activity ($a_w$). It is important to recognise the interactions between the physicochemical factors influencing growth (Adams & Moss, 2000). Thus tables such as Table 14.1, giving the minimum water activity at which growth can occur, imply that all other factors are optimal. The optimal temperature for the growth of *Aspergillus flavus* (an important producer of aflatoxins) is about 30°C, at which it can grow at $a_w$ as low as 0.81, and that for *Penicillium expansum* (an important producer of patulin) is about 20°C, at which it can grow at $a_w$ as low as 0.85.

It is very important to note that the nature of the humectant or solute influencing the $a_w$ of a medium can itself influence growth. In general, ionic solutes such as salts are more inhibitory than carbohydrates and polyols at the same $a_w$.

Some yeasts, such as *Zygosaccharomyces bailii* can grow over a range of water activities, perhaps as low as 0.8, and may be described as xerotolerant, whereas *Z. rouxii* is truly xerophilic and able to grow at $a_w$ 0.62–0.65. The interaction between $a_w$ and the optimum temperature for growth can be clearly illustrated with this species. In 10% (w/w) glucose ($a_w$ 0.99) the

<table>
<thead>
<tr>
<th>Table 14.1 Minimum $a_w$ for the growth of a selected range of moulds</th>
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<tbody>
<tr>
<td><strong>Mould species</strong></td>
</tr>
<tr>
<td><em>Rhizopus nigricans</em></td>
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<tr>
<td><em>Penicillium expansum</em></td>
</tr>
<tr>
<td><em>Penicillium aurantiogriseum</em></td>
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<tr>
<td><em>Aspergillus flavus</em></td>
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<tr>
<td><em>Aspergillus ochraceus</em></td>
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<tr>
<td><em>Eurotium chevalieri</em></td>
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<tr>
<td><em>Wallemia sebi</em></td>
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<td><em>Xeromyces bisporus</em></td>
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optimum temperature is 24 °C whereas in 60% (w/w) glucose ($a_w$ 0.87) it is 33 °C (Jermini & Schmidt-Lorenz, 1987). Not many detailed studies involving the interaction of three environmental factors on the growth of moulds are available but Magan & Lacey (1984) presented such data for a wide range of field and storage fungi and Table 14.2 shows data for two species extracted from this paper.

In general, members of the genus *Aspergillus* are isolated more often from tropical and subtropical regions and *Penicillium* is more frequently isolated from temperate parts of the world, reflecting their respective optimum temperature ranges.

The limiting value of water activity for the growth of any microorganism is about 0.6 (Xeromyces bisporus) and at lower water activities food spoilage is not microbiological but may be due to chemical reactions, such as oxidations, or insect damage. If the water activity of the environment is 0.6, corresponding to a water potential of −68 MPa, the cytoplasm will need to be at an even lower water activity to maintain the positive turgor pressure required for active growth. To achieve this requires that the cytoplasm has very high concentrations of appropriate compatible solutes (glycerol, erythritol and mannitol in many moulds) and it is probable that macromolecules such as DNA would change their configuration and no longer function properly. Of course, although growth is not possible at such low water activities there is usually no problem for the survival of mould propagules as is apparent in the use of freeze drying for the maintenance of many moulds in culture collections.

Because most fungi can grow over a wide range of pH (3–8) it is likely that pH itself will have little effect on the growth of fungi on foods and water activity is going to be a much more important factor at neutral to alkaline pH (Wheeler et al., 1991). Although they have a high water activity, fresh fruits and vegetables are relatively resistant to microbial spoilage because of the natural defences of living plant tissue. However, harvesting inevitably compromises the integrity of plant tissue and microorganisms can gain access. The low pH of many fruits gives moulds a competitive advantage over bacteria but one frequently sees considerable specificity in the species of

<table>
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<tr>
<th>Table 14.2</th>
<th>Influence of pH and temperature on the minimum $a_w$ for germination of <em>Penicillium brevicompactum</em> and <em>Aspergillus amstelodami</em> (Magan &amp; Lacey, 1984)</th>
</tr>
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<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td><strong>pH 6.5</strong></td>
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<td></td>
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<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><strong>P. brevicompactum</strong></td>
<td>0.9</td>
</tr>
<tr>
<td><strong>A. amstelodami</strong></td>
<td>NG</td>
</tr>
<tr>
<td><strong>NG</strong></td>
<td>= no germination.</td>
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mould able to spoil different fruits. Thus a blue mould on apples causing an extensive soft rot is almost inevitably *Penicillium expansum* and the green and blue moulds of citrus fruits are usually *Penicillium digitatum* and *P. italicum* respectively. Fruits and vegetables, being living plant tissue, have complex mechanisms for inhibiting invasion by moulds, and other microorganisms, and the specificity seen in those species able to invade specific plant tissues must reflect the ability to avoid one or more of these inhibitory factors. Many yeasts are also able to grow at very low pH, although the nature of the acidulant may influence the apparent minimum pH. Thus *Saccharomyces cerevisiae* can grow at a pH as low as 1.6 using HCl to adjust the pH, 1.7 using phosphoric acid and 1.8–2.0 using a range of organic acids, and *Brettanomyces bruxellensis* can grow at pH 1.8 in a medium acidified with HCl but only 2.3 if citric acid is used. Of course it will take much higher concentrations of organic acids to achieve these low pH values.

Low temperatures would normally inhibit the growth of many moulds, but there are a significant number that grow well at the temperatures of the domestic refrigerator. Growth of blue moulds on refrigerated cheese will often be *Penicillium commune*, the damp walls of cold rooms can become black with growths of *Cladosporium herbarum*, and the pink slimes on refrigerated foods may be due to the yeast *Rhodotorula glutinis*.

*Geotrichum candidum* is an interesting mould with closer affinities to some of the true yeasts than to other moulds. It has a rare teleomorph, *Galactomyces geotrichum*, which produces naked asci each with a single ascospore. Although it is associated with the spoilage of a number of foods it is also a very good indicator of declining hygiene in a food factory or preparation area as it frequently contaminates processing lines and methods have been published for estimating such contamination (Cichowicz & Eisenberg, 1974). It can sometimes happen that a particular product of food manufacture acts as a selective agent for a potential spoilage organism. Thus, rich fruit cake has such a low water activity that it might seem to be immune from microbiological spoilage but the mould *Wallemia sebi* can grow over a wide range of $a_w$ from 0.997 to 0.69 and it produces small brown colonies which may be difficult to spot on a commodity such as fruit cake until it reaches the consumer. It produces large numbers of small (3–4 µm) dry spores well adapted for air dispersal within a factory and it is possible for numbers to build up to levels that may require closure of the factory if due diligence is not observed. It requires the routine use of low $a_w$ media to monitor its presence.

Although the media and methods available for the routine isolation and identification of spoilage fungi are not as widely used as those for bacteria, a quantitative and qualitative knowledge of mould contaminants in a food production environment can be useful in resolving a potential mould problem (Samson *et al.*, 1992). It may be possible to judge, for example, whether an increased level of mould contamination within a factory arises from moulded raw materials stored within access of air vents leading into the
A number of antifungal agents such as sorbate and propionate can be incorporated into foods to prevent mould growth where \( a_w \) and temperature alone are not appropriate. Some moulds, such as *Penicillium roqueforti*, can degrade sorbate and are resistant to all but unacceptably high levels. The inappropriate application of fungicide can also lead to unexpected side effects. Thus, during the use of propionate and acetate for the protection of high moisture barley, stored as a nutritious winter feed, insufficiently high levels may allow the growth of moulds, such as *Aspergillus flavus*, in a temperate climate where it would not normally be competitive. Although not growing very well, under these circumstances, this species produces elevated levels of aflatoxin.

With the exception of the ascospores of species of *Byssochlamys* and *Talaromyces* mould propagules are generally sensitive to heat treatment, although it is worth noting that they become more resistant at low water activities. Corry (1987) provided an extensive table illustrating the influence of water activity on decimal reduction time (\( D \)) of fungi including a list of literature sources. Pitt & Hocking (1997) also provide values of both \( D \) and \( z \) values (the \( z \) value represents the effect of temperature on the \( D \) value) for some species of moulds and yeasts.

For example, using sucrose as the solute, the \( D_{75^\circ C} \) of ascospores of *Byssochlamys nivea* can vary between 60 min at \( a_w \) 0.98 and 470 min at \( a_w \) 0.84, and the \( D_{51^\circ C} \) of vegetative cells of the yeast *Saccharomyces cerevisiae* varies from 21 min at \( a_w \) 0.99 to 53 min at \( a_w \) 0.89. Vegetative cells of *S. cerevisiae* are up to 100 times more sensitive than ascospores. Thus the \( D_{50^\circ C} \) of vegetative cells may be 0.1–0.3 min compared with a \( D_{60^\circ C} \) of 5.1–17.5 min for ascospores. The \( z \) values for fungi are generally about 4 \(^\circ C\) but a value of 7.2 \(^\circ C\) has been given for ascospores of *S. cerevisiae* in 0.05 M phosphate citrate buffer compared with 3.8 \(^\circ C\) in apple juice.

In general the prevention of mould spoilage requires good hygiene during both the production and storage of foods, the separation of areas where raw materials are handled from those where final products occur, and early warning of any build-up of mould spores in production lines and storage facilities.

The appearance of mould on a food usually makes it look unappetising and often confers a musty odour and flavour, which results in mouldy foods being discarded, leading to significant economic losses. Although there are some exceptions, moulds generally require oxygen for growth and may require a gaseous phase for active sporulation. Products packaged in an oxygen-permeable material in the absence of an air space will often produce extensive white mycelium but no spores. It is frequently the spores of genera such as *Aspergillus* and *Penicillium* that are pigmented and confer the coloured powdery appearance on the surface of a mouldy food. Thus, although *Penicillium expansum* can grow extensively in the tissue of an intact apple, producing a characteristic yellow soft rot, it usually sporulates only on the surface of the
fruit, producing the characteristic blue mould. However, some cultivars of apple, such as Charles Ross, mature to produce a more open texture and the author has found such an apple with a blue soft rot, the blue colour being due to sporulation within the flesh.

The visible size of a mould colony may not reflect the quantity of mould biomass. Thus a single viable propagule in a full bottle of a liquid such as a beverage where CO₂ levels may be high and O₂ levels limited, may produce quite a large colony of sparse mycelium with little branching which, on filtering, washing and drying, will be found to weigh very little. In contrast, a number of propagules germinating on the surface of a partially filled bottle may produce small compact colonies with a larger biomass.

14.7 Sources of further information and advice


Mycological media for the study of foodborne and food spoilage fungi: see Beuchat (1987); Pitt & Hocking (1997); Samson et al. (1992, 1995).

Cultures and identification:
- CABI Bioscience, Bakeham Lane, Egham, Surrey, TW20 9TY, UK.
- Centraalbureau voor Schimmelcultures, PO Box 85167, 3508 AD Utrecht, The Netherlands

14.8 References


15

Zygomycetes

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15.1 Introduction

The Zygomycetes, popularly known as the ‘pin molds’, are fungi belonging to the Eumycota, the true fungi that form extended mycelia and diverse asexual and sexual spore structures. The Zygomycetes are fungi that thrive in soil and dead plant material. They also have an exquisite taste for dung. They are often seen as rapid growers that quickly devour simple carbon sources and therefore in ecological perspective are seen as typical r-strategists. These organisms follow a sort of ‘hit and run’ strategy, but never form long-term relationships with their substrata as a result of their incapability to degrade complex polymers. This does not imply that their metabolism is always very simple. The fungus *Rhizopus oryzae* can exhibit versatility in the transformation of a wide range of xenobiotics as steroids, terpenes and aromatic compounds (Martin *et al*., 2004). The fungus *Cunninghamamella elegans* biotransforms the triphenylmethane dye Malachite green (Chang *et al*., 2001) and the polycyclic carcinogen and pollutant fluorathene (Pothuluri *et al*., 1990). The fungus *Mucor rouxii* was used for the stereoselective reduction of β-keto-esters (Mangone *et al*., 2002). Many Zygomycetes are associated with living organisms as parasites or mycorrhiza-forming fungi. As parasites they infect numerous species of insects and to a lesser extent eelworms or other microscopic animals. Insects infected with *Entomophthora* climb to extended parts of plants where they die and when the fungus breaks open the cuticle, spores are released from the mycelium and freely disperse into the open air. *Entomophthora grylli* causes synchronized death of infected grasshoppers so that spore dispersal is at the most humid time of the day.
Another important group of the Zygomycetes forms an intimate association with the roots of plants in vesicular–arbuscular mycorrhyza where both the plant and the fungus benefit. The order Glomales harbours fungi that are among the most important mycorrhyza-forming fungi and as such promote the growth of many plant species. Generally, the Zygomycetes are seen as more primitive fungi compared with the dominant other Eumycota, namely the Ascomycetes and the Basidiomycetes. They lack septa as an important organization structure in hyphae, although these can be formed, for instance as a delineation between mycelium and spore-forming structures. At our laboratory during numerous years of sampling of soils, food and plant material, we observed during numerous occasions overgrowth of malt extract agar surfaces with a Zygomycete that masked the other fungal species. One can speculate that the absence of septa has a bearing on cytoplasmic movement or intrahyphal transport, which can be faster, and henceforth that there is a link between the fast growth generally observed among the Zygomycete hyphae. *Rhizopus stolonifer* has an extension zone (the place where the hyphae extends) of 29µm and a peripheral growth zone (where the fungal colony extends) of 8700 µm, the ratio is 300 (see Trinci, 1971). For *Actinimucor repens* these are 14, 2500 and 180 respectively. Such ratios are lower in Ascomycetes. There is readily cytoplasmic streaming in *Rhizopus* and cells can survive some damaging even without the dominant presence of septa. In Zygomycetes, anastomoses are sparse; these are interhyphal connections, numerous among the Asco- and Basidiomycetes. This may be associated with the organization of fungal tissue formation as occurs in extensive fruit bodies, which are absent among the Zygomycetes.

Further, the composition of the Zygomycete cell wall is different from that of the major Eumycota as the function of the β-glucans as important structural components is here exerted by chitosan. Together with chitin, chitosan forms the backbone of the cell wall and these compounds are embedded in polyglucuronic acid and mannoproteins.

### 15.2 The morphology of Zygomycetes

In the litterbin for your kitchen waste, Zygomycetes appear with abundant mycelium in which black dots appear. These black dots are spore-forming structures of the Mucorales, the group of Zygomycetes with the largest number of species and by far the most relevant for food–fungus interactions. The black dots are the so-called sporangia (Fig 15.1). Initially, these are white, but they darken during further spore formation and maturation. Sporangiospores are formed inside sporangia by a cleaving process of a multi-nuclei-containing cytoplasm. This is an intricating process that makes proper distribution of nuclei to spores vital. With *Rhizopus oligosporus*, this domesticated species seems to have developed a defect in the production of rounded spores as if the cleavage process is not completed. Nearly 20% of the formed
sporangiospores are very big and have irregular shapes. In non-domesticated strains of the *R. microsporus* group to which *R. oligosporus* belongs, this number is much lower. It is tempting to think that the transport of fungi for many centuries by the human hand has such a bearing on the formation of the natural transport vehicle (unpublished results, J. Jennessen *et al.*, SLU, Uppsala, Sweden). The sporangium is borne on a stalk and together these form the sporangiophore, which can reach macroscopic dimensions. In the case of *Phycomyces*, sporangiophores are formed that are easily 10 cm long. These structures react to gravity (negative response, they grow upwards), but this response is overruled by light (a positive response). During the stage of elongation of the stalk, extension rates are observed that are among the fastest in the fungal kingdom. Most sporangiophores of the Zygomycetes range between 500 µm and 2 mm in length.

The variety of the sporangiophore structure is bewildering among the Zygomycetes, but generally the following structures can be recognized:

- **Columella**, a vesicle or central part inside the sporangium and continuous with the sporangiophore. Sometimes the columella has one or more projections (see Fig. 15.2a,b).
- **Sporangiole**, a small and usually globose sporangium with one or a few spores.
- **Apophysis**, a swelling of the sporangiophore just below the sporangium.
- **Merosporangium**, a cylindrical sporangium which breaks into a row of merospores (see Figs 15.2 and 15.3, *Mycotypha* sp.).
- **Rhizoids**, root-like structures that adhere so-called stolons to a substratum (such as agar surfaces, see Fig. 15.2d).

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**Fig 15.1** Sporangia of different *Rhizopus* species: (a) Sporangia, stolon and rhizoid of *R. stolonifer*; (b) *R. microsporus* group; (c) *R. oryzae* (after Schippers, 1984).
Fig 15.2 Light microscopy of asexual spore forming structures among the genus *Mucorales*: (a) sporangiophore of *Mucor plumbeus*; (b) when the sporangiospores are removed, the typical columella with extensions is visible; (c) sporangiophore of *Absidia corymbifera*; (d) sporangiophores and stolons of *Rhizopus stolonifer*; (e) sporangiospores of *R. stolonifer* with striations (ornamentation); (f) sporangiophore of *Syncephalastrum racemosum* with merosporangia that contain merospores; (g) merospores of *S. racemosum*. 
The zygospore is the hallmark and the name-giving structure of the Zygomycetes (see Fig. 15.3, Rhizopus). It is the only sexual spore that is made within the group. It is usually formed among different mycelia of opposite mating type (i.e. heterozygous, although homozygous species are known) and during the process of communication chemical hormones such as trisporic acid play a role. Initially, two specialized branches grow towards each other and fuse. The fused cell is called the zygote and is delineated by two septa. The zygote develops into a large zygospore; often two suspensor cells hold the zygote, and these can be unequal in size. Both suspensor cells as zygospores can be ornamented, which can be important for recognition of the fungal species. Zygospores possess thick cell walls and perform dormancy, where long periods of time are necessary before germination occurs. Zygospores of Mucor hiemalis show no germination within 30 days and only 1% after 90 days. In Phycomyces blakesleeanus 80% of the zygospores germinate between 80 to 120 days after their formation.

15.3 Growth conditions of Zygomycetes

One would think that the Zygomycetes are fungi that are not able to withstand extreme conditions because of their rapid growth and the relatively simple nutrients they use for growth. They grow only at relatively high water activities, the minimal water activities ($a_w$) for growth for the fungi M. circinelloides, M. racemosus, M. spinosus and Rhizopus stolonifer, Thamnidium elegans are 0.90, 0.94, 0.93, 0.93 and 0.94 respectively (Samson et al., 2004). However,
some Zygomycetes are able to grow in solutions with rather high salt concentrations as are used during the preparation of the Chinese food product su-fu (Han et al., 2004). For instance, Mucor circinelloides grows in 15% NaCl, $a_w = 0.9$ (Tresner and Hayes, 1971).

The Zygomycetes also cover a fairly large range of temperatures. Rhizomucor pusillus formerly designated as Mucor pusillus, is a thermophilic fungus that can grow above 50°C. According to the definition, thermophilic fungi have a growth temperature optimum above 50°C and cannot develop below 20°C (Cooney and Emerson, 1964). Mucor hiemalis can grow below 0°C (Joffe, 1962), while Thamnidium elegans grows during cold-storage of meat.

Some Mucorales develop under anaerobic conditions and can perform a phenomenon that is observed also within the Asco- and Basidiomycetes, namely dimorphism. This includes a shift from filamentous growth (formation of hyphae) to the development of single cells by bud formation (as in yeasts). Under high carbon dioxide and absence of oxygen, Mucor rouxii and racemosus form buds and grow as yeasts. Mycotypha and Rhizopus species show also anaerobic development. Rhizopus grows in such a case as hyphae and Mycotypha grows as a yeast.

Growth parameters are extensively measured and modeled for R. oligosporus, the fungus used for the preparation of the food tempeh (Sparringa et al., 2002). Growth of the fungus was predicted to be optimal at 42°C, pH 5.85, $a_w$ approx. 1 and 0.03% CO$_2$ and was 1.7 mm/h (that is 28 µm/min). Growth of the fungus was sensitive to pH and was very low below pH 3.5 and above pH 7.5. Decreased growth was observed with lowered water activity (from $a_w$ 1.00 to 0.96) and increased CO$_2$ level (from 0.03% to 12.5%). At a temperature of 30°C and further ‘midpoint’ conditions, growth was 60% of the optimal temperature. With water activity this was 74% at $a_w = 0.96$ compared with growth at $a_w = 1.0$. With CO$_2$ 70% of the growth at 12.5% of the gas was seen compared with ambient conditions. Below pH 3.5 and above 7.5 it was 27% of the optimal growth. With R. oligosporus the optimal temperature became lower at unfavourable pH values, which is more commonly observed. The fungus is also very sensitive to free ammonia (NH$_3$) with slowed growth at concentrations of 0.42 and 0.84 mmol and inhibition above 1.3 mmol. Sporulation was prevented above 0.42 mmol of the compound (Sparringa and Owens, 1999). Measurements at our laboratory with R. oryzae showed that this fungus had optimal growth at 36°C on potato dextrose agar with an average growth speed of 3 mm/h. No growth was observed at 12°C after 48 h of incubation. Growth speed was higher on malt extract and rye-medium and lower on corn meal agar and oatmeal agar, but here an average speed of 1.5 mm/h was also observed (J. Houbraken, unpublished results).

In Section 15.5 different factors are summarized that influence the development of Mucorales in spoilage situations with an emphasis on R. stolonifer. These include the effects of antibiotic compounds, essential oils, temperature treatments and the use of controlled atmosphere treatments.
15.4 Zygomycetes in food and industry

Among the class Zygomycetes, the order *Mucorales* has the highest number of species that are related to food and industry. These fungi are known for the production of organic chemicals as fumarate, lactate and enzymes. *Rhizopus oryzae* can produce high levels of lactate from glucose, which is used as a food additive or as a precursor for the production of degradable plastics (Longacre *et al.*., 1997; Skory, 2004). The fungus also is able to produce L(+)-lactic acid from recycled office paper, suggesting an excellent way for bioconversion of used paper (Park *et al.*, 2003).

With respect to lipid biotechnology *Mucor* species (*javanica* and *circinelloides*) and *Mortierella isabellina* are promising candidates for the production of polyunsaturated fatty acids (Ratledge, 1989; Hiruta *et al.*., 1996; Xian *et al.*, 2003) including γ-linoleic acid, a compound that can suppress inflammation. Gema *et al.* (2002) report that these compounds can be produced on biological waste products as orange peel by the fungus *Cunninghamamella elegans*.

The thermophilic fungus *Rhizomucor miehei* forms a thermostable lipase in amounts interesting for industrial use for waste treatment and cheese ripening. Lipases are important in food industry for the production of fruit juices, baked foods and modification of fats and oils (Adrio and Demain, 2003). Acid proteases (which are also used from the fungus *Rhizomucor pusillus*) are used for meat and fish processing. *Rhizopus oryzae* forms pectinases for production of wines and preserves.

Recently, a phytase of potential commercial interest and even better thermostability than commercial phytases was reported from a *R. oligosporus* strain (Casey and Walsh, 2004). Phytase is an enzyme that degrades phytic acid and its salts (phytates), the latter representing the major storage form of phosphorus in cereal grains and legumes. Phytate is not degraded by monogastric animals and feed must be supplemented with inorganic phosphate rendering the manure phosphorus-rich, which results in eutrophication of surface waters. Phytase mobilizes the phosphorus from phytate and hence counteracts adverse environmental effects. Finally, enzymes produced by a *Rhizopus* starter culture during a malting process resulted in increased β-glucanase and xylanase contents and improved endosperm cell-wall degradation (Noots *et al.*, 2001).

Besides the production of organic chemicals or enzymes many products that consist of soya or rice colonized with Mucorales as *Mucor* and *Rhizopus* are used as a food product, mostly in South-East Asia. For instance tempeh kedele is a soya bean cake covered with white mold into which proteases and lipases are released by the fungus *Rhizopus oligosporus*. Enzymes released by the fungus play an important role in the process of tempeh formation. Tempeh has a high protein content (40–50%) and the essential amino acids are present in the food product. It contains a mixture of filamentous fungi, yeasts and bacteria, the latter at high levels. These include the dominant *R. oligosporus*, but also the filamentous fungi *R. chinensis*, *R. oryzae*, *Mucor*
and the yeasts *Trichosporon beigeli*, *Clavispora lusitaniae*, *C. maltosa*, *C. intermedia* and *Yarrowia lipolytica* are found, as are the bacterial species *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Lactobacillus* spp. The hyphae penetrate 2 mm inside the soya cake during a period of 40 hours and the fungal mass is not sporulating at harvest. So, the next time when you eat tempeh, remember that you are eating a complete microbial community.

Another product, tofu, is the result of fermentation of soya milk curd with *Mucor racemosus*, *Rhizopus chinensis* and *Actinomucor elegans*. Sufu is ‘molded’ tofu (also designated as ‘pehtze’) that is matured in a brine solution and contains fungi of the Zygomycete genera *Actinomucor* (repens and taiwanensis), *Mucor* (circinelloides, hiemalis and racemosus) and *Rhizopus microsporus* var. *microsporus* (Han et al., 2004). The Rhizopus species used in tempeh production usually are less proteolytic than the species of *Aspergillus* used for koji, which is related to soya sauce. There are also indications that an unidentified species of *Mucor* improved the sensory properties of dry fermented sausages from Spain (Garcia et al., 2001a).

### 15.5 Zygomycetes and spoilage

#### 15.5.1 Spoilage by several Mucorales

The Zygomycetes that are most relevant for food spoilage belong to the genera *Absidia*, *Mucor* and *Rhizopus*. Ecologically, these fungi grow widely in soil, plant debris and dung. It will not be a surprise that species of *Rhizopus* and *Absidia* predominantly occur on stored grain, fruit and vegetables. They, however, are also observed on meat and salted or unsalted fish (Santoso et al., 1999; Mizakova et al., 2002) and in dairy products (e.g. Aboul-Khier et al., 1985). Zygomycetes could also be isolated from pasta products (Halt et al., 2004).

There is a difference in spore distribution that may be relevant here: *Mucor* (Absidia) forms sporangiospores that are embedded in a mucus-like material, while *Rhizopus* forms sporangiospores that are dry. This can mean that the distribution of *Rhizopus* through the air should be easier and spore numbers may be higher and therefore substrates will be contaminated more extensively by these fungi. This difference in spore release is only partly confirmed by the data, the most dominant Zygomycetes in the air are *Mucor plumbeus*, *R. stolonifer* and *Absidia corymbifera* that commonly occur in indoor environments (Samson et al., 2004). Other Zygomycetes, namely *Thamnidium* and *Cunninghamamella*, do occasionally contaminate food. *T. elegans* is especially known for growth on cold stored beef, and it is suggested that it has an effect on the ‘ageing’ of beef due to enzymic activity or that its presence could inhibit bacterial growth (Kotula et al., 1988; Campano et al., 1985).

Table 15.1 summarizes many observations on Zygomycetes that contaminate food. One fungus is left out of the table and that is *Rhizopus stolonifer*. 
Table 15.1  Zygomycetes as food spoilers. These data are a compilation of Pitt and Hocking, (1997) and Samson et al. (2004) and more recent papers as summarized beneath the table

<table>
<thead>
<tr>
<th>Species</th>
<th>Spoiling action</th>
<th>Isolated from</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Absidia corymbifera</em></td>
<td>Pathogen of peaches.</td>
<td>Wheat, barley, flour, bran, stored grain, sunflower seeds, pecan nuts, hazelnuts, peanuts, decaying vegetables, canned tomatoes, cassava, (rehydrated dried) fruit, meat products, biltong, soil, air, compost, animals and man. Can develop on wounds in humans. Decayed root tubers.</td>
</tr>
<tr>
<td><em>Cunninghamella elegans</em></td>
<td>Rotting in kola nuts.</td>
<td>Tropical and subtropical soils, low levels in maize. Brazil nuts. Herbs and spices. Decayed root tubers</td>
</tr>
<tr>
<td><em>Mucor circinelloides</em></td>
<td>Spoils cheese and yams. Pathogen of mangoes.</td>
<td>Isolated from meat, cereals (maize, barley) and (hazel) nuts. Decaying potatoes, mung beans, soybeans. Human and animals.</td>
</tr>
<tr>
<td><em>Mucor racemosus</em></td>
<td>Spongy soft rot of cool stored sweet potatoes, potatoes and citrus. Spoils yoghurt and cheese.</td>
<td>Isolated from meat, nuts, cereals (seeds of wheat, barley, rice), tea, tomatoes, soil, dung, food (often in milk).</td>
</tr>
<tr>
<td><em>Mucor hiemalis</em></td>
<td>Rots in guavas, carrots and cassava. Spoilage of yoghurt.</td>
<td>One of the commonest soil fungi. Isolated from dung and air. Fresh vegetables, cereals (stored grain, rice, corn), (hazel) nuts, cotton seed, flour, (soy) beans, bananas, sugar cane, vegetables, potatoes. Wheat-based fast foods (Nigeria). Pre-prepared line food in Egypt.</td>
</tr>
<tr>
<td><em>Mucor plumbeus</em></td>
<td>Spoils cheese, anaerobic spoilage of apple juice in the lab.</td>
<td>Reported from meat, nuts and cereals. Black rice, soy beans and coriander. Food, indoor, often as air contaminant. Worldwide, soils, hay, dung, stored seeds of wheat, oats.</td>
</tr>
<tr>
<td><em>Mucor piriformis</em></td>
<td>Rotting of cold stored pears.</td>
<td>Maize samples.</td>
</tr>
<tr>
<td><em>Rhizopus microsporus</em></td>
<td></td>
<td>Food, peanuts, wheat and tropical food. Brazil nuts.</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizomucor pusillus</em></td>
<td></td>
<td>Cereals, fermented foods, nuts and processed meats, spices and capsicums. Cold stored meat (long whiskers), suppressed by modern storage methods</td>
</tr>
<tr>
<td><em>Syncephalastrum racemosum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thamnidium elegans</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Absidia, peanuts (Botswana) (Mphande et al., 2004); Absidia, canned tomatoes (Nigeria) (Efuiwewwere and Atirike, 1998); Absidia, rehydrated dried prunes and raisins, (El-Halouat and Debevere, 1997); Rhizopus oryzae, Cunninghamella elegans, Brazil nuts (Freire et al., 2000); Mexican herbs and spices, Cunninghamella and Rhizopus (Garcia et al.; 2001b). Cunninghamella and Rhizopus sp. kola nuts (Nigeria) (Adebajo, 2000), Absidia corymbifera, Cunninghamella elegans, decayed root tubers (cassave, potatoes) (Fawole and Odunfa, 1992).
15.5.2 **Rhizopus stolonifer**

This fungus is the most notorious Zygomycete with respect to food spoilage and occurs in many cases of ‘box-rot’ or ‘transit-rot’ of many different crops. It is by far the most commonly occurring species on food of the Mucorales, has a worldwide distribution, and causes rot in ripe and harvested fruit and vegetables. At and before 1985 this fungus was also called *R. nigricans*. It is also dominant in the air and occurs more often in warmer zones. *R. stolonifer* grows also on MY50 agar with a water activity of 0.89 and under anaerobic conditions.

*Rhizopus*-soft rot is a threat in all post-harvest situations including storage, marketing and transport of crops. It causes soft rot of avocados, cassava, crucifers, pulses, yams and sweet potatoes. In eggplant, grape, pepper (capsicum), stone fruit, strawberry and tomato the disease is dubbed *Rhizopus*-rot. Snowdon (1990) mentions *Rhizopus*-rot in mandarins, avocados (where a peculiar and unpleasant odor evolves), bananas, guavas, mangoes and pineapples. Injury of fruit is important, if not a prerequisite, for successful colonization of the crop. These can, for instance, be the result of insect attacks, for instance by Hawaii fruit flies on guava. Stone fruit (including peaches, nectarines, apricots, plums and cherries) show important post-harvest losses as a result of *Rhizopus*-rot, with also *R. oryzae* as a contaminant. The fungus causes destructive rot, also designated as ‘leak’ of fresh berries and is the cause of a major part of the marketing losses on all berry fruits. It is also of special importance in ripe melons and also cucumbers and related vegetables are attacked. Different other vegetables are sensitive to the disease as well.

Sweet potatoes show heavy losses due to *Rhizopus*-rot. Carrots are attacked when they lose 8% or more of their moisture, with cooling as an important control treatment. Also peas and all types of green beans can be rotted, as well as tomatoes, peppers and eggplants. Remarkably, green tomatoes can also be invaded by the fungus. Furthermore, the fungus rots sugar beets, cauliflower, onions and related crops and potatoes. In a relatively small number of cases other Zygomycetes are described that cause ‘*Rhizopus*-rot’. For instance, *Mucor piriformis* is important as a cause of soft rot in the UK, while in other countries *Rhizopus* is more prevalent. In case of rotten peaches, *Gilbertella persicaria* dominated during two seasons in South Carolina peach production areas (Ginting et al., 1996). *Rhizopus oryzae* was aggressively pathogenic towards sweet potato (Ray and Byju, 2003). *Choanephora cucurbitarum* is a beautiful Zygomycete that affects marrows and pumpkins.

Affected stone fruit becomes surrounded by a coarse, loose ‘nest’ of mycelium. Infected areas of fruits appear water soaked at first and very soft and initially give a mildly pleasant smell. Soft rot is characterized by the loss of cohesion among cells due to the action of pectinolytic enzymes. For instance during ‘box rot’ of dried French prunes, soft sticky macerated areas on the fruit and slippage of the skin were observed. During further stages of infection, the fungus spreads rapidly, engulfing several fruit adjacent to the
originally infected one in a few days. Hyphae also develop over healthy tissue and even on the surface of containers. It is isolated from many other sources including cereals, vegetables, nuts and meats (Pitt and Hocking, 1997; Samson et al., 2004).

After infection of the fruit, zygospores could be formed when different mating types of the fungus meet, and these structures may survive periods of starvation including the winter in decayed (dried) tissue (Agrios, 1997). The fungus also causes spoilage on food when it is no longer present! Some enzymes produced by this fungus (probably pectinases) may cause softening and spoilage in canned fruit. This has occurred in apricot samples (Sommer et al., 1974). Pectin degrading enzymes are widely studied in the genus *Aspergillus* and many of their characteristics are summarized by de Vries and Visser (2001). Pectin lyases produced by *Aspergillus* species generally have optima between 40 and 55°C and polygalacturonases between 35 and 60°C. The optimal pH of the latter enzymes varies between 3.5 and 6.0, while pectin lyases have optima between 6.0 and 9.0. Although the optima of these enzymes are rather high, these enzymes are more stable at lower temperatures. With respect to the stability of these enzymes, some of them may survive short pasteurization treatments, but pectin-degrading enzymes of *R. stolonifer* are only scarcely studied in this respect. Saito et al. (2004) describe a 31 kDa enzyme from *R. oryzae* with an optimum near pH 4.5 at 45°C. Compared with *Aspergillus* this could be a polygalacturonase since these have lower pH optima. In the study of Sommer et al. (1974) retention of enzymic (pectolytic) activity as judged by viscosity changes in pectin of pieces of apricot tissue occurred after heat treatments at 100°C. In juice from apricot rotted by *R. stolonifer* pectolytic activity still was detectable after 30 and 40 min of heat treatments.

### 15.6 How to prevent spoilage

Infection of fruits and vegetables is essentially a post-harvest problem and here different strategies can be followed. *Rhizopus stolonifer* infection (or, in some cases, other Zygomycetes) typically develops during storage, transport of the product, during commercialization or at the consumer’s home. Careful handling of the crop is very important since the pathogens enter the product through natural or evoked wounds (see for instance Holmes and Stange, 2002, for sweet potatoes). Traditionally treatments of the crop with antifungals compounds such as iprodione (Ginting et al., 1996; Bonaterra et al., 2003) are used to control the disease; however, with *Rhizopus*-soft rot these treatments are not always successful. Trials with ammonium-molybdate exhibit a strong inhibitory effect when *R. stolonifer* caused lesions on apples (Nunes et al., 2001a).

Hot water dips could have results, but this depends very much on the type of fruit treated. The range of temperatures has to be chosen very precisely to
prevent damage to the fruit and simultaneously prevent fungal development. Development of the fungi *Botrytis cinerea* and *Monilinia fructigena* was lowered after treatment at 45 to 48°C on cherries, but there was a dramatic effect on the organoleptic properties of strawberries after treatment (Marquenie *et al.*, 2002). Use of hot water with 10% ethanol resulted in reduction of rot and firmer flesh of peaches and nectarines (Margosan *et al.*, 1997) although spores of *R. stolonifer* are less resistant to ethanol/temperature combinations as *Botritys cinerea* and *Alternaria* sp. (Mlikota-Gabler *et al.*, 2004). Dipping of stone fruits in peracetic acid (250 mg/l) for 8 min reduced *Rhizopus*-rot to zero (Mari *et al.*, 2004). Sporangiospores of *R. stolonifer*, however, can escape the action of inhibitory compounds (chlorine) when they are carried through wounds into intercellular spaces in tomato (Bartz *et al.*, 2001).

Packaging of the fruit under a modified atmosphere is another option given the fruit survives it. Hoogerwerf *et al.* (2002) observed a clear reduction (56%) in growth of *R. stolonifer* under 20% O₂/20% CO₂ at 20°C. Additional high (80%) oxygen storage did not add much further to the inhibition effect. Storage of wounded and inoculated peaches under ozone resulted in inhibited aerial development and sporulation in the case of *Mucor piriformis*, but not in a reduction of the severity of fungal decay (Palou *et al.*, 2002).

Thirdly, natural compounds produced by plants often have antifungal activity; different references have shown effects against *R. stolonifer*. Cinnamaldehyde, a compound in cinnamon has clear antifungal activity against many fungi including *Rhizopus* in the submillimolar range (Utama *et al.*, 2002; Made *et al.*, 2002), while essential oil of *Thymus vulgaris* is effective against *Botrytis cinerea* and *R. stolonifer* in strawberry fruits where decay was reduced from 55.3% in untreated fruit to 15.7 and 17.5% in two experiments (Bhaskara Reddy *et al.*, 1997). Vapors of thyme oil, oregano oil, thymol and carvacrol inhibited *R. stolonifer* (Plotto *et al.*, 2003). An active compound of mustard oil can possibly be used to prevent development of fungi on bread (Nielsen and Rios, 2000). Remarkably, another natural compound, chitosan, a building block of the Zygomycete cell wall has an inhibitory effect on *Mucor racemosus* and also prevents development of *Byssoschlamys* spp., other fruit spoiling fungi (Roller and Covill, 1999). Vapors of short-chain organic acids such as acetic, formic and propionic acids showed control of post-harvest decay of cherry (Sholberg, 1998). Natamycin, a compound isolated from streptomycetes, has a broad-spectrum inhibitory effect on numerous food-relevant fungi and has a more lasting antifungal action than potassium sorbate in the case of *Mucor racemosus* on smoked sausages (Stiebing *et al.*, 2001).

Different microorganisms that live on fruit are studied for their potential to antagonistic activity against soft rot. The bacterium *Enterobacter cloacae* antagonizes *R. stolonifer* on peaches (Wisniewski *et al.*, 1989). Lesion development of wounded pears by *R. stolonifer* was dose-dependent with the amount of spores added to 3 × 3 × 3 mm³ wounds; 20 spores resulted in a lesion diameter of 1.5 cm after 7 days while 20 000 spores resulted in a
12 cm lesion. Infection was prevented totally by the bacterium *Pantoea agglomerans* when added in 25 times higher numbers (or more) than the fungal spores (Nunes *et al*., 2001b). Nectarine and apricots (stone fruit) punctured and immersed in the bacterial suspension and after 2 hours immersed in a 1000 spores/ml solution of *R. stolonifer* gave a dramatic decrease in lesion size and incidence of fruit rot (Bonaterra *et al*., 2003). A minimum of $10^5$–$10^6$ bacteria were needed for an antagonizing effect. The bacteria need a close physical interaction with the fungal spores, a phenomenon more often observed in fungal bacteria interactions (Dijksterhuis *et al*., 1999; Hogan and Kolter, 2002). Magnusson and Schnürer (2001) identified a broad spectrum proteinaceous and antifungal compound produced by the bacterium *Lactobacillus coryniformis* that inhibited growth of *Mucor hiemalis*.

In addition, yeast species such as *Pichia membranefaciens* can antagonize *R. stolonifer* spores inside wounds on nectarine fruits (Qing and Shiping, 2000). *Candida sake* prevented lesion development during 5 days in wounded Golden Delicious apples entirely, but the cells needed to be in at least a 240-fold majority compared with the sporangiospores of *R. stolonifer* (Vinas *et al*., 1998).

Finally, combinations of different methods can be effective in attacking the *Rhizopus* problem. Hongyin-Zhang *et al.* (2004) found a combination of microwave treatment and an antagonistic yeast (*Cryptococcus laurentis*) promising for the control of *R. stolonifer* on peaches.

### 15.7 Zygomycetes and mycotoxins

Fungi can produce a bewildering variety of chemical compounds and, as we saw earlier, Zygomycetes also have the capacity to degrade many different compounds. Ascomycetes of different genera are able to form complex toxic compounds than are harmful to humans. For instance, the carcinogenic compound aflatoxin has been acutely toxic to victims in the third world after consumption of molded food. The toxin is produced, among other fungi, by *Aspergillus flavus*, but there are a number of reports that state that the toxin was also produced by Zygomycetes, but this observation has never been confirmed.

There are two compounds, however, that are produced by members of the genus *Rhizopus* that are of interest here. When *Rhizopus microsporus* was grown on maize and brown rice, a compound was found dubbed rhizonin (A and B) that caused kidney and liver lesions in rats (Wilson *et al*., 1984). The related *R. chinensis* and *R. oligosporus*, which are used for food production (e.g. tempeh), did not form notable amounts of the toxin (Jennessen *et al*., 2005). In addition, *R. microsporus* (but in literature stated as *R. chinensis*; Aoki *et al*., 2003) forms rhizoxin, a potent angiogenesis inhibitor. This is a compound that blocks blood vessel formation through a combined action on
endothelial cells. Therefore, this compound could have a role in the treatment of different diseases, including cancer.

### 15.8 Zygomycetes and spore formation and germination

The production of sporangiophores on mycelium deals with specific requirements. When sporangiospores of *R. oryzae* were added to wetted rice and subsequently inoculated, it became clear that water activity is a vital factor for the formation of these structures. In addition, oxygen is important, for a rather small amount of inoculated rice (200 g) a headspace of 16 litres of air was certainly not enough for sporangiophore formation. Remarkably in this fungus, the formation of sporangiophores was optimal at 30°C, which was lower than the optimal growth temperature (35°C).

The germination of many types of fungal spores includes swelling and germ tube formation and is followed by colony formation. All these stages can be discerned and may be under the control of different factors. Zygomycetes are dispersed through a variety of spore structures as oidia, sporangiospores, conidia, arthrospores, chlamydospores and the sexual zygospores. The first four types of cells are important for dispersal. The latter two are more or less survival capsules in time and stay in their position when the rest of the colony has disappeared.

Germination of *R. oligosporus* sporangiospores is optimal at 37°C and pH 4 and stimulated by glucose and L-alanine. Starter cultures of *R. oligosporus* used for the production of tempeh are grown on rice and then dried and pulverized, but still could germinate after an activation treatment in malt extract broth. The potential of the spores to form a colony decreases during prolonged storage of the dried sporangiospores. To a certain extent, sporangiospores of *R. oligosporus* exhibit metabolic activity when they are wetted in the absence of any nutrients namely in buffer, but colony formation under these conditions is not observed. When more and more compounds are added (phosphate, amino acids, glucose, combinations of the compounds), germination increased (Thanh and Nout, 2004; Thanh, 2004). Presence of sporangiospores in malt extract for 4 h, 37°C strongly increased the carboxyfluorescein diacetate (cFDA) staining of spores that were dried for 2 months. cFDA uptake by spores indicates metabolic activity of the spores and a beginning of the germination process. Spores stored for 11 months showed no colony formation on 2% glucose alone (<1%). A sharp increase (33–36% of the spores) in colony formation was observed on malt extract, peptone, yeast extract and glucose/peptone agar medium. After an initial activation treatment on malt extract broth this had increased to 42–46%. Casamino acids gave an intermediate value.

Use of three fluorescent probes (propidium iodide (PI) \{1′-(4,4,7,7,-tetramethyl-4,7-diazaundecamethylene)-bis-[3-methyl-2,3dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(3′-trimethylammoniumpropyl)-pyridinium...
tetraiodide) (TOTO) and cFDA) suggested that a large population of dried spores show PI-related membrane permeabilization, but not TOTO binding to the DNA. This is remarkable while both PI and TOTO are regarded as an indicator of cell death. After activation of the PI population, the majority of the spores stained with cFDA and therefore were metabolically active. This could be caused by an effect called imbibitional damage where damage to the membrane occurs as a result of rewetting at a low temperature. However, there is evidence that a slightly damaged cell population is regenerating after an activation treatment. The fraction of PI positive, TOTO negative (thus damaged) spores increased with storage time.

With *R. oryzae*, sporangiospores germinate readily in malt peptone medium, but germination decreases below a pH of 4.8. Again, best germination was observed at 30°C, while the highest radius of colonies is observed at 35°C. So, the development of fungi shows different stages, germination, mycelial growth and sporangiophore formation, and each stage can have typical requirements.

There are a number of notable effects during germination of sporangiospores:

- Sporangiospores exhibit the so-called crowding effect. Germination of spores of *R. oryzae* show lower germination when they are present in higher densities. Germination lowers in *R. oryzae* from 67 to 22% of the cells after 4 hours of incubation.
- Sporangiospores are influenced by so-called self-inhibitors. Fungal compounds that reversibly inhibit germination as for instance in fruit bodies. Nonanoic acid is such a compound (Breeuwer et al., 1997), which results in both a decrease in internal pH as a lower number of fluorescent (= metabolic active) cells. The mode of action of this compound may be as a weak organic acid such as sorbate, propionic acid and acetic acid.
- Sporangiospores of *Phycomyces blakesleeanus* are activated to germinate by a heat treatment (van Assche et al., 1972). These spores are dormant and increasingly germinate after a short heat treatment at 50°C.

### 15.9 Medical aspects of Zygomycetes

Generally Zygomycoses (infection of humans with a Zygomycete) occur in cases of immunodeficiency or in patients with diabetes mellitus. *Absidia (corymbifera)* and *Sacksenae vasiformis* are associated with injuries for example as a result of traffic accidents (Ribes et al., 2000; Horre et al., 2004) and this can occur in immunocompetent persons. When infection becomes systemic (disseminated through the body) they can become very progressive even in immunocompetent individuals and infection of any body part is possible (e.g. Solano et al., 2000).

Often the terms zygomycosis or mucormycosis are used, but the most important pathogenic fungi are *Rhizopus oryzae* and *R. microsporus*. One-
third to one-half of all cases of zygomycosis result in an infection of the sinuses (rhinocerebral disease) and when not treated accurately, from this location further fungal development can occur with fatal consequences (Ribes et al., 2000). The second important group of infections is related to pulmonary disease. These two groups suggest that the most important route of infection is via airborne spores that enter the respiratory tract. Zygomycetes can also cause cutaneous infection, but gastro-intestinal disease is uncommon. Another source of infection is a non-sterile bandage or via infusion tubes.

The most important fungi by far of medical importance are members of the genus *Rhizopus*, accounting for 90% of all cases of rhinocerebral disease and 75% of all cases of zygomycosis. *R. oryzae* and *R. microsporus* var. *rhizopodiformis* are the dominant pathogenic fungi in this respect, but it has to be stressed that these fungi are opportunistic and that they infect in the context of weakened patients. Two important features of infection are the colonization of blood vessels and the ability of the fungus to grow well at body temperature.

Other Zygomycetes are of less medical importance. *Apophysomyces elegans* is related to cutaneous (skin) infections. *Cunninghamella bertholetiae* is also a cause of zygomycosis and associated with immunocompromised patients. The entomopathogenic fungus *Basidiobolus ranarum* is related to a number of cases of skin infection, albeit only in (sub)tropic areas. A number of vicious infections is related to *Conidiobolus coronatum*.

### 15.10 Specific detection, identification and enumeration methods

The detection of Zygomycetes on food and food products is mainly based on visual development of the fungus on the crop or on the food products. While the Zygomycetes often grow very fast, cultivation and identification can be finished earlier (within a few days) than in the case of other food-relevant fungi. One could develop methods to measure specific volatiles formed by the fungus, but no reports of *Rhizopus* are available. Gokmen and Acar (2004) report that fumaric acid in apple juice can be a potential indicator of microbial spoilage (caused by, among others, *R. stolonifer*) of the apples used, but this is after the product has been processed. Although molecular detection methods are potentially powerful, many drawbacks occur in food situations that have to be dealt with. In fact proper sampling will be of major importance here. How can an infection with *R. stolonifer* be identified in a large crop of stone fruit? How many samples are needed and how big is the sample size? At the moment careful handling and visual inspection are still of vital importance for the detection of Zygomycetes on food. Furthermore, specific molecular data on the taxonomy of the Zygomycetes are still very scarce and as such also the development of specific primers for the species of the taxon (Seif et al., 2005).
15.11 Sources of further information and advice

Because of the small size of the fungal group compared to other fungi, information about the Zygomycetes is scattered throughout the textbooks. There are a number of monographs of different genera of the Mucorales in the series Studies in Mycology, which are available on the internet (Schippers, 1984; Stalpers and Schippers, 1972). There are coherent chapters on the phylum Zygomycotina in Introductory Mycology (Alexopoulos, Mims and Blackwell, 1996) where the different groups of the Zygomycetes are described very well. Food-relevant Zygomycetes are portrayed very well in Pitt and Hocking’s Fungi and Food Spoilage (1997) and in Introduction to Food- and Airborne Fungi (Samson et al., 2004).

For post-harvest diseases, the color atlas of post-harvest diseases and disorders (two volumes) of Snowdon (1990 and 1992) is still unsurpassed. For medical problems, the Atlas of Clinical Fungi by Hoog et al. (2000) provides very good descriptions of different medical important Zygomycetes. In addition the review of Ribes et al. (2000), gives a lot of background analysis on zygomycosis.

15.12 Future trends

Consumers in western countries more often demand fresh products that are minimally processed. As this trend continues, the risk of (fungal) deterioration is increasing and Zygomycetes, especially R. stolonifer may become increasingly important as a spoilage organism. In post-harvest situations, fungicides are restrictively used and possibly the future laws will become even more restrictive to the application of these compounds. Currently, new alternatives in food processing are being investigated. For example, high-pressure treatment of food products, treatments with natural compounds (essential oils) to prevent fungal development or packaging of fresh product under controlled atmospheres. These novel techniques even may be combined in order to eradicate fungal propagules as is for example done for bacteria (Karatzas et al., 2001; Pol et al., 2000) or with sporangiospores of Mucor plumbeus (Fenice et al., 1999). In addition, biocontrol techniques and traditional physical methods such as hot water dips may be addressed. In all cases careful handling of the crop is important since these fungi enter the crop via cracks or wounds. While more and more crops are harvested and treated mechanically and not by the human hand there is a risk that post-harvest infection will increase. For example, mechanical removal of the roots of tulip bulbs increase the wounds and possible pathogens are distributed efficiently to other bulbs helping the disease to spread through the crop.

The increase in the number of diseases that afflict the immune system (e.g. HIV AIDS) may lead to an increase of the number of cases of infection with Zygomycetes. While these infections are particularly severe, increased
efforts have to be made to find special weak points of the Zygomycetes, for instance enzymes that are involved in chitosan formation.

15.13 References


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16

Penicillium and related genera

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16.1 Introduction

Fungi belonging to the genus *Penicillium* are among the most ubiquitous organisms on earth. They have been found wherever they have been sought, from the Antarctic across the tropics to Greenland. It is hard to find a sample of soil or decaying vegetation free of *Penicillium* spores. Because of this, the common viewpoint is that Penicillia are just contaminants, present in low numbers, difficult to speciate and probably not worth speciating.

Work carried out during the past 20 years, however, has shown that *Penicillium* species are often remarkably substrate-specific, though usually for reasons we do not understand. Improvements in taxonomy have shown that certain commonly isolated species are really soil fungi, seen in foods only as contaminants, while others are consistently foodborne, with a high potential to cause spoilage if conditions prove favourable.

Most *Penicillium* species do not cause such overt spoilage as *Aspergillus* species often do, as growth of *Penicillium* species is less rampant and usually self-limiting. Nevertheless, *Penicillium* species are an important cause of food spoilage. The ability to recognize common spoilage species can be very helpful, both in efforts to elucidate the problem, and in answering the perennial questions about possible mycotoxin contamination.

In general terms, speciation of *Penicillium* isolates is not easy. However, if account is taken of the substrate on which a species is found, a great deal of information can often be obtained relatively easily. So in the sections that follow, species have been grouped according to the substrate or substrates on which they are likely to cause spoilage.
16.2 Taxonomy

*Penicillium* is a large genus. One hundred and fifty species were recognized in the last complete taxonomy (Pitt, 1979), but subsequent studies indicate that this number is conservative. The most recent general compilation of species names (Pitt *et al*., 2000) lists about 220 species, and since that time 30 or more further species have been published (Samson and Frisvad, 2004). At least 50 species are of common occurrence (Pitt, 2000). All common species grow and sporulate well on synthetic or semisynthetic media, and are usually readily recognizable to genus level.

Classification within *Penicillium* is based primarily on microscopic morphology (Fig. 16.1). The genus is divided into subgenera based on the number and arrangement of phialides (elements producing conidia) and metulae and rami (elements supporting phialides) on the main stalk cells (stipes). The classification of Pitt (1979) includes four subgenera: *Aspergilloides*, where phialides are borne directly on the stipes without intervening supporting elements; *Furcatum* and *Biverticillium*, where phialides are supported by metulae; and *Penicillium*, where both metulae and rami are usually present. The majority of important food spoilage (and toxigenic) species are found in subgenus *Penicillium*.

16.3 Enumeration

General enumeration procedures suitable for foodborne moulds are effective

![Fig. 16.1 Penicillium types in Penicillium (a, b) monoverticillate; (c) terverticillate; (d, e) biverticillate, subgenus Furcatum; (f) biverticillate, subgenus Biverticillium.](image-url)
for enumerating all common *Penicillium* species. Many antibacterial enumeration media can be expected to give satisfactory results. However, some *Penicillium* species grow rather weakly on very dilute media such as potato dextrose agar. Dichloran rose bengal chloramphenicol agar (DRBC) and dichloran 18% glycerol agar (DG18) are recommended for enumerating *Penicillium* species (Pitt and Hocking, 1997). The most appropriate methodology is outlined elsewhere (King et al., 1986; Hocking et al., 1992; Pitt and Hocking, 1997; Samson et al., 2004).

### 16.4 Identification

Identification of *Penicillium* isolates to species level is only effectively carried out under carefully standardized conditions of media, incubation time and temperature. The media to be used are Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N; Pitt, 1979; Pitt and Hocking, 1997). Inoculated Petri dishes are incubated at 25 °C for 7 days before examination. Additional Petri dishes of CYA incubated at 5 °C and 37 °C are recommended. In addition to microscopic morphology, gross physiological features, including colony diameters, colours of conidia and colony pigments, are used to distinguish species (Pitt, 1979, 2000).

### 16.5 *Penicillium* species causing food spoilage

*Penicillium* species causing food spoilage can be divided into three major groups: those infecting fresh foods, especially fruits; those infecting cereals, principally after harvest and during drying; and those likely to be found in processed foods. These groups are treated separately below.

#### 16.5.1 *Penicillium* species spoiling fresh fruit and vegetables

*Penicillium* species are the major fungi causing spoilage of citrus and pome fruits. These species cause visible rots on the fruit and are responsible for enormous losses around the world. The species involved are readily recognized *in situ* and readily identifiable in culture.

**Pome fruits**

Apples and pears are commonly spoiled by *Penicillium expansum*, which causes a characteristic brown, spreading rot. In culture, after 7 days incubation at 25 °C, *P. expansum* produces relatively quickly growing, deep colonies with a broad white margin (diameters 30–40 and 20–40 mm on CYA and MEA respectively). Brown exudate and soluble pigment are usually produced on CYA. This species is classified in subgenus *Penicillium*, so penicilli are
terverticillate, borne on stipes with smooth, slender walls. Conidia are ellipsoidal, 3.0–3.5 µm long, and smooth walled.

*Penicillium expansum* grows between $-2^\circ C$ or even lower, up to 35$^\circ C$, and down to 0.83 water activity ($a_w$) (Pitt and Hocking, 1997). It has been isolated from a wide range of other fruits, including tomatoes, strawberries, avocados, mangoes and grapes, indicating that it is a broad spectrum pathogen on fruits (Snowdon, 1990). Control of infection of fruit begins in the orchard, with the use of fungicides to prevent infections which permit entry by *P. expansum*. Prevention of damage to fruit and rapid processing are also important (Snowdon, 1990).

*Penicillium expansum* is commercially important both as a major spoilage fungus, and as the main source of the mycotoxin patulin, which occurs in apple and pear juice as the result of rotting of fruit due to *P. expansum*. Patulin levels in juices can be effectively controlled by culling rotting fruit before crushing. Many juice factories use high-pressure water sprays to cut out rotting areas of fruit before processing.

*Penicillium solitum* is a less common, but also important, pathogen of pomaceous fruit. It is resistant to the fungicides used to control growth of *P. expansum* and so its role in apple spoilage has increased in recent years (Pitt *et al*., 1991; Sanderson and Spotts, 1995). This species also causes spoilage of cheese (Hocking and Faedo, 1992; Lund *et al*., 1995a).

*Penicillium solitum* produces dark green, moderately sized colonies (20–28 mm diameter on CYA and MEA after 7 days at 25$^\circ C$), and shows little other colouring. Penicilli are terverticillate, typical of species in subgenus *Penicillium*, borne on rough stipes and conidia are smooth and spherical, 3.0–4.0 µm in diameter. Mycotoxins are not produced. Little has been recorded on the physiology of this species, but it probably has similar growth characteristics to *P. expansum*.

Apples can also be spoiled by *Penicillium funiculosum*, which causes core rot (Pitt and Hocking, 1997). This species produces loose-textured, dark green colonies, 25–40 mm diameter on CYA and MEA after 7 days at 25$^\circ C$, which usually show deep red reverse colours on CYA. Penicilli are biverticillate, typical of those formed by species in subgenus *Biverticillium*, and borne on short, smooth stipes. Conidia are ellipsoidal, 2.2–3.0 µm long. *P. funiculosum* grows from about 8–42$^\circ C$, with an optimum near 30$^\circ C$. It grows down only to 0.9 $a_w$ but is acid tolerant, growing down to pH 2 or lower (Pitt and Hocking, 1997). No mycotoxins are produced.

**Citrus fruits**

A different range of *Penicillium* species is responsible for rotting of citrus fruits, but the economic losses are also very high. *Penicillium digitatum* produces destructive brown rots on oranges and less frequently other types of citrus. In culture it is readily recognizable by the formation of rapidly growing olive colonies on both CYA and MEA (35–55 and 35–70 mm diameter, respectively). Penicilli are very large, biverticillate to tverticillate, and
produced on smooth stipes. It is classified in subgenus *Penicillium*, but is not typical of species in that subgenus. Conidia are distinctive, ellipsoidal to cylindroidal, olive in colour, and 6–8 (–15) µm long, larger than those of other *Penicillium* species. Growth of *P. digitatum* lies between 6 and 37 °C, with a minimum aw for growth near 0.9.

Initial control involves reducing spore build up in the orchard by removing fallen fruit, and in the factory by removing culled fruit (Snowdon, 1990). Control on harvested fruit relies on fungicidal sprays or dips, but resistance to thiabendazole, benomyl and imazalil has developed in most countries to a greater or lesser degree (Eckert *et al*., 1994).

*Penicillium italicum* also causes rots in citrus, principally in lemons. Fungal growth in the rots is blue or blue green. In culture, *P. italicum* produces rapidly growing green colonies, 30–40 and 35–55 mm diameter on CYA and MEA, respectively. Colony margins are often irregular, and a dark brown reverse colour is produced on CYA. Penicilli are terverticillate, borne on smooth stipes. Conidia are characteristically produced as short cylinders from the phialides, rounding up somewhat with maturity, and measuring 3.0–5.0 µm in length. In consequence it is classified in subgenus *Penicillium*, section *Cylindrospora*. Growth occurs down to 0 °C or slightly below, and up to 32–34 °C, with an optimum near 24 °C. Growth occurs down to 0.87 aw, and down to pH 1.6 (Pitt and Hocking, 1997). Control measures are essentially the same as those for *P. digitatum*.

Under conditions where *P. italicum* has been controlled, the much less common and relatively newly described species *P. ulaiense* can be a problem. This species is closely related genetically to *P. italicum*, recognizably different by much slower growth on CYA and MEA. Colony appearance is very similar (Holmes *et al*., 1994). Like *P. italicum*, *P. ulaiense* is pathogenic on citrus fruits, especially oranges and lemons. In the short time since its recognition, isolates have come from most citrus growing areas around the world. Most isolates are resistant to imazalil, the chemical of choice for control of *P. italicum*. However, *P. ulaiense* is a weaker pathogen than *P. italicum* (Holmes *et al*., 1994). Growth parameters, so far as they are known, are similar to those of *P. italicum*.

These three species of citrus rotted *Penicillium* species are found only rarely from other food sources. None of these three species is known to produce any mycotoxins.

**Other fruits and vegetables**

Other species of *Penicillium* that cause spoilage of fruits and vegetables are much less important economically than those already described.

*Penicillium brevicompactum* is commonly isolated from a wide range of fruit, where it is a weak pathogen. It has been reported to cause spoilage in stored apples and grapes, mushrooms, cassava and potatoes (Pitt and Hocking, 1997), and ginger (Overy and Frisvad, 2005). It is a slowly growing species on both CYA and MEA, forming dull green colonies, 20–30 and 12–22 mm
diameter, respectively after 7 days at 25°C, and which sometimes show brown exudate and reverse colours. Penicilli are large and terverticillate, typical of subgenus *Penicillium*, borne on smooth stipes. Conidia are ellipsoidal, 2.5–3.5 µm long, with smooth to very finely roughened walls. *P. brevicompactum* grows between −2°C and 30°C with an optimum near 23°C, and at it can grow down to 0.78 a_w, it is one of the more xerophilic Penicillia (Pitt and Hocking, 1997). *P. brevicompactum* produces the weak mycotoxin mycophenolic acid, unlikely to be important in foods. *Penicillium aurantiogriseum* is a broad spectrum weak pathogen on fruits, having been reported to have caused spoilage of a variety of stored fruits and vegetables, including apples, pears, strawberries, grapes, melons, tomatoes, cassava and potatoes (Snowdon, 1990, 1991). Not all such reports have been authenticated, and it is likely that closely related species are responsible for some of these cases. *P. aurantiogriseum* grows slowly on CYA and MEA, producing colonies 30–37 and 24–35 mm diameter after 7 days at 25°C, respectively. Colonies are distinctly blue. Penicilli are terverticillate, typical of subgenus *Penicillium*, and borne on smooth to finely roughened stipes, conidia have smooth walls and are spherical, 3.0–4.0 µm in diameter. Some modern authors (e.g. Samson and Frisvad, 2004) consider that *P. aurantiogriseum* should be separated into several species; however, from the food technologist’s point of view, all have similar physiology. Identification of these as *P. aurantiogriseum* will usually be sufficient. The physiology of *P. aurantiogriseum* is similar to that of many other species classified in subgenus *Penicillium*: growth from about −2 to 30°C, with an optimum near 23°C. It grows down to 0.81 a_w (Pitt and Hocking, 1997). This species, or collection of species, produces several mycotoxins: penicillic acid, roquefortine C and verrucosidin. These are usually of minor significance.

A destructive rot of garlic is caused by *Penicillium allii*, which is otherwise a very uncommon species (Vincent and Pitt, 1989; Snowdon, 1991, as *P. corymbiferum*). It grows quite rapidly on CYA and MEA, producing dull green colonies, often with deep reddish brown pigments in exudate and reverse. Penicilli are terverticillate, borne on long, rough walled stipes. It is not known to produce mycotoxins.

The ubiquitous species *Penicillium chrysogenum* has been reported to cause spoilage of various fruits, but it is not a major pathogen. It is notable that the strain of *P. chrysogenum* originally used for penicillin production came from a spoiled melon.

*Penicillium oxalicum* has been reported to be a major pathogen on yams, to be the main spoilage fungus on cassava, and to cause spoilage of greenhouse cucumbers (Pitt and Hocking, 1997). This species is recognizable by fast, dark green growth on CYA and MEA, with colonies 35–60 and 20–50 mm in diameter, respectively, and a salmon reverse on CYA. This species produces very large numbers of conidia, which leave the colony in sheets if the plate is jarred. Stipes are smooth, and penicilli are terminal biverticillate and large, indicating classification in subgenus *Furcatum* section *Furcatum*. Conidia
are large and ellipsoidal, 3.5–5.0\(\mu\)m long, with smooth walls. Growth occurs between 8 and above 37\(^\circ\)C, perhaps as high as 42 \(^\circ\)C. It grows down to 0.87\(a_w\) (Pitt and Hocking, 1997). \textit{P. oxalicum} produces the mycotoxin secalonic acid D, which may be important in grain dust toxicity (Ehrlich \textit{et al.}, 1982).

16.5.2 \textit{Penicillium} species causing spoilage of cereals

Cereals are a major habitat for \textit{Penicillium} species, particularly those belonging to subgenus \textit{Penicillium}. Most species classified in this subgenus are uncommon in soil or decaying vegetation, major ecological niches for species in other subgenera. However, no real evidence exists that \textit{Penicillium} species invade cereal plants before harvest, so they are considered to be neither pathogens nor commensals, but postharvest invaders. The reason for the affinity with cereals remains unknown.

In European cereals, the most important species is \textit{Penicillium verrucosum}. This is a slowly growing species which produces bright green colonies, measuring 15–25 and 12–15 mm diameter on CYA and MEA, respectively. Colonies often show clear or pale yellow exudate, and a brown reverse. Stipes are robust and rough walled, penicilli are terverticillate, and conidia are spherical, smooth walled and 2.5–3.0\(\mu\)m diameter. \textit{Penicillium verrucosum} does not usually cause obvious spoilage, but it is responsible for the production of ochratoxin A. Tight limits have been set on levels of this mycotoxin in Europe. Recent work has shown that this species invades wheat and barley only after harvest.

Physiologically, \textit{P. verrucosum} is a xerophile, capable of growth down to 0.8 \(a_w\). Its more important physiological parameter, however, is that it grows only below 31 \(^\circ\)C, so that it is confined to cool temperate climates, is unknown in the tropics and rare in warmer temperate zones such as the United States and Australia. In consequence, ochratoxin A occurs in cereals only from Europe and northern North America.

A century ago, ‘yellow rice syndrome’ was important in Japan and other oriental countries. The disease acute cardiac beri beri had been recognized for three centuries and was an often fatal disease of young healthy people. The disease was attributed to the consumption of yellow rice more than 100 years ago (Saito \textit{et al.}, 1971; Uraguchi \textit{et al.}, 1972). The main species associated with this syndrome was \textit{Penicillium citreonigrum}, which produces the very toxic citreoviridin. \textit{P. citreonigrum} produces slowly growing colonies, 20–28 mm diameter on CYA and MEA, and colonies often show yellow soluble pigment and an intensely yellow reverse. This species is classified in subgenus \textit{Aspergilloides}, as it produces small, monoverticillate penicilli on delicate, smooth walled stipes. Conidia are small, spherical and smooth, 1.8–2.8\(\mu\)m diameter. Few studies on physiology have been reported: growth probably occurs from below 5 \(^\circ\)C to 37 \(^\circ\)C, and it may be xerophilic (Pitt and Hocking, 1997). With the banning of the sale of yellow rice in Japan in 1915, the disease vanished. \textit{P. citreonigrum} is now regarded as an uncommon species.
Other *Penicillium* species may have had a role in this syndrome, particularly *P. islandicum*, which produces a battery of mycotoxins, including islanditoxin, cyclochlorotine, luteoskyrin and erythroskyrin. Their role in acute cardiac beri beri is not certain, however. *P. islandicum* is a readily recognized species, as it forms small but deep colonies on CYA and MEA, 17–22 mm diameter, with orange brown mycelium and deep brown reverse colours. This species is classified in subgenus *Biverticillium*, and produces biverticillate penicilli on smooth walled stipes. Conidia are broadly ellipsoidal, 3.0–3.5 µm long. It grows between about 10 and 40°C, with 0.83 $a_w$ as its minimum. *P. citrinum* also occurs on rice, and may have been involved in this syndrome also, as it produces the mycotoxin citrinin, which is bright yellow.

As noted above, a number of other species belonging to subgenus *Penicillium* occur primarily on cereals, and from time to time cause spoilage. For example, spoilage of maize can occur in the United States due to a disease called ‘blue eye’ which results from the growth of *P. aurantiogriseum* on maize harvested damp and cool stored (Ciegler and Kurtzman, 1970). *Penicillium oxalicum* sometimes infects maize preharvest, due to insect damage or wounding.

### 16.5.3 Processed foods

**Refrigerated foods**

Many *Penicillium* species have the ability to grow at low temperatures, even below 0°C, and so are a prime cause of spoilage of refrigerated foodstuffs. A few species also have the ability to grow under reduced oxygen tensions, and so are the major cause of spoilage of packaged, refrigerated goods. Penicillia can be seen on goods in nearly any refrigerator. Perhaps the most vulnerable food of all is cheese, with a generally high water activity, and generally dependent on refrigeration and packaging for microbial stability.

Factors enabling fungi to cause spoilage of cheese include the ability to grow at refrigeration temperature, growth at low oxygen tensions, lipolytic activity and resistance to weak acid preservatives. A number of different species of *Penicillium* possess these properties, and *Penicillium* species are the major cause of cheese spoilage. *P. commune* is the most common cause of spoilage in European cheeses (Lund et al., 1995a). This species produces dull green colonies on CYA and MEA, measuring 30–37 and 23–30 mm in diameter on CYA and MEA respectively, with clear or pale yellow exudate, no soluble pigment and a pale or light brown reverse. Penicilli are large and terverticillate (typical of subgenus *Penicillium*), borne on rough walled stipes. Conidia are relatively large, spherical and smooth, 3.5–4.0(–5.0) µm diameter. *P. commune* is the wild type of the domesticated cheese mould *P. camemberti*, and as such has no doubt been associated with cheeses for centuries.

In Australia, spoilage of cheese by *P. roqueforti* is also common. This species is used in cheese manufacture, but also acts as a significant spoilage
agent on products such as cheddar where mould growth is undesirable. *P. roqueforti* produces rapidly growing, dark green, flat colonies on CYA and MEA, measuring 40–70 mm diameter. Dark green reverse colours are characteristic of this species, as are long stipes with very rough walls, terverticillate penicilli and large, spherical smooth walled conidia, 3.5–4.0 (–6.0) µm diameter. The basic physiology of this species is similar to that of many other species in subgenus *Penicillium* so far as temperature growth ranges and growth at reduced water activity. However, *P. roqueforti* is distinguished by having the lowest growth requirement for oxygen of any *Penicillium* species. It also is uncommonly resistant to weak acid preservatives. This combination means that *P. roqueforti* is a major spoilage fungus in a wide variety of processed foods as well as cheese. It has caused a particular problem with packaged rye bread in Europe, owing to its preservative resistance (Lund *et al*., 1995b).

Cheeses do not usually contain preservatives, but some types and some cheese products, such as cheese spread, contain sorbate. *P. roqueforti* and some other species can cause spoilage by decarboxylating sorbate to trans-1,3-pentadiene, causing a flavour defect known as ‘kerosene’ flavour.

Other species commonly involved in cheese spoilage include *P. chrysogenum*, *P. expansum*, *P. solitum*, *P. verrucosum*, *P. viridicatum* and *P. brevicompactum* (Pitt and Hocking, 1997). All of these species are classified in subgenus *Penicillium*.

The main cheese spoilage *Penicillium* outside subgenus *Penicillium* is *P. glabrum*, a very common species able to spoil a wide range of foods (Pitt and Hocking, 1997). *P. glabrum* produces dark green, low, relatively fast growing colonies on CYA and MEA, 40–50 mm diameter after 7 days at 25 °C, and produces clear to brown exudate and reverse colours. It is classified in the subgenus *Aspergilloides*, as it produces large, terminally swollen monoverticillate penicilli. Conidia are spherical, 3.0–3.5 µm in diameter. *P. glabrum* grows down to 0 °C, and up to about 35 °C. It is probably xerophilic (Pitt and Hocking, 1997). It does not produce mycotoxins.

### High-fat foods and jams

The properties of high-fat foods and jams are quite different, so it is surprising that one *Penicillium* species causes spoilage of both margarine and jam. *P. corylophilum* grows relatively rapidly on both CYA and MEA, 25–35 and 30–45 mm diameter, respectively, forming dull green, flat colonies with little pigmentation other than sometimes a green reverse. Stipes are smooth walled, and penicilli are biverticillate, characteristic of subgenus *Furcatum* section *Furcatum*, and often show metulae of uneven length. Conidia are spherical, smooth walled and 2.5–3.0 µm in diameter. The minimum growth temperature for *P. corylophilum* is near 5 °C, with a maximum below 37 °C. It is a xerophile, capable of growth down to 0.80 a_w (Pitt and Hocking, 1997). This species is not known to produce mycotoxins.
Control of spoilage in margarine is best accomplished by raising the salt content or the addition of permitted preservatives. If jams are hot filled and containers are inverted before cooling, spoilage is rare.

**Pasteurized juices and drinks**

Pasteurization is lethal to conidia (asexual spores) of most kinds of fungi, including *Penicillia*. However, some *Penicillium* species, and some from other closely related genera, produce ascospores (sexual spores), which have much higher heat resistance. Some fungal ascospores can remain viable for appreciable times at 90°C or more, sufficient to survive normal pasteurization processes. Any species producing ascospores is a potential spoilage fungus in pasteurized products. However, most pasteurized products are packaged under oxygen-limiting conditions, so in practice spoilage fungi are limited to those few species with low oxygen requirements.

The principal genus causing spoilage of pasteurized juices is *Byssochlamys*, a small genus rarely seen away from pasteurized products. *Byssochlamys* species produce ascospores on open wefts of hyphae, rather than in the closed bodies typical of sexual stages of *Penicillium* or *Aspergillus*. The asexual stages of *Byssochlamys* species are classified in the genus *Paecilomyces*, which produces conidia in penicilli, rather like those of *Penicillium* but much less structured. Conidia are always elongate, ellipsoidal or cylindrical.

Two species of *Byssochlamys* are important, *B. fulva* and *B. nivea*. *B. fulva* produces colonies on CYA and MEA of at least 60 mm diameter after 7 days at 25°C. Colonies are brown. Ascospores are produced in culture at temperatures only near 30°C, and are formed in uncovered asci on wefts of fine yellow hyphae. Ascospores are smooth, hyaline, ellipsoidal, and 5–7 µm long. Penicilli are large and irregular, and conidia are brown, cylindrical or barrel shaped, 7–10 µm long.

Extensive heat resistance studies indicate that ascospores of *B. fulva* have a $D$ value between 1 and 12 minutes at 90°C with a $z$ value of 6–7°C (Pitt and Hocking, 1997). *B. fulva* also grows at very low oxygen tensions, so occurs in Tetrapacks and similar products from time to time.

On CYA, *B. nivea* grows to 40–50 mm diameter, but on MEA colonies cover the whole Petri dish after 7 days at 25°C. Colonies are white with no other colours. Asci are borne like those of *B. fulva*, and are slightly smaller, 4–6 µm in diameter. Penicilli are less complex than those of *B. fulva*, and conidia are white, ellipsoidal to pear shaped, 3–6 µm long. The physiology of *B. nivea* is similar to that of *B. fulva* with regard to both heat resistance and ability to grow at low $a_w$ (Pitt and Hocking, 1997).

Ecologically, *B. nivea* has usually been reported only from Europe, whereas *B. fulva* has a worldwide distribution. Both of these species occur in foods only as the result of contamination by soil, so fruits such as strawberries, pineapples and other fruits readily contaminated by rain splash, or which are harvested from the ground, are most likely to have a problem with these fungi. Control can be very difficult: laboratory checks for the presence of
heat resistant ascospores, followed by diversion of suspect raw materials to uses other than pasteurized juices, is the route followed by the food industry. It is worth noting that these fungi do not produce ascospores in the food factory: their presence in a product is always an indication of contamination by soil.

16.5.4 Ubiquitous spoilage species

A few Penicillium species are so common in foods that they must be considered to be ubiquitous general agents of spoilage. The most readily recognized of these is P. citrinum. Colonies of P. citrinum grow slowly, especially on MEA, with diameters 25–30 and 14–18 mm, respectively after 7 days at 25°C. In their typical appearance, colonies have yellow exudate, soluble pigment and reverse colours, due to production of copious quantities of citrinin. Penicilli are produced on smooth walled stipes and are biverticillate, typical of subgenus Furcatum section Furcatum, and consist of a well-defined terminal verticil of metulae. Conidia are spherical, smooth walled and 2.5–3.0 µm diameter.

Penicillium citrinum grows from 5 to 38°C, and down to a little above 0.80 aw (Pitt and Hocking, 1997). P. citrinum occurs in almost every kind of food: small grained cereals, maize and wheat flour; nuts, including peanuts, pecans, pistachios and hazelnuts; fermented and cured meats such as hams; cocoa and coffee beans, and other types of beans including soybeans. It is to be expected that low levels of citrinin will often be present in foods. However, P. citrinum does not often cause overt spoilage, so citrinin is not considered to be a serious hazard in foods. It is much more toxic to birds than to mammals, and has caused sickness in poultry from time to time.

A second ubiquitous species is P. chrysogenum. It often occurs with P. citrinum, and is found in the same wide range of foods. However, as mentioned above, it is also a weak pathogen on fresh fruits of several types. P. chrysogenum produces blue-green colonies, on CYA 35–45 mm and on MEA 25–40 mm diameter, and often shows yellow pigments in exudate, soluble pigment and reverse. Penicilli are terverticillate, typical of subgenus Penicillium, and produced on smooth walled stipes. Conidia are smooth walled and ellipsoidal, 2.5–4.0 µm long. This species grows from 4 to 37°C, with an optimum at 23°C. It is a xerophile, germinating down to 0.78 aw (Pitt and Hocking, 1997). No mycotoxins are produced as a rule.

Pitt and Hocking (1997) remarked that P. crustosum ‘had been isolated from the majority of cereal and animal feed samples examined by us over two decades’. They noted that confusion over the correct name for this species had meant that it was seldom mentioned in the older literature. They further noted that ‘our examination of isolates published under a range of names indicates that P. crustosum has been responsible for spoilage of maize, processed meats, cheese, biscuits, cakes and fruit juices’ (Pitt and Hocking, 1997). It is also a weak pathogen on citrus fruits and melons (Snowdon,
Therefore *P. crustosum* can occur in both raw materials and finished products. Like other asexual fungi, spores of *P. crustosum* are not heat resistant, and are inactivated by pasteurization. The occurrence of *P. crustosum* in processed foods may be due to any number of factors, such as inadequate processing, post-processing recontamination, defective packaging, excess water activity, or reliance on refrigeration for stability. *Penicillium crustosum* produces rather rapidly growing dull green, flat colonies, on CYA and MEA measuring 30–40 mm diameter, always heavily sporing, and often with a distinctly granular surface texture. If mature colonies on MEA (7 days old or more) are struck sharply, masses of conidia break off. A member of subgenus *Penicillium*, *P. crustosum* produces large, terverticillate penicilli on long, rough walled stipes. Conidia are large, spherical and smooth walled, 3.0–4.0 µm in diameter. The physiology of this species is little studied, but will be similar to closely related species such as *P. expansum*.

It is important that *P. crustosum* be recognized when it occurs, as it is the major source of penitrem A, a potent neurotoxic mycotoxin. Penitrem given to animals causes sustained trembling in low doses, but is rapidly lethal in higher concentrations. It has occasionally caused sickness in humans (Lewis *et al.*, 2005).

Another ubiquitous species has already been described. *P. glabrum* occurs in a very wide range of foods: it has caused cheese and margarine spoilage, and is found in dried and concentrated foods of all types.

### 16.6 Future trends

As food scientists seek to reduce the use of preservatives, decrease process severity, and extend shelf-life, so the probability of spoilage by *Penicillium* species will continue to increase. Low-temperature storage in the absence of other preservation techniques is inadequate to control the growth of many *Penicillium* species, so the increasing reliance on low temperature for stability of products is ineffective. In the absence of a sexual state, *Penicillium* spores are inactivated by pasteurization, so a heat treatment followed by aseptic packaging is probably the best way to control *Penicillium* spoilage.

A second development is also apparent. The recent trend to revise taxonomy of fungi based on molecular studies will result in an increasing number of *Penicillium* species being named. Taxonomic treatments based on these revisions will often be too complex for the food microbiologist to be able to use, and this threatens to reduce their ability to identify, and attempt to control, Penicillia in foods.

The only logical predictions of the future for *Penicillium* spoilage are that it will not decrease, and that identification is unlikely to become easier for the food microbiologist.
16.7 Conclusions

*Penicillium* species are very common in foods and feeds. As nearly everything is contaminated with *Penicillium* spores, it is important to be able to recognize the difference between incidental contamination and actual spoilage, because spoilage brings with it the potential for mycotoxin production. Fortunately, spoilage by *Penicillium* species is usually readily recognized by visible growth of a single colony type. The most common foodstuffs spoiled by *Penicillium* species are fresh fruits and cheese. In the case of fresh fruit, avoidance of mycotoxin problems is accomplished by rejection of mouldy fruit. For apple juice manufacture, culling of mouldy fruit is important to prevent contamination by patulin. In cheeses, it is considered sufficient to remove 1 cm or so from the surface of the mouldy product before processing further or consumption.

16.8 References


17

Aspergillus and related teleomorphs
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17.1 Introduction

The genus Aspergillus was first described almost 300 years ago and is an important genus in foods, both from the point of view of spoilage, and because many species produce mycotoxins. Although a few species have been used in production of fermented food (e.g. Aspergillus oryzae in soy sauce manufacture), most Aspergillus species occur in foods as spoilage or biodeterioration fungi. They are extremely common in stored commodities such as grains, nuts and spices, and occur more frequently in tropical and subtropical than in temperate climates (Pitt and Hocking, 1997).

Aspergillus species compete with Penicillium and Fusarium species for dominance in foods and food plants. Aspergilli generally grow at higher temperatures or lower water activities than Penicillia. Aspergilli also usually grow more rapidly than Penicillia, although they take longer to sporulate, and produce spores which often are more resistant to light and chemicals. Aspergillus species dominate spoilage in the tropics, whereas in temperate zones, Penicillium species are more common.

Aspergillus is a genus of Hyphomycetes. The fruiting structures are distinctive: the conidiophores have large, heavy walled stipes and swollen apices, termed ‘vesicles’, which are usually roughly spherical. The cells from which spores are formed, phialides, or metulae and phialides, are produced from the vesicle (Fig. 17.1). The simultaneous production of these rows of spore-bearing cells distinguishes Aspergillus from Penicillium, as phialide production in Penicillium and related genera is always successive, not simultaneous.
Two other useful features are characteristic of most *Aspergillus* species. First, stipes are usually formed from a short cell termed a *footcell* within a fertile hypha. Second, stipes are usually nonseptate, so that the vesicle, stipe and footcell all form a very large single ‘cell’. *Penicillium* stipes are usually septate and footcells are very unusual.

### 17.2 Taxonomy

*Aspergillus* is a large genus containing more than 100 recognized species, most of which are easily grown in the laboratory. A recent taxonomy to the most common *Aspergillus* species, including those important in foods, is provided by Klich (2002), although the classic taxonomic reference by Raper and Fennell (1965) is still the most comprehensive, even though some of their concepts are now out of date (Samson and Pitt, 1985, 1990), and many new species have since been described (Pitt and Samson, 1993). A nomenclaturally correct classification for species within the genus *Aspergillus* was proposed by Gams *et al.* (1985), grouping species into six subgenera which are subdivided into sections.

In addition to traditional morphological taxonomic techniques, secondary metabolites (Frisvad 1985, 1989), and molecular techniques (Moody and Tyler, 1990; Mullaney and Klich, 1990; Geiser *et al.*, 1998; Tran-Dinh *et al.*, 2000).

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**Fig. 17.1** A typical head of *Aspergillus ochraceus* showing the structural elements: the stalk (known as the stipe) from which is formed the central vesicle, which in turn produces a layer of cells (metulae) which support the phialides from which conidia are produced.
Aspergillus and related teleomorphs

1999; Schmidt et al., 2003; Abarca et al., 2004; Varga et al., 2004; Esteban et al., 2005, and many others) have been used to clarify relationships within the genus Aspergillus.

17.2.1 Teleomorphs
Teleomorphs are sexual, or perfect, states of fungi. Aspergillus anamorphs (imperfect states) are found in at least eight teleomorphic Ascomycete genera; however only three of these, Eurotium, Neosartorya and Emericella, occur in foods. All form cleistothecia.

Eurotium species (previously known as the ‘Aspergillus glaucus group’) are the most common and significant of the foodborne genera with Aspergillus anamorphs. They produce bright yellow cleistothecia and pale yellow ascospores. Heads producing conidia are formed from phialides only. All species are xerophilic, and are important in the spoilage of low water activity (aw) foods and stored commodities. Neosartorya is the second most common of the Aspergillus teleomorphs found in foods. It also produces heads formed from phialides alone, but cleistothecia are white and ascospores are uncoloured. The ascospores of Neosartorya are heat resistant, so species in this genus are important in spoilage of heat-processed foods particularly fruit products (Pitt and Hocking, 1997). Emericella species are less frequently encountered in foods, and rarely cause spoilage. They produce heads with both metulae and phialides, and white cleistothecia producing red or purple ascospores. The cleistothecia are surrounded by Hülle cells, which are thick walled, highly refractile, roughly spherical cells resembling chlamydoconidia. Growth patterns in Emericella species are similar to those of Neosartorya.

17.2.2 Classification
Aspergillus is classified into subgenera and sections. The separation of these relies primarily on four features: the presence of a teleomorph and its characteristics; the presence or absence of metulae; the arrangement of metulae or phialides on the vesicle; and colony colours. In species without teleomorphs, Aspergillus colony colours are dominated by conidial colour. These colours are consistently associated with particular species.

A number of Aspergillus species are capable of producing mycotoxins and, of these, the aflatoxins are by far the most widely known. Many papers have been published dealing with chemistry, detection, toxicology, production, genetic pathway, occurrence in foods, and regulatory aspects of aflatoxins. The existence of other toxigenic Aspergillus species, particularly those capable of producing ochratoxin A, means that correct identification of isolates from foods and knowledge of the ecology of these moulds is particularly important.
17.3 Significant Aspergillus mycotoxins

Over 40 species of Aspergillus have been listed as capable of producing toxic metabolites (Cole and Schweikert, 2003; Cole et al., 2003), but the Aspergillus mycotoxins of greatest significance in foods and feeds are aflatoxins (produced by Aspergillus flavus, Aspergillus parasiticus and relatively few, less common, species which are not important in foods), ochratoxin A from Aspergillus ochraceus and related species and from Aspergillus carbonarius and occasionally Aspergillus niger, sterigmatocystin, produced primarily by Aspergillus versicolor but also by Emericella species, and cyclopiazonic acid (A. flavus is the primary source, but it is also reported to be produced by Aspergillus tamarii). Citrinin, patulin and penicillic acid may also be produced by certain Aspergillus species, and tremorgenic toxins are produced by Aspergillus terreus (territrems), Aspergillus fumigatus (fumitremorgens) and Aspergillus clavatus (tryptoquivaline) (Hocking, 2001).

Toxins of Aspergillus species exhibit a wide range of toxicities, with the most significant effects being long term. Aflatoxin B₁ is the most potent liver carcinogen known for a wide range of animal species, including humans. Ochratoxin A and citrinin both affect kidney function. Cyclopiazonic acid has a wide range of effects (Cole, 1986), and tremorgenic toxins such as territrems affect the central nervous system. Table 17.1 lists the most significant toxins produced by Aspergillus species and their toxic effects.

17.4 Isolation, enumeration and identification

Techniques for the isolation and enumeration of Aspergillus species from foods are the same as those used for other foodborne fungi and have been described in detail in Pitt and Hocking (1997) and Samson et al. (2004a). Antibacterial media containing compounds to inhibit or reduce spreading growth of moulds, such as dichloran rose bengal chloramphenicol (DRBC) agar or dichloran 18% glycerol (DG18) agar (Pitt and Hocking, 1997) are recommended for enumerating fungi in foods (Samson et al., 1992; Hocking et al., 2006). There is one medium, Aspergillus flavus and parasiticus agar (AFPA), designed specifically for detection of potentially aflatoxigenic species (Pitt et al., 1983; Pitt and Hocking, 1997).

Keys and descriptions of the most common foodborne Aspergillus species can be found elsewhere (Pitt and Hocking, 1997; Klich 2002; Samson et al., 2004a). Identification of Aspergillus species requires growth on media developed for this purpose, including Czapek agar, a defined medium based on mineral salts, or a derivative such as Czapek yeast extract agar (CYA), and malt extract agar. Growth on Czapek yeast extract 20% sucrose agar (CY20S) can be a useful aid in identifying species of Aspergillus (Pitt and Hocking, 1997).
Unlike *Penicillium* species, *Aspergillus* species are conveniently ‘colour-coded’, and the colour of the conidia can be a very useful starting point in identification, at least to the Section level. As well as conidial colour, microscopic morphology to determine the presence of phialides only, or metulae plus phialides, and shape and size of vesicle, etc., is important in identification. Correct identification of *Aspergillus* species is an essential prerequisite to assessing the potential for spoilage and mycotoxin contamination in a commodity, food, or feedstuff.

The *Aspergillus* species, including teleomorphic species, that are commonly found in foods, are described in detail below.

### 17.5 Teleomorphic genera with *Aspergillus* anamorphs

#### 17.5.1 Genus *Emericella* Berk

*Emericella* forms white cleistothecia surrounded by Hüle cells (thick walled refractile cells like chlamydoconidia) and produces purple ascospores.

#### Table 17.1 Significant mycotoxins produced by *Aspergillus* species and their toxic effects (from Hocking 2001)

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Toxicity</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt; and B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Acute liver damage, cirrhosis, carcinogenic (liver), teratogenic</td>
<td><em>A. flavus,</em> A. parasiticus, A. nomius</td>
</tr>
<tr>
<td>Aflatoxin G&lt;sub&gt;1&lt;/sub&gt; and G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Similar effects to B aflatoxins: G&lt;sub&gt;1&lt;/sub&gt; toxicity less than B&lt;sub&gt;1&lt;/sub&gt; but greater than B&lt;sub&gt;2&lt;/sub&gt;</td>
<td><em>A. parasiticus,</em> A. nomius</td>
</tr>
<tr>
<td>Cyclopiazonic acid</td>
<td>Degeneration and necrosis of various organs, tremorgenic, low oral toxicity</td>
<td><em>A. flavus,</em> A. tamarii</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>Kidney necrosis (especially pigs), teratogenic, immunosuppressive, probably carcinogenic</td>
<td><em>A. ochraceus</em> and related species, A. carbonarius, A. niger (occasional)</td>
</tr>
<tr>
<td>Sterigmatocystin</td>
<td>Acute liver and kidney damage, carcinogenic (liver)</td>
<td><em>A. versicolor,</em> Emericella spp.</td>
</tr>
<tr>
<td>Fumitremorgens</td>
<td>Tremorgenic (rats and mice)</td>
<td><em>A. fumigatus</em></td>
</tr>
<tr>
<td>Territrems</td>
<td>Tremorgenic (rats and mice)</td>
<td><em>A. terreus</em></td>
</tr>
<tr>
<td>Tryptoquivalines</td>
<td>Tremorgenic</td>
<td><em>A. clavatus</em></td>
</tr>
<tr>
<td>Cytochalasins</td>
<td>Cytotoxic</td>
<td><em>A. clavatus</em></td>
</tr>
<tr>
<td>Echinulins</td>
<td>Feed refusal (pigs)</td>
<td><em>Eurotium chevalieri,</em> E. amstelodami</td>
</tr>
</tbody>
</table>

Unlike *Penicillium* species, *Aspergillus* species are conveniently ‘colour-coded’, and the colour of the conidia can be a very useful starting point in identification, at least to the Section level. As well as conidial colour, microscopic morphology to determine the presence of phialides only, or metulae plus phialides, and shape and size of vesicle, etc., is important in identification. Correct identification of *Aspergillus* species is an essential prerequisite to assessing the potential for spoilage and mycotoxin contamination in a commodity, food, or feedstuff.

The *Aspergillus* species, including teleomorphic species, that are commonly found in foods, are described in detail below.
Conidiophores usually have short brown stipes, bear both metulae and phialides, and produce columns of dark green conidia. Only one species, *E. nidulans*, is common in foods.

**Emericella nidulans** *(Eidam) Vuill.* **Anamorph:** Aspergillus nidulans *(Eidam) G. Winter*

*Emericella nidulans* (Figure 17.2e) colonies may be dark grass green if conidial heads are abundantly formed, or creamish if cleistothecia and Hülle cells are present. Violet or pink soluble pigment may be produced, and the reverse of the colonies is usually brightly coloured in shades of pink, orange, brown or violet brown. This species grows rapidly at 37°C, and has occasionally been reported to be pathogenic to humans (Horre *et al.*, 2002) and is the cause of guttural pouch mycosis in horses (Kosuge *et al.*, 2000; Cabanes *et al.*, 2002).

**Ecology**

*Emericella nidulans* is not particularly common in foods, and has not been implicated in actual spoilage, but has been isolated from a wide variety of sources. The most common reports are from cereals and cereal products (wheat, flour and bread, barley, rice, maize and sorghum), nuts (peanuts, hazelnuts), dried beans and spices (see Pitt and Hocking, 1997).

**Physiology**

*Emericella nidulans* grows between 6–8°C and 46–48°C or even 51°C, with an optimum at 35–37°C (Panasenko, 1967; Lacey, 1980). The minimum $a_w$ for germination is 0.80 $a_w$ at 37°C (Ayerst, 1969), so this species is both marginally thermophilic and xerophilic, a most unusual combination. The heat resistance of *E. nidulans* ascospores does not appear to have been studied, but we have not encountered any instances of spoilage in heat processed foods by this species.

**Mycotoxins**

*Emericella nidulans* has been reported to produce sterigmatocystin, a mycotoxin more commonly associated with *Aspergillus versicolor* (Horie *et al.*, 1989; Kawahara *et al.*, 1994) and emestrin (Terao *et al.*, 1988, 1990). Emestrin is highly toxic, with an LD$_{50}$ of 13 mg/kg when given to mice intraperitoneally (Terao *et al.*, 1988). Natural occurrence of mycotoxins in foods contaminated by this species has not been reported.

17.5.2 Genus *Eurotium* Link: Fr.

*Eurotium* is widespread and a very well-known genus of Ascomycetes, forming yellow cleistothecia with smooth, cellular walls, often encrusted in red, orange or brown hyphae. The *Aspergillus* anamorphs have heads with phialides only, producing dull grey-green, spinose conidia. All species of *Eurotium*
Fig. 17.2 Sporing structures of some common foodborne *Aspergillus* species: (a) *A. carbonarius*; (b) *A. flavus*; (c) *A. fumigatus*; (d) *A. clavatus*; (e) *A. nidulans*; (f) *A. terreus*. 
Food spoilage microorganisms

are xerophilic, so grow and sporulate best on reduced $a_w$ media such as Czapek yeast extract agar with 20% sucrose (CY20S; Pitt and Hocking, 1997). This medium (0.98 $a_w$) encourages development of both anamorphs and teleomorphs (ascospores) plus mycelial colours which are used for species identification.

Four species, *Eurotium amstelodami*, *E. chevalieri*, *E. repens* and *E. rubrum*, are exceedingly common in all kinds of reduced $a_w$ environments. Less commonly encountered are *E. herbariorum*, which resembles *E. rubrum*, and *E. cristatum*, which resembles *E. amstelodami*. All four common *Eurotium* species may be isolated from a similar range of commodities in which they are often the principal spoilage organisms: stored cereals and cereal products, nuts, spices, dried meat and fish products, dried fruits, bakery goods, cheese and jam (see Pitt and Hocking, 1997). The common *Eurotium* species generally grow optimally between 0.90 and 0.96 $a_w$, and their minimum $a_w$ for growth is in the region 0.70–0.74. They are mesophiles, growing best between 25–30°C, with temperature maxima just over 40°C (see Pitt and Hocking, 1997).

*Eurotium repens* and *E. rubrum* have been used as starter cultures in the manufacture of katsuobushi from bonito (Dimici and Wada, 1994), and *E. repens* has been used for production of fish sauce from fish meal (Hayakawa *et al.*, 1993).

*Eurotium* species produce chloroanisoles which may cause off-odours in food carried in contaminated shipping containers (Hill *et al.*, 1995), isoprene, a cause of off-odour in a bakery product (Berenguer *et al.*, 1991), ketonic rancidity in coconut (Kinderlerer, 1984; Kinderlerer and Kellard, 1984), proteolytic activity on meat (Binzel, 1980) and lipolytic activity on vegetable oils (Kuku, 1980). They may be relatively resistant to weak acid preservatives such as propionic acid and sorbate (Viñas *et al.*, 1990; Guynot *et al.*, 2002).

A high percentage (80–100%) of *Eurotium* ascospores survive heating at 60°C for 10 min, at $a_w$ 0.98 and pH 3.8, although at 70°C for 10 min only 0.5–25% survive, and at 75°C for 10 min, most ascospores are inactivated (Pitt and Christian, 1970). However, *E. chevalieri* appears to be slightly more heat resistant than the other species, with up to 0.5% surviving 10 min at 80°C (Pitt and Christian, 1970). Conidia of these species are much less heat resistant: only 8% survived 10 min at 50°C, 3% 10 min at 60°C and none at 70°C under the same conditions (Pitt and Christian, 1970). Ascospores are also more resistant than conidia to high pressure (Karatas and Ahi, 1992), microwaves (Dragoni *et al.*, 1990) and smoke (Kersken, 1974).

The four species are distinguished by their ascospore size and ornamentation, and the colours of the hyphae enveloping the cleistothecia, features which are best seen on CY20S agar after 10–14 days incubation at 25°C. They also vary slightly in their physiology and biochemistry.
Eurotium amstelodami L. Mangin, Anamorph: Aspergillus vitis Novobr.

Physiology
The minimum $a_w$ for growth has been reported as 0.70 at 25 °C (Armolik and Dickson, 1956), or 0.75 $a_w$ (Wheeler and Hocking, 1988), with an optimal $a_w$ near 0.96 (Scott, 1957), or 0.90 to 0.93 $a_w$ (Avari and Allsopp, 1983). *Eurotium amstelodami* grows optimally at 33–35 °C (Domsch *et al*., 1980), with a maximum at 43–46 °C (Blaser, 1975).

Mycotoxins
No known toxins have been identified from *Eurotium amstelodami*.

Identification
Ascospores of *Eurotium amstelodami* have wide, irregular ridges or flanges and rough walls. Colony colours consist only of yellow from the cleistothecia and dull green from the conidia.

Eurotium chevalieri L. Mangin, Anamorph: Aspergillus chevalieri L. Mangin

Physiology
*Eurotium chevalieri* grows optimally at 30–35 °C (Domsch *et al*., 1980) and 0.94–0.95 $a_w$ (Pitt and Hocking, 1977). Its maximum temperature is 40–43 °C (Blaser, 1975), and minimum $a_w$ is near 0.71 at 33 °C (Ayerst, 1969), although a higher minimum $a_w$ (0.74 $a_w$ at 25 °C at pH 3.8) was reported by Pitt and Christian (1968).

Mycotoxins
*Eurotium chevalieri* has been reported to produce echinulin and neoechinulin, which cause feed refusal in swine (Vesonder *et al*., 1988). However, other tests for toxicity of *Eurotium chevalieri* have been negative (Frisvad and Samson, 1991; Adebajo and Oyesiku, 1994).

Identification
The cleistothecia of *E. chevalieri* are enveloped in yellow to orange vegetative hyphae, and produce distinctive ascospores shaped like pulley wheels, 4.5–5.0 µm long, smooth walled, with two prominent, parallel, sometimes sinuous, longitudinal flanges.

Eurotium repens de Bary, Anamorph: Aspergillus reptans Samson & W. Gams

Physiology
*Eurotium repens* grows from 4–5 °C to 38–40 °C, with an optimum near 25–27 °C (Panasoniko, 1967; Gonzalez *et al*., 1988). The minimum $a_w$ for
germination of E. repens may be as low 0.69 $a_w$ in glucose/fructose media (Andrews and Pitt, 1987), or 0.72 $a_w$ on media of neutral pH, at temperatures of 20 to 25°C (Snow, 1949; Armolik and Dickson, 1956; Magan and Lacey, 1984a) with optimal growth between 0.90 and 0.95 $a_w$ (Andrews and Pitt, 1987).

Mycotoxins
Mycotoxins are not known to be produced by this species (Frisvad and Samson, 1991).

Identification
Eurotium repens is distinguished from other Eurotium species by its smooth walled ascospores that have no ridges or flanges, and usually have no longitudinal furrow. Hyphal and reverse colours are yellow to orange, never red.


Physiology
Growth temperatures for Eurotium rubrum are probably similar to those reported for E. repens. The minimum $a_w$ for germination has been reported as 0.70–0.73 $a_w$ (Snow, 1949; Armolik and Dickson, 1956; Wheeler et al., 1988), with an optimum $a_w$ for growth near 0.94 $a_w$ (Avari and Allsopp, 1983).

Mycotoxins
Several reports have been published which indicate that Eurotium rubrum produces a range of toxic compounds. However well-documented confirmation of toxicity is still lacking (Frisvad and Samson, 1991).

Identification
Eurotium rubrum colonies on CY20S usually show areas of brilliant red or rusty colours after 10 days or more incubation. Ascospores are 5.0–6.0 µm long, and show a definite longitudinal furrow and low, minutely roughened ridges.

Eurotium herbariorum Link, Anamorph: Aspergillus glaucus Link
This resembles E. rubrum in many features, and although far less common than Eurotium rubrum, is nevertheless widespread. E. herbariorum is more xerophilic, and produces larger ascospores (commonly 6–8 µm) than E. rubrum. On media of pH 3.8 containing glucose/fructose as the controlling solute, ascospores of E. herbariorum germinated at 0.74 $a_w$ after 19 days, the shortest lag time of any of the common species. Conidia of Aspergillus glaucus germinated at 0.75 $a_w$ in 14 days (Pitt and Christian, 1968). E. herbariorum ascospores are more heat resistant than the common, smaller-spored Eurotium
species. When heated in 5° Brix grape juice, *E. herbariorum* ascospores showed a $D_{70}$ of 2.5 min and a $z$ value of 9.1°C; in 65° Brix concentrate, the $D_{70}$ was 5.2 min and $z$ value 7.1°C (Splittstoesser et al., 1989).

### 17.5.3 Genus *Neosartorya* Malloch & Cain

*Neosartorya* produces cleistothecia with cellular walls like *Eurotium*, however, the walls and ascospores are colourless or white, not yellow. Like those of *Eurotium*, *Neosartorya* anamorphs produce vesicles bearing phialides only, but vesicles are small and pyriform (pear-shaped), enlarging towards the apices. About 10 species and varieties are currently accepted (Malloch and Cain, 1972; Kozakiewicz, 1989), which mainly inhabit soil and decaying vegetation. All are thermoduric or thermophilic, none is a xerophile. The well-known human pathogen *Aspergillus fumigatus* is closely related (Girardin et al., 1995), and occasional pathogenicity to humans has been reported. *Neosartorya* isolates should be handled with care.

The main importance of *Neosartorya* species in food spoilage is the very high heat resistance of their ascospores. *N. fischeri* is the main *Neosartorya* species significant in foods.

*Neosartorya fischeri* (Wehmer) Malloch & Cain

**Ecology**

*Neosartorya fischeri* was reported from cans of strawberries which had been opened and incubated at 25°C for 5 days, and this was the first indication that this species was a potential problem in acid canned foods (Kavanagh et al., 1963). No visible spoilage had occurred, although spores had withstood the canning process of 12 min at 100°C. This species had been isolated from canned strawberries in Ireland for 9 years out of 10 between 1958 and 1968, despite increases in the length and severity of the canning process used (McEvoy and Stuart, 1970). In our laboratory, *N. fischeri* has been isolated on numerous occasions from heat-treated strawberry purée and also from spoiled canned strawberries, and a sports drink. It was the most common species isolated in a survey of heat-resistant fungi in fresh Australian strawberries (Hocking, unpublished). Spotti et al. (1992) also found *N. fischeri* in heat-treated fresh fruit. Isolation of *Neosartorya fischeri* from pasteurized fruit juices (Jesenska and Petrikova, 1985; Scott and Bernard, 1987) and fruit powders (Beuchat, 1992) has been reported several times, but only occasionally from spoiled product (Splittstoesser and Splittstoesser, 1977).

*Neosartorya fischeri* is rarely reported from foods which have not been heat treated or processed.

**Physiology**

Ascospores of this species are very heat resistant. Kavanagh et al. (1963) reported that ascospores of an isolate more recently identified as *Neosartorya*
Neosartorya fischeri withstood boiling in distilled water for 60 min. More detailed studies of the heat resistance of Neosartorya fischeri ascospores indicate $D_{88^\circ C}$ values of 1.2–7.5 min (Quintavalla and Spotti, 1993), 4.2–16.2 min (Beuchat, 1986) or 1.4 min with a $z$ value of 5.6 $^\circ C$ (Scott and Bernard, 1987), indicating a higher heat resistance than Byssoschlamys fulva or B. nivea (Beuchat, 1986). A $D_{86^\circ C}$ value of 8 min, with a $z$ value of 6.9 $^\circ C$ in phosphate buffer at pH 7 has also been reported (Suresh et al., 1996). Milder heat treatments, e.g. 15 min at 75–80 $^\circ C$, cause activation of ascospores (Suresh et al., 1996). A mathematical model for the combined effect of $a_w$, pH and redox potential on the heat resistance of N. fischeri has been published (Reichart and Mohacsi-Farkas, 1994).

High-pressure treatments (600–900 MPa) for 20 min at 20 $^\circ C$ in apricot nectar reduced counts of Neosartorya fischeri 100-fold. At 50 $^\circ C$, inactivation required less than 4 min at 800 MPa, and at 60 $^\circ C$, 1–2 min at 700 MPa. Pressure resistance in distilled water was lower (Maggi et al., 1994). In our laboratory, we have observed activation of Neosartorya ascospores by pressure treatment of 600 MPa for 10 min at ambient temperature. Pressure resistance of N. fischeri ascospores varied with age, with older ascospores (7–15 weeks) showing greater resistance than younger ascospores (3–7 weeks) (Chapman et al., in press). Growth has been reported in O$_2$ levels as low as 0.1% at 25 $^\circ C$ (Nielsen et al., 1989).

Neosartorya fischeri is used to produce acid proteases which are widely used in cheese-making, baking and meat tenderization (Wu and Hang, 2000).

Mycotoxins and pathogenicity
This species produces fumitremorgens A and C and verruculogen (Beuchat et al., 1988; Nielsen et al., 1988; Frisvad and Samson, 1991). However, these compounds have not been reported in food. N. fischeri has been reported as an agent of pulmonary aspergillosis in a liver transplant recipient (Gori et al., 1998). In view of its close affinity with Aspergillus fumigatus, and its strong growth at 37 $^\circ C$, perhaps this is not surprising.

Identification
Colonies of Neosartorya fischeri spread rapidly at both 25 and 37 $^\circ C$, and are white; white cleistothecia and inconspicuous grey green Aspergillus heads are produced. The Aspergillus heads have pyriform vesicles bearing phialides only, with small, smooth-walled conidia. The ascospores are distinctive: ellipsoidal, 7–8 $\mu$m long, with two prominent longitudinal flanges, which may appear as crests under the microscope. The ascospore walls may be smooth, irregularly roughened or sometimes spinose.

17.6 Genus Aspergillus Fr.: Fr.
The species of Aspergillus that cause spoilage in, or are regularly isolated from, food are described below. For simplicity, the species are dealt with in alphabetical rather than taxonomic order.
17.6.1 **Aspergillus aculeatus** Iizuka

*Aspergillus aculeatus* produces dark brown to black conidia. It belongs in Section *Nigri*, and is closely related to *A. niger*. It is distinguished from *A. niger* by *Aspergillus* heads bearing only phialides.

**Ecology**

*Aspergillus aculeatus* causes post-harvest dry rot of tomatoes (Fajola, 1979) and, with other members of Section *Nigri*, is involved in *Aspergillus* bunch rot of grapes (Jarvis and Traquair, 1984; Leong *et al.*, 2004). It has been isolated in our laboratory from both fresh and dried grapes.

**Taxonomy**

*Aspergillus aculeatus* has sometimes been considered to be a variety of *A. japonicus* (Al-Musallam, 1980), but is considered distinct due to consistent differences in morphology and secondary metabolite production.

**Physiology**

*Aspergillus aculeatus* grows between about 10 and 42 °C, with an optimum near 30 °C (Leong *et al.*, 2004). It has a high pectinolytic enzyme activity (Adisa, 1989).

**Mycotoxins**

This species produces secalonic acid D (Anderson *et al.*, 1977) and some other minor compounds (Frisvad and Samson, 1991). Secalonic acid D has significant animal toxicity (Ciegler *et al.*, 1980), but a role in human or animal disease, especially from this species, has not been shown.

**Identification**

Colonies are dark brown to black. *Aspergillus* heads of *A. aculeatus* bear phialides only. It is distinguished from *A. japonicus*, which also produces phialides only, by its larger vesicles and ellipsoid conidia. The conidia are ellipsoid, sometimes subspherical, 4–5 µm long, spinose, borne in radiate heads.

17.6.2 **Aspergillus candidus** Link

*Aspergillus candidus* is the only species of *Aspergillus* with persistently white conidia. It is readily distinguished from all species other than *A. niveus*. It differs from *A. niveus* by producing vesicles fertile over the entire area and metulae usually more than 10 µm long. The conidia are mostly spherical, 2.5–3.5 µm diam, with smooth walls, borne in radiate heads.

**Ecology**

*Aspergillus candidus* occurs commonly in stored cereals, particularly wheat, and cereal products such as flour and bread. It has also been isolated from
milled rice (it was particularly common in samples from Indonesia and the Philippines; Pitt et al., 1998), and various nuts including peanuts, hazelnuts, walnuts and pecans. *A. candidus* frequently occurs on salamis and other processed meats and dried fish (see Pitt and Hocking, 1997).

*Aspergillus candidus* has been recommended as a starter culture of low toxicity for processed meat manufacture (Grazia et al., 1986; Spotti et al., 1994).

**Physiology**
A range of growth temperatures has been reported for *A. candidus*, perhaps reflecting that more than one species is encompassed by this name. Tansey and Brock (1978) reported an optimum at 45–50°C, with a maximum of 50–55°C, whereas Panasenko (1967) reported a minimum, optimum and maximum near 3–4, 20–24 and 40–42°C respectively, similar to those reported by Domsch et al. (1980) (11–13, 25–28 and 41–42°C). We have recently studied a group of isolates from tropical dried fish, clearly identifiable as *A. candidus*, unable to grow at 37°C.

*Aspergillus candidus* is a xerophile, with a minimum $a_w$ for growth of 0.75 and an optimum greater than 0.98 (Ayerst, 1969). *A. candidus* is more tolerant of low $O_2$ than most *Aspergillus* species, being capable of growth in 0.45% $O_2$ (Magan and Lacey, 1984b). It is also tolerant to propionic acid used as a grain preservative (Müller et al., 1981).

**Mycotoxins**
*Aspergillus candidus* produces a range of secondary metabolites (Frisvad and Samson, 1991), but of these only kojic acid has significant toxicity. Its common occurrence in grain dust may pose a respiratory hazard (Krysinska-Traczyk and Dutkiewicz, 2000).

17.6.3 *Aspergillus carbonarius* (Bain.) Thom

*Aspergillus carbonarius* (Fig. 17.2a), a member of Section *Nigri*, has only come to prominence in the past 10 years or so because of its ability to produce ochratoxin A in grapes (Abarca et al., 1994). *A. carbonarius* is distinguished from the more common *A. niger* by its larger, blacker, conidia and generally larger heads. It can also be distinguished by molecular techniques (Esteban et al., submitted).

**Ecology**
Most recent reports of *A. carbonarius* relate to its occurrence in grapes and its role in production of ochratoxin A (see Leong et al., 2004), although it was first reported as a cause of *Aspergillus* bunch rot in grapes by Gupta (1956). It has also been reported from coffee beans where it may also be a source of ochratoxin A (Taniwaki et al., 2003).
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Physiology
Aspergillus carbonarius grows between 10 and 42°C, with a maximum near 30–35°C (Leong et al., 2004; Mitchell et al., 2004; Belli et al., 2005). The optimum \( a_w \) range for growth is 0.93–0.98, depending on strain (Mitchell et al., 2004; Belli et al., 2005). However, the optimum temperature for ochratoxin A production is lower than for growth, around 15–20°C (Esteban et al., 2004; Leong, 2005).

Mycotoxins
Aspergillus carbonarius produces ochratoxin A, and is the primary source of this toxin in grapes and grape products (wine, grape juice, wine vinegar, dried grapes) (see Leong et al., 2004; Leong, 2005).

17.6.4 Aspergillus clavatus Desm.
Aspergillus clavatus (Fig. 17.2d) produces blue grey colonies. Its large, long, clavate (club-shaped) vesicles bearing only phialides are distinctive.

Ecology
Aspergillus clavatus is particularly common in barley during malting, and can build to unacceptably high levels if malting temperatures are elevated or spontaneous heating occurs (Flannigan et al., 1984; Flannigan, 1986). In extreme cases blue green mats of A. clavatus may form on grain during malting (Shlosberg et al., 1991). Under these conditions it may produce mycotoxins and can be allergenic. It is reported to be the cause of ‘malt workers’ lung’ (Riddle et al., 1968; Flannigan, 1986). It is mostly associated with cereals, and has been reported from wheat, flour, bread and maize, but occasionally from other sources (see Pitt and Hocking, 1997).

Physiology
Aspergillus clavatus grows optimally near 25°C, with a minimum of 5–6°C, and a maximum near 42°C (Panasenko, 1967; Northolt et al., 1978). The minimum reported \( a_w \) for growth is 0.88–0.87 (Northolt et al., 1978).

Mycotoxins
This species produces patulin (Frisvad and Samson, 1991), which has the potential to cause ill health in animals (Gilmour et al., 1989; Shlosberg et al., 1991) and may have harmful effects in humans. Aspergillus clavatus also produces tryptoquivalones, toxins isolated from a mouldy rice sample implicated in death of a child (Büchi et al., 1977) and cytochalasin E (Glinsukon et al., 1974). Some of the effects of these toxins seen in laboratory animals are similar to those observed in farm animals. Outbreaks of mycotoxicoses associated with A. clavatus have been reported in stock fed on culms from distillery maltings in Europe, the United Kingdom, and South Africa (Flannigan and Pearce, 1994).
17.6.5  *Aspergillus flavus* Link, *A. parasiticus* Speare and *A. oryzae* (Ahルン) Cohn

Undoubtedly, the most important group of toxigenic Aspergilli are the aflatoxinogenic fungi *A. flavus* (Fig. 17.2b), *A. parasiticus* and the recently described but much less common species *A. nomius*, all of which are classified in *Aspergillus* Section *Flavi* (Gams et al., 1985). *Aspergillus flavus* and *A. parasiticus* are distinguished by their bright yellow green (or less commonly yellow) conidal colour and rapid growth at both 25 and 37°C. *A. flavus* produces conidia which are rather variable in shape and size, with relatively thin, smooth to moderately rough, walls, with most being finely roughened. Conidia of *A. parasiticus* are spherical and have relatively thick, rough walls. In addition, vesicles of *A. flavus* are larger, up to 50μm in diameter, and usually bear metulae, while vesicles of *A. parasiticus* rarely exceed 30μm in diameter and metulae are uncommon.

The suite of toxins produced by these three species is species-specific (Klich and Pitt, 1988; Kozakiewicz, 1994). *A. flavus* can produce aflatoxins B₁, B₂ and cyclopiazonic acid (CPA), but only a proportion of isolates are toxigenic. *A. parasiticus* produces aflatoxins B₁, B₂, G₁ and G₂, but not cyclopiazonic acid, and almost all isolates are toxigenic. *A. nomius* is morphologically similar to *A. flavus*, but like *A. parasiticus*, produces B and G aflatoxins without cyclopiazonic acid.

*Aspergillus flavus* and *A. parasiticus* are closely related to *A. oryzae* and *Aspergillus sojae*, species that are used in the manufacture of fermented foods and do not produce toxins (Egel et al., 1994). Accurate differentiation of related species within Section *Flavi* is important in order to determine the potential for toxin production, and the types of toxins likely to be present.

Detection and identification

Rapid detection of aflatoxinogenic fungi is possible using Aspergillus flavus and parasiticus agar (AFPA) (Pitt and Hocking, 1997), a medium formulated specifically for this purpose. When incubated on AFPA at 30°C for 48–72h, *A. flavus*, *A. parasiticus* and *A. nomius* produce a bright orange-yellow colony reverse which is diagnostic and readily recognized. *A. flavus* and related species also grow well on general purpose yeast and mould enumeration media such as DRBC agar or DG18 agar, and are relatively easily recognized. Screening isolates for aflatoxin production can also be used to differentiate the species. Cultures can be grown on coconut-cream agar and observed under ultraviolet light (Dyer and McCammon, 1994) or a simple agar plug technique coupled with thin layer chromatography (Filtenborg et al., 1983) can be used to screen cultures for aflatoxin production as an aid to identification. The combination of characteristics most useful in differentiation between the three aflatoxinogenic species are summarized in Table 17.2.

Ecology

*Aspergillus flavus* is cosmopolitan, but *A. parasiticus* is less widespread.
A. flavus and A. parasiticus have a strong affinity with nuts and oilseeds. In a survey of the mycoflora of commodities in Thailand (Pitt et al., 1993, 1994), A. flavus was one of the most commonly occurring fungal species in nuts and oilseeds, but A. parasiticus was rarely encountered. Corn, peanuts and cottonseed are the most important crops invaded by these fungi, and invasion often takes place before harvest, rather than during storage. Peanuts may be invaded while still in the ground if the crop suffers drought stress or related factors (Sanders et al., 1981; Cole et al., 1982; Pitt et al., 1991). In corn, insect damage to developing kernels allows entry of aflatoxigenic moulds, but invasion can also occur through the silks of developing ears (Lillehoj et al., 1980). Cotton seeds are invaded through the nectaries (Klich et al., 1984).

Cereals and spices are common substrates for A. flavus (Pitt and Hocking, 1997), but aflatoxin production in these commodities is almost always a result of poor drying, handling, or storage, and aflatoxin levels are rarely significant. Significant amounts of aflatoxins can occur in peanuts, corn, and other nuts and oilseeds, particularly in some tropical countries where crops may be grown under marginal conditions, and where drying and storage facilities are limited (Arim, 1995; Dhavan and Choudary, 1995; Lubulwa and Davis, 1994).

**Physiology**

*Aspergillus flavus* has been reported to have a minimum temperature for growth near 10–12 °C, a maximum near 43–48 °C, and an optimum near 33 °C, with aflatoxins being produced from 12–40 °C (Diener and Davis, 1967; Northolt et al., 1977; Domsch et al., 1980; ICMSF, 1996). Its optimal $a_w$ for growth is 0.996 (Gqaleleni et al., 1997), with a minimum $a_w$ for growth variously reported as 0.78 $a_w$ at 33 °C (Ayerst, 1969) and 0.82 at 25 °C, 0.81 at 30 °C and 0.80 at 37 °C (Pitt and Miscamble, 1995). A predictive model for A. flavus growth in relation to $a_w$ and temperature has been published (Gibson et al., 1994). Growth of A. flavus occurred over the pH range 2.1 to 11.2 (the entire range examined) at 25, 30 and 37 °C, with optimal growth

<table>
<thead>
<tr>
<th>Species</th>
<th>Conidia</th>
<th>Sclerotia</th>
<th>Toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. flavus</em></td>
<td>Smooth to moderately roughened,</td>
<td>Large, globose</td>
<td>Aflatoxins B$_1$ and B$_2$,</td>
</tr>
<tr>
<td></td>
<td>variable in size</td>
<td></td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td><em>A. parasiticus</em></td>
<td>Conspicuously roughened, little</td>
<td>Large, globose</td>
<td>Aflatoxins B and G</td>
</tr>
<tr>
<td></td>
<td>variation in size</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. nomius</em></td>
<td>Similar to</td>
<td>Small, elongated (bullet-shaped)</td>
<td>Aflatoxins B and G</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
over a broad range from pH 3.4–10 (Wheeler et al., 1991). Conidia of *Aspergillus flavus* are not heat resistant, with a \( D_{60^\circ C} \) of 1 min at neutral pH and high \( a_w \), with \( z \) values from 3.3 to 4.1°C (ICMSF, 1996).

**Mycotoxins**

*Aspergillus flavus* is the main source of aflatoxins, the most important mycotoxins in the world’s food supplies. Aflatoxins are produced in foods only by *A. flavus* and *A. parasiticus*. Although *A. nomius* and a few other *Aspergillus* species can produce aflatoxins, they do not occur in food. The four major naturally produced aflatoxins are known as aflatoxins B₁, B₂, G₁ and G₂, with ‘B’ and ‘G’ referring to the blue and green fluorescent colours produced under UV light. Aflatoxins are both acutely and chronically toxic to animals, including man. They produce four distinct effects: acute liver damage; liver cirrhosis; induction of tumours; and teratogenic effects (Stoloff, 1977).

More detailed information about aflatoxins and their effects can be found elsewhere (see Pitt and Hocking, 1997; Hocking, 2001). Because of their high toxicity, low limits for aflatoxins in foods and feeds have been set by many countries (van Egmond and Jonker, 2004).

Cyclopiazonic acid is produced by some strains of *Aspergillus flavus*. CPA is an indole tetramic acid which can occur in naturally contaminated agricultural commodities and compounded animal feeds. It is acutely toxic to rats and other test animals, causing severe gastrointestinal and neurological disorders (Nishie et al., 1985). Its role in human health remains unclear.

*Aspergillus oryzae* (Ahlburg) Cohn is closely related to *A. flavus*, and produces colonies of similar or slightly smaller size on the standard media. However, colonies of *A. flavus* remain green as they age, whereas those of *A. oryzae* are more floccose and turn olive brown as they age. *Aspergillus oryzae* is of great economic importance, as it forms the basis of much of the fermented food industry in Japan and other parts of Asia. Tane koji, prepared by growing *A. oryzae* on cooked rice, provides a source of enzymes used in the production of shoyu (soy sauce), miso, hamanatto and other important Oriental products, which are mostly used as food flavourings (Hesseltine, 1965; Hesseltine and Wang, 1967; Beuchat, 1987; Cook and Campbell-Platt, 1994). *A. oryzae* does not produce aflatoxins, but some strains may produce cyclopiazonic acid (Orth, 1977; EPA, 1997).

### 17.6.6 *Aspergillus fumigatus* Fresen.

*Aspergillus fumigatus* (Fig. 17.2c) forms velvety, bluish colonies bearing characteristic, well-defined columns of conidia. Growth at 37°C is exceptionally rapid. The conidial heads are also diagnostic: elongated vesicles bear crowded phialides which tend to be roughly parallel to the stipe axis. Conidia are spherical to subspheroidal, 2.5–3.0μm in diameter, with finely roughened or spinose walls. Care should be exercised in handling cultures of this species because it is a recognized human pathogen.
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Ecology
The prime habitat for *A. fumigatus* is decaying vegetation, in which it causes spontaneous heating (Cooney and Emerson, 1964). Reported food-related sources include stored commodities in the tropics, cocoa beans during and after fermentation, oilseeds, cereal grains, spices, stored eggs and spices. It is quite common on cured and processed meats, especially in the tropics (see Pitt and Hocking, 1997). It has been reported as one cause of ‘Rio’ off-flavours in coffee (Liardon et al., 1992).

Physiology
The most important physiological character of *A. fumigatus* is its thermophilic nature: its minimum growth temperature is near 12°C, the optimum 40–42°C and maximum near 55°C (Panasenko, 1967; Ayerst, 1969; Evans, 1971; Domsch et al., 1980). *A. fumigatus* is a marginal xerophile, with a minimum $a_w$ for growth of 0.82 $a_w$ near 40°C (Ayerst, 1969).

Mycotoxins
*Aspergillus fumigatus* produces fumitremorgens, verruculogen and gliotoxin and although a role in animal disease seems likely (Frisvad and Samson, 1991; Land et al., 1993; Boudra and Morgavi, 2005; Frisvad et al., 2006), little has been published about the possible production or significance of these toxins in foods. Gliotoxin may be important in the invasion of human, animal and bird lungs by *A. fumigatus* (Richard et al., 1994; Lewis et al., 2005).

17.6.7 *Aspergillus niger* Tiegh.
*Aspergillus niger* forms easily recognized black or dark brown colonies, as do the closely related species *A. carbonarius*, which produces conidia 7–10 µm in diameter and *A. awamori*, which produces finely roughened conidia. *A. awamori* is used in food fermentations and is perhaps a domesticated form of *A. niger*. *A. niger* and closely related species (*A. carbonarius*, *A. awamori*, *A. aculeatus* and *A. japonicus*) all belong in *Aspergillus* Section Nigri.

Ecology
*Aspergillus niger* is more prevalent in warmer climates, both in field situations and stored foods. The black spores apparently provide protection from sunlight and UV irradiation, providing a competitive advantage in such habitats. *A. niger* is very frequently isolated from sun-dried products such as vine fruits (King et al., 1981; Leong et al., 2004) where it may produce ochratoxin A. *Aspergillus niger* is by far the most common *Aspergillus* species responsible for post-harvest decay of fresh fruit, including grapes, in which it is a principal agent of *Aspergillus* bunch rot (Nair, 1985; Snowdon, 1990), apples, pears, peaches, citrus, figs, strawberries, mangoes and melons (Barkai-Golan, 1980;
Snowdon, 1990) and black mould rot of onions (Snowdon, 1991).

Aspergillus niger is among the most common fungi isolated from nuts (peanuts, pecans, pistachios, hazelnuts, walnuts, kola nuts, coconut and copra). Cereals and oilseeds are also frequent sources, especially maize, but A. niger can be isolated from almost any type of stored commodity. Dried, smoked and cured fish and meat products are other common sources (see Pitt and Hocking, 1997).

Physiology
Growth temperatures for Aspergillus niger are minimum, 6–8°C, maximum, 45–47°C and optimum 35–37°C (Panasenko, 1967). A. niger is a xerophile: Ayerst (1969) reported germination at 0.77 aw at 35°C. A. niger is able to grow down to pH 2.0 at high aw (Pitt, 1981).

Mycotoxins
Until relatively recently, Aspergillus niger was regarded as a benign fungus, and has been widely used in food processing. It is categorized as ‘generally regarded as safe’ by the US Government. However, in 1994 Abarca et al. (1994) reported that some A. niger isolates can produce ochratoxin A. Although ochratoxin A production is not common, it has been confirmed by other researchers (Accensi et al., 2004; Belli et al., 2004; Leong, 2005).

17.6.8 Aspergillus ochraceus K. Wilh.
Aspergillus ochraceus (Fig. 17.1) is the most commonly occurring species in Aspergillus Section Circumdati. A. ochraceus produces yellow brown (ochre) colonies. The vesicles are spherical, bearing densely packed metulae and phialides with small, smooth, pale brown conidia. This species rarely grows at 37°C (Pitt and Hocking, 1997).

Ecology
Aspergillus ochraceus is widely distributed, particularly in dried foods, including dried fish, various dried beans and pulses, nuts and oilseeds (see Pitt and Hocking, 1997). It is less frequently reported from cereals (rice, barley, wheat, maize). The presence of this species in green coffee beans may lead to ochratoxin A production (Mantle and Chow, 2000; Taniwaki et al., 2003). Rots in garlic have been reported to be caused by Aspergillus ochraceus, but the species responsible is A. alliaceus (Snowdon, 1991).

Physiology
Aspergillus ochraceus grows between 8 and 37°C, with the optimum 24–31°C (ICMSF, 1996). The minimum aw for growth is 0.77 at 25°C (Pitt and Christian, 1968), with the optimum 0.95–0.99 (ICMSF, 1996). NaCl concentrations up to 30% (w/v), equivalent to 0.77 aw, are reportedly tolerated (Domsch et al., 1980). On coffee-based media, optimal conditions for germination and growth were observed at 0.95–0.99 aw and 20–30°C, with
Aspergillus and related teleomorphs

Aspergillus ochraceus was discovered to produce a mycotoxin, named ochratoxin A by van der Merwe et al. in 1965. Other minor toxins of lower toxicity were ochratoxins B and C. A. ochraceus may be a significant source of ochratoxin A in coffee (Taniwaki et al., 2003). Pardo et al. (2005) reported that maximum ochratoxin A production in coffee beans occurred at 20°C and 0.99 a_w, whereas at 10°C, ochratoxin was not produced, regardless of a_w. No ochratoxin was detected by Pardo et al. (2005) at 0.80 a_w; however, Milanez and Leitao (1994) reported ochratoxin production down to 0.80 a_w on beans at 25°C.

Aspergillus ochraceus also produces penicillic acid, a mycotoxin of lesser importance (Northolt et al., 1979), xanthomegnin and viomellein (Frisvad and Thrane, 2000).

Recent molecular studies by Frisvad et al. (2004) indicate that a new species, Aspergillus westerdijkiae, may be a more important ochratoxin A producer than A. ochraceus. Other Aspergillus species in Section Circumdati can also produce ochratoxin A. Aspergillus sclerotiorum, Aspergillus alliaceus, Aspergillus melleus and other less common species have all been reported to produce ochratoxins (Ciegler, 1972; Varga et al., 1996; Frisvad and Samson, 2000; Frisvad et al., 2006).

17.6.9 Aspergillus penicillioides Spegazzini and A. restrictus G. Sm.

Aspergillus penicillioides and A. restrictus are extremely xerophilic species. Because they grow poorly on most microbiological media, they are often overlooked. Both species grow very slowly under all standard conditions, and produce green conidia. A. penicillioides is more xerophilic than A. restrictus, and forms spathulate vesicles, fertile over more than the upper half, whereas those of A. restrictus are more rounded, and usually fertile only over the upper half, with conidia borne in distinct columns.

Ecology

Aspergillus penicillioides and A. restrictus are both common in a variety of low a_w foods including flour, dried fruit and dried fish, spices, including pepper and dried chillies, confectionery, and low a_w baked goods such as fruit cakes and puddings (see Pitt and Hocking, 1997). They are probably among the pioneer species to establish in commodities stored at low a_w, and may pave the way for fungal succession by less xerophilic species such as Eurotium species (Hocking, 2003). A. restrictus is frequently isolated from house dust and may play a role in fungal allergies (Toyazaki, 2002).
Physiology
Both species grow very poorly at high $a_w$. The optimal $a_w$ for growth of *A. penicillioides* is 0.91–0.93 (Andrews and Pitt, 1987). At 25°C, *Aspergillus penicillioides* is capable of germination down to 0.73 $a_w$ in laboratory media containing glucose/fructose or glycerol as principal solute. In natural substrates, *A. penicillioides* grows at much lower water activities: we have repeatedly isolated this species from grain and dried pet food stored at 0.68 $a_w$ for up to 6 months (our unpublished observations). Growth temperatures have not been accurately reported but growth occurs at 37°C at lower $a_w$ (Wheeler *et al.*, 1988), with a minimum near 15°C at 0.95–0.90 $a_w$ (Wheeler *et al.*, 1988). Smith and Hill (1982) reported the temperature range for growth of *Aspergillus restrictus* was minimum, 9°C, optimum, 30°C and maximum, 40°C. Growth of this species has been observed down to 0.75 $a_w$ (Snow, 1949; Pelhate, 1968).

Mycotoxins
Neither species has been reported to produce mycotoxins.

17.6.10 *Aspergillus sydowii* (Bainier & Sartory) Thom & Church
*Aspergillus sydowii* forms dark turquoise colonies, often with a brown reverse. It grows slowly at 25°C and often not at all at 37°C, produces heads with both metulae and phialides, and dark blue-green conidia. Vesicles on the larger stipes are small and club-shaped, and diminutive penicilli are also formed.

Ecology
*Aspergillus sydowii* is a widely distributed storage fungus, isolated from a variety of dried foods, particularly nuts, but also from rice, beans, spices and dried meat products (see Pitt and Hocking, 1997).

Physiology
*Aspergillus sydowii* is closely related to *Aspergillus versicolor* so can be expected to have similar physiological properties. The minimum for growth is near 0.78 $a_w$ (Snow, 1949; Wheeler and Hocking, 1988).

17.6.11 *Aspergillus tamarii* Kita
*Aspergillus tamarii* belongs in *Aspergillus* Section *Flavi*, and resembles *A. flavus* and *A. parasiticus*, but conidia of *A. tamarii* are coloured olive to brown, and are larger, with thick, conspicuously roughened walls. On AFPA, *A. tamarii* produces a deep brown reverse coloration, in contrast to the orange yellow of *A. flavus* and *A. parasiticus*. This is a useful diagnostic aid.
Aspergillus and related teleomorphs

Ecology
Aspergillus tamarii occurs most commonly in tropical and subtropical regions, in nuts (peanuts, pistachios, pecans, hazelnuts, walnuts, kola nuts and betel nuts), oilseeds and copra. It is occasionally isolated from other sources such as wheat and other small grains, coffee beans, soybeans, spices and dried meat and fish products (see Pitt and Hocking, 1997).

Physiology
The physiology of Aspergillus tamarii is similar to that of A. flavus. Ayerst (1969) reported that A. tamarii was capable of growth down to 0.78 $a_w$ at 33°C.

Mycotoxins
Aspergillus tamarii does not produce aflatoxins, but it does produce some other toxic compounds, including cyclopiazonic acid (Dorner, 1983; Ito et al., 1998), and kojic acid, a less toxic compound (Ito et al., 1998). The presence of A. tamarii in high concentrations in foodstuffs is clearly undesirable. A. tamarii may have been the cause of ‘kodua poisoning’ from kodo millet seed (Paspalum scrobiculatum) in India (Rao and Husain, 1985). The alkaloid fumiclavine A was also found in kodo millet seed from that outbreak, and it may have contributed to the toxicity (Janardhanan et al., 1984).

17.6.12 Aspergillus terreus Thom
Aspergillus terreus (Fig. 17.2f), Section Terrei produces rapidly growing pale brown colonies, with Aspergillus heads bearing densely packed metulae and phialides with minute conidia borne in distinctive long columns.

Ecology
Aspergillus terreus occurs commonly in soil and in foods, particularly stored cereals and cereal products, beans, pulses and nuts, but is not regarded as an important spoilage mould (Pitt and Hocking, 1997).

Physiology
Aspergillus terreus grows much more strongly at 37°C than at 25°C (Pitt and Hocking, 1997), but little information has been published on its physiology. The reported minimum $a_w$ for growth is 0.78 at 37°C (Ayerst, 1969).

Mycotoxins
Aspergillus terreus produces a wide range of metabolites (Frisvad and Samson, 1991), the most significant of which are probably a group of tremorgenic toxins known as territrems (Ling et al., 1979). These toxins have not been implicated in human disease (Cole and Dorner, 1986; Ling, 1994).
Aspergillus versicolor

Aspergillus versicolor grows slowly, produces both metulae and phialides from small vesicles, and green conidia. Growth at 37°C is weak or absent. This species may produce a wide range of mycelial and reverse pigmentation, especially if cultures are incubated for 14 days or so.

Ecology

Aspergillus versicolor is the most important food spoilage and toxigenic species in Aspergillus Section Versicolores. It is widely distributed and has been reported from most kinds of foods. Major sources include cereals, oilseeds, nuts and pulses from both tropical and temperate regions, but it has also been found in dried meat products and hard cheeses (see Pitt and Hocking, 1997). It is one fungus responsible for decay in fresh breadfruit (Omobuwajo and Wilcox, 1989), and is also one cause of the ‘Rio’ off-flavour in coffee due to the formation of trichloroanisoles (Liardon et al., 1992).

Physiology

Aspergillus versicolor is a mesophile, with a minimum temperature for growth of 9°C at 0.97 aw, a maximum of 39°C at 0.87 aw, and an optimum of 27°C at 0.98 aw (Smith and Hill, 1982). It is also xerophilic: its minimum aw for growth is 0.78–0.80 (see Pitt and Hocking, 1997). A. versicolor has a pH minimum above pH 3.1, and a pH maximum above pH 10.2 (Wheeler et al., 1991).

Mycotoxins

Aspergillus versicolor is the major producer of sterigmatocystin, a carcinogen which is a precursor of the aflatoxins (Cole and Cox, 1981), but aflatoxins are not produced by this species. Natural occurrence of sterigmatocystin has been reported in rice in Japan, wheat and barley in Canada, cereal-based products in the United Kingdom (Scott, 1994) and Ras cheese (Abd Alla et al., 1996). Sterigmatocystin has also been reported from mould affected buildings and building materials (Nielsen et al., 1999; Tuomi et al., 2000; Nielsen 2003).

Aspergillus wentii Wehmer

Aspergillus wentii (Section Wentii) produces golden brown colonies. It grows faster on reduced aw media than on CYA. It produces Aspergillus heads with both metulae and phialides on long stipes, and the conidia are relatively large (3–5 µm).

Ecology

Aspergillus wentii has only rarely been reported as a spoilage fungus, possibly because of its xerophilic nature. However, it appears to be relatively common.
on stored commodities, including dried fish, from tropical countries (Wheeler et al., 1986; Pitt et al., 1993, 1994, 1998).

Physiology
*Aspergillus wentii* is a xerophile that exhibits strong growth in both sugar and salt environments. The optimum $a_w$ for growth is near 0.94, with a minimum $a_w$ for germination at 25°C of 0.73 $a_w$ in glucose/fructose, 0.75 $a_w$ in glycerol and 0.79 $a_w$ in NaCl (Andrews and Pitt, 1987).

Mycotoxins
Emodin is produced by *Aspergillus wentii* (Wells et al., 1975); however, there are no reports of toxicoses associated with *A. wentii* in foods or animal feeds.

17.7 *Aspergillus* as spoilage fungi

Why are *Aspergillus* species so ubiquitous and successful as spoilage fungi? Their ability to grow at reduced $a_w$ and in warmer climates, on a wide range of substrates, ensures that they can be found in almost all food storage facilities. Although they are not the most xerophilic fungi (*Xeromycetes bisporus* holds that honour), they are non-fastidious and will grow on a wide range of food and non-food substrates, including leather, paper, museum artefacts, etc. (Pitt and Hocking, 1997). In addition, the ascospores of *Eurotium* species are more resistant than conidia to heat, UV irradiation and high pressure, although they not as heat-resistant as ascospores of *Neosartorya, Byssoclamys* or *Talaromyces*.

17.8 Control measures

Spoilage of foods by *Aspergillus* species falls into two general categories: specific food–fungus associations and non-specific spoilage. Where there is an association between particular species of *Aspergillus* and a food crop, such as *A. flavus* and peanuts, or *black Aspergilli* and grapes, measures can be taken during the growing phase of the crop to minimize fungal populations and thus contamination levels. In the case of peanuts, a biocontrol strategy involving competitive exclusion by non-toxigenic strains of *A. flavus* can reduce aflatoxin levels in peanuts (Cleveland et al., 2003; Pitt and Hocking, 2003). For grapes, current research is targeting reduction of inoculation levels in the vineyard by management strategies such as mulching vines, pruning to improve canopy and bunch architecture and suitable irrigation systems (Leong et al., accepted).

*Aspergillus penicillioides* and *Eurotium* species are pioneer species in the
colonization of stored commodities. Control of spoilage of stored commodities relies largely on control of water activity. Maintenance of a dry environment (below 0.6 $a_w$) will ensure that mould growth cannot develop. Temperature gradients and large diurnal fluctuation in temperature can cause moisture migration resulting in localized areas of high $a_w$ where fungal growth can develop. Adequate ventilation of storage structures can minimize temperature gradients and consequent moisture migration. Regular cleaning of storage facilities to remove grain residues and prevent insect infestation will minimize inoculum levels of spoilage fungi.

Control of spoilage of reduced $a_w$ foods such as bakery goods and confectionary also relies on $a_w$ control, but there are other hurdles than can be used to prevent spoilage. In the food industry, weak acid preservatives such as sorbates, benzoates and propionates are commonly used to extend shelf-life of cakes and bread. Preservatives are used in combination with acid pH, reduced $a_w$ and modified atmosphere packaging to extend shelf-life of baked goods (Abellana et al., 2000; Guynot et al., 2002; Gock et al., 2003).

Models for growth of Aspergillus species and mycotoxin production, based on combinations of $a_w$ and temperature, such as those of Rosso and Robinson (2001), Parra and Magan (2004) and Pardo et al. (2004, 2005) can be used to predict safety and stability of foods that are susceptible to spoilage by Aspergilli.

17.9 Future trends

Molecular techniques have brought great changes to our understanding of the fungal genome and to fungal taxonomy. Gene sequences are now routinely being used to establish relationships within the various sections of Aspergillus and to describe new species that are morphologically indistinguishable (Frisvad et al., 2004; Samson et al., 2004b). This will make identification of some Aspergillus species very difficult for the food microbiologist at the bench level. However, fungal genomics opens the way for a better understanding of pathways leading to the production of enzymes, useful secondary metabolites and mycotoxins. This knowledge can be exploited, either for enhanced enzyme and secondary metabolite production, or in control of fungal growth and mycotoxin production.

17.10 Conclusions

Aspergillus is one of the most important fungal genera in the spoilage of foods and animal feeds, particularly in warm-temperate climates and the tropics. Eurotium species along with A. restrictus and A. penicillioides are significant because they are the pioneering species in spoilage of stored
commodities (grains and nuts), and their metabolic activities pave the way for fungal succession by less xerophilic species such as mycotoxigenic Aspergilli and Penicillium species (Hocking, 2003).

Unlike Penicillium, there are few instances of associations between spoilage of specific foods and particular Aspergillus species. Although species-substrate associations such as Aspergillus flavus with peanuts and A. carbonarius with grapes are well recognized, these two species are also very common in other substrates, and conversely, other fungi are also known to cause spoilage in these commodities.

The genus Aspergillus contains a number of economically significant, highly mycotoxigenic species, the aflatoxigenic species clearly being the most important from the point of view of human health, although ochratoxin production by a number of Aspergillus species is becoming increasingly important. Many of the other mycotoxins produced by Aspergillus may have roles in cancer induction and immunosuppression, but these are yet to be elucidated.

17.11 Sources of further information

There is a plethora of literature on Aspergillus species and their metabolic activities that is of relevance to food microbiologists. However, the following texts are recommended as useful compilations of information on this genus and its importance to the food industry.


17.12 References


Aspergillus and related teleomorphs


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Food spoilage microorganisms


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18

Other types of spoilage moulds

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18.1 Introduction

Food spoilage moulds (excluding *Penicillium*, *Aspergillus* and the Mucorales which are dealt with in Chapters 15–17) form a diverse group of organisms. Until recent years moulds were treated simply as ‘moulds’ and recognition of specific genera and species was generally considered unnecessary. However, it has more recently been accepted that many foods have a specific spoilage mycoflora, sometimes known as the ‘associated mycobiota’ or ‘funga’ (Filtenborg *et al.*, 1996). Equally it has been recognised that many moulds may be present on and isolated from foods in which they never or rarely cause spoilage. In other words interpretation of isolation and identification results is an essential feature of food mycology.

The detailed classification of the diverse spoilage moulds is outside the scope of this chapter, although almost all are Deuteromycetes (Fungi Imperfecti) that only form an asexual stage (Anamorph) but are presumed to be Ascomycetes that have lost the capacity to form a sexual stage (Teleomorph). The classification of these fungi is dealt with in detail in two excellent texts: Pitt and Hocking (1997) and Samson *et al.* (2002), each species being illustrated by photographs of colonies of the moulds and photomicrographs or line drawings of the microscopic structures.

Food mycology, being predominantly concerned with spoilage and spoilage potential, differs from food bacteriology in one major respect. Food bacteriologists tend to seek either specific spoilage and indicator groups or individual named pathogens and toxinogens. The food mycologist, however, starts from the food and investigates its composition, processing factors and storage conditions. This leads to groups of organisms that might, for example,
survive pasteurisation, grow in deep chill, resist or degrade preservatives, grow at low water activity, etc. Appropriate isolation and identification procedures are then followed and, after identification, the properties of the mould along with its limits for growth can be elucidated using reference texts and, nowadays, the Internet.

Some fungi are naturally found on crops growing in the field and are known as ‘field fungi’. Others are more usually associated with stored crops and are known as ‘storage fungi’. There are also fungi associated with many classes of manufactured food, either as process survivors or as post-process contaminants.

### 18.2 Field fungi and storage fungi

Growing crops are generally high in moisture until the time of harvest and contain starches or sugars. This is reflected in the associated mycobiota: they are mostly hygrophiles (lovers of moisture) rather than xerophiles (lovers of dryness), and produce enzymes that break down carbohydrates to obtain energy as well as, in many cases, enzymes such as cellulases and pectinases that break down structural components and permit invasion of plant tissues. Among the field fungi, species of *Fusarium* are considered to be the most significant. Aside from their role as major producers of mycotoxins they also cause a wide range of plant diseases such as rots, blights and vascular wilts. Once a field crop is mature and starting to dry pre- or post-harvest, these Fusaria, being hygrophiles, cease to grow and generally begin to die, so they are rarely encountered by students of manufactured foods. An exception is *Fusarium oxysporum*, which has been isolated from a variety of manufactured foods, including UHT fruit juice and a ‘complete’ food for use in hospitals (author’s own laboratory; see also Samson *et al.*, 2002). In stored high-moisture foods, e.g. fruit and vegetables, Fusaria are an important cause of storage rots.

Another group of field fungi, often associated together because they produce dark green to brown pigments in their structures is known as Dematiaceous Hyphomycetes. This group includes a variety of well-known genera, including *Alternaria*, *Cladosporium*, *Curvularia*, *Drechslera*, *Stemphylium* and *Ulocladium*. Of these, *Alternaria* probably causes most concern, because of its production of a number of highly toxic mycotoxins. On the other hand, its presence in stored seed and brewing grains without growth can be an indicator of freshness, as by the time it dies, seeds are becoming stale and are less effective at germinating, a trait needed for seed crops and, in the case of barley, for germinating to produce the amylase needed to release sugars for brewing beers of various kinds.

### 18.3 Spoilage fungi

The majority of fungi relevant in the storage of dried crops are Aspergilli and
related teleomorphs, to be considered elsewhere in this book. However, stored produce and manufactured foods often have their own distinct mycoflora (associated mycobiota) comprising a limited and distinctive range of organisms. The same applies to moulds present in the environment in which food is manufactured and produced as these can be distinctive by virtue of both the type of food manufactured and the location, structure and maintenance of the building.

18.4 Common foodborne moulds

A brief description of some of the most common food spoilage moulds excluding *Aspergillus* and *Penicillium* is given below (Table 18.1). It follows the ‘mini-definitions’ format often used in food bacteriology, and is intended to outline the chief characteristics of the most frequently isolated spoilage moulds. Complete descriptions with photographs and appropriate drawings can be found in the food mycology literature (Pitt and Hocking, 1997; Samson *et al.*, 2002).

### 18.4.1 *Alternaria* spp.

*Alternaria* forms distinctive dark brown or black colonies with club-shaped spores under the microscope. Spores have horizontal and vertical cross-

<table>
<thead>
<tr>
<th>Table 18.1</th>
<th>Common fungi and their food types or characteristic environments, excluding <em>Aspergillus, Penicillium</em> and related organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food type</td>
<td>Some examples of typical spoilage and environmental moulds</td>
</tr>
<tr>
<td>Cheese</td>
<td><em>Scopulariopsis</em> (usually on the rind), <em>Cladosporium</em> spp.</td>
</tr>
<tr>
<td>Condensed milk (sweetened)</td>
<td><em>Wallemia sebi</em></td>
</tr>
<tr>
<td>Dried herbs and spices</td>
<td><em>Wallemia sebi, Chaetomium globosum</em></td>
</tr>
<tr>
<td>Fats and low fat spreads</td>
<td><em>Cladosporium</em> spp.</td>
</tr>
<tr>
<td>Garlic and onions</td>
<td><em>Botrytis aclada (B. allii)</em></td>
</tr>
<tr>
<td>Low water activity foods</td>
<td><em>(cereals, confectionery, jams and preserves)</em> <em>Chrysosporium</em> spp., <em>Xeromyces bisporus</em></td>
</tr>
<tr>
<td>Nuts</td>
<td><em>Alternaria</em> spp.</td>
</tr>
<tr>
<td>Pasteurised foods</td>
<td><em>Fusarium oxysporum</em></td>
</tr>
<tr>
<td>Tomatoes</td>
<td><em>Alternaria</em> spp., <em>Stemphylium</em> spp.</td>
</tr>
<tr>
<td>Soft fruit and vegetables</td>
<td><em>Botrytis cinerea, Alternaria</em> spp., <em>Geotrichum candidum</em></td>
</tr>
<tr>
<td>Air in production areas</td>
<td>Many different mould species but especially <em>Cladosporium</em> spp.</td>
</tr>
<tr>
<td>Machinery and pipework</td>
<td><em>Geotrichum candidum</em> (formerly known as <em>Oidium lactis</em> and <em>Oospora lactis</em>)</td>
</tr>
</tbody>
</table>
walls and are formed in chains. They are mainly associated with growing plants and fresh stored cereals but can also cause rots in fresh fruit and vegetables.

**18.4.2 Aureobasidium pullulans**
This is a black yeast, with colonies at first pink and mucoid, usually developing dark to black areas within 1–2 weeks. Large numbers of yeast-like conidia are formed from hyphae or short projections from hyphae. Hyphae may be colourless (hyaline) or pigmented deep greenish brown (Dematiaceous), the latter usually being somewhat distorted. This organism is a widespread saprophyte that is often found in foods but does not often cause spoilage. It seems to be prevalent in some frozen foods but does not cause spoilage at freezer temperatures.

**18.4.3 Botrytis cinerea**
Colonies on agar fill the Petri dish with fluffy mycelium that becomes grey with sporulation. Spores are produced on branched heads, in clusters like small bunches of grapes. It is a vigorous rotting agent of many fruit, especially grapes and soft fruit, as well as vegetables. Its rot is sometimes encouraged in grapes, being the ‘noble rot’ desired in the manufacture of some high-quality sweet dessert wines.

**18.4.4 Chaetomium spp.**
This is an Ascomycete that produces prominent small black sporing structures on agar. These perithecia are surrounded by dark radiating hyphae and contain ascospores that are oval and brown pigmented. It is frequently found in cereals, rice, pulses and nuts from tropical regions and also spices such as pepper, where it has occasionally caused spoilage when moisture control problems have occurred.

**18.4.5 Chysonilia sitophila** *(formerly Monilia sitophila)*
Often known as ‘red bread mould’, *C. sitophila* was well known in bakeries and on bakery products, causing an invasive pink-coloured spoilage. Growth generally fills a Petri dish and spores tend to be shed outside the lid rim, resulting in massive contamination of laboratory incubators. Spores are yeast like and formed on undifferentiated hyphae (arthroconidia). Following the widespread introduction of preservatives such as calcium propionate in baked goods, during the latter half of the 20th Century, this mould became rare. More recently, however, with the elimination of preservatives as a result of consumer pressure, it is reappearing with increasing frequency.
18.4.6 **Cladosporium spp.**

Cladosporia are very distinctive moulds found in a wide variety of locations. In culture they form dark green, velvety colonies. Sporing structures are delicate tree-like branching hyphae, terminating in smooth or roughened spherical or oval spores but structures usually disintegrate in microscopic mounts so that individual spores and elongated supporting cells are seen mixed together. Cladosporia are widespread on plant materials and in the air, from where they can be conveniently isolated and used as indicators to track air flow in food businesses. They cause rots of some fruit and vegetables and as they have the capacity to grow at temperatures several degrees below zero Celsius (psychrotrophs), they can grow in deeply chilled products. Spoilage is typically on fatty or proteinaceous foods such as carcass meat (black spot), cheese and low-fat butter substitutes.

18.4.7 **Chrysosporium spp.**

Species of *Chrysosporium* are often reported as rare fungi but actually cause spoilage of rather a wide variety of low-moisture foods, including cereals, dried fruit and confectionery. One reason for their reported rarety is certainly that (as with *Wallemia sebi*) they grow slowly and inconspicuously on most commonly used laboratory media. Indeed some species do not grow at all. When grown on suitable media (e.g. containing 50% w/w glucose) growth is usually close to the agar and coloured white or in pale yellowish to brown shades. Under the microscope a variety of spore types is seen (depending on species), including solitary conidia (aleurioconidia), resting spores (chlamydoconidia) and fragmented hyphae (arthroconidia).

18.4.8 **Endomyces fibuliger and Hyphopichia burtonii**

These two very similar yeast-like fungi produce low white colonies and when seen under the microscope consist of a mixture of uncoloured hyphae, bearing yeast-like conidia from short projections. As with *Chrysonilia sitophila* (above) they are mainly associated with bakeries where they are the cause of ‘chalky mould’, especially in packaged and sliced bread. Uncommon when bread was highly preserved, they are reappearing now that fewer preservatives are being used.

18.4.9 **Fusarium spp.**

This genus contains a large number of species and is best known for the range of mycotoxins produced. Most species are plant associated, often causing wilts, blemishes and blights. *Fusarium* is notoriously difficult to grow for identification in culture as it degenerates rapidly. Successful isolation and growth for identification involves isolation from single spores, use of special media such as potato sucrose agar or tap water agar overlaid with sterile
carnation leaves, and incubation for 12/24 hours under a light bank of fluorescent and ‘black light’ tubes. Colonies are fluffy (floccose) when young, in colours varying from white through cream, pink, orange, mauve or magenta. Depending on species, under the microscope either or both of two types of spore are formed, sometimes accompanied by resting spores known as chlamydoconidia. The normal spores are larger macroconidia which are usually banana shaped with several cross-walls in the cell and a distinctive hook or ‘foot cell’ at the base, and smaller microconidia, which may be spherical to elliptical with 0–1 cross-walls.

18.4.10  Geotrichum candidum
In culture, Geotrichum candidum produces spreading, white, yeast-like colonies. Under the microscope, acutely branching hyphae are seen, which disintegrate almost entirely into brick-shaped conidia (arthroconidia) that become somewhat rounded in age. It has a worldwide distribution and, although causing rots on a number of fruits, it has a particular affinity for processing equipment and dairies. It is used as a starter culture in the manufacture of some cheese of the Brie and Camembert types (along with Penicillium camemberti) but in pipework can cause fouling and even blockage if allowed to grow.

18.4.11  Moniliella acetoabutans
This is another mould that produces yeast-like spores from relatively undifferentiated hyphae in colonies with a persistently white obverse (top) colour. Although relatively uncommon, it has found a distinct niche in acetic acid preserves due to its ability to grow at acetic acid concentrations of up to 4% and survive at levels up to 10%.

18.4.12  Phoma spp.
Phoma is a large genus whose species are difficult to distinguish. It produces distinctive pycnidia (dark round or oval structures with one or more openings and exuding masses of small conidia in slime). The pycnidia are just visible to the naked eye. Although present on cereals and causing black rot of a number of fruit and vegetables, Phoma spp. are observed from time to time in vacuum packaged block cheese, where they are one cause of a defect called ‘thread mould’ when growing across the cheese surface, usually where whey has collected following pressing and in any folds in the vacuum bag.

18.4.13  Stemphylium and Ulocladium
These two moulds produce growth superficially resembling that of Alternaria but microscopically the spores are more rounded, lacking the beaked club-
shape seen in *Alternaria*. Although largely associated with plants and stored materials, rots of stored fruit and vegetables occasionally occur.

**18.4.14 Trichoderma harzianum and T. viride**
These closely related moulds grow vigorously in culture, usually filling the Petri dish with irregular and tufted growth in white to cream shades, turning to a yellow green or ‘verdigris’ green in patches. When grown in an enclosed incubator, there is often a smell of coconut when the incubator door is opened. Sporing structures are highly branched like candelabra, bearing single and small conidia which are either smooth (*T. harzianum*) or roughened (*T. viride*). Although frequently present on stored products without causing spoilage, Trichodermas are able to spoil a number of stored fruit and vegetable types because they are able to produce a number of powerful enzymes, especially cellulase. They have also been isolated from a number of manufactured foods, including fats and margarines. According to Samson *et al.* (2002) they are somewhat preservative resistant, although figures are not given.

**18.5 Characteristics and conditions for growth/death**

**18.5.1 Nutrients**
Nutritionally, moulds are heterotrophs, in fact having nutritional preferences that are remarkably similar to humans, and hence causing ready spoilage of human foods. By comparison, yeasts are often rather fastidious and may be able to assimilate only a limited range of, for example, sugars. As a consequence yeasts, like bacteria, having limited morphological variety, are characterised largely on substrate utilisation, whereas moulds, having morphological variety but limited nutritional variation, are characterised by colonial and microscopic morphology. One particular feature of moulds, used in their characterisation, is the colour of their colony obverse and reverse. This has led to difficulties in recent years, as the quality and purity of manufactured mycological media have improved to the extent that trace metals (mainly copper and zinc) are no longer present at sufficient levels to allow typical growth and the pigments necessary for identification to be expressed. As a result, it is now necessary to amend all mycological media by adding 1 ml per litre of trace element solution (1 g ZnSO₄·7H₂O + 0.5 g CuSO₄·5H₂O in 100 ml distilled water). Where trace metal solution is not available, making media with tap water rather than distilled water is usually a suitable alternative, although this is not normally acceptable to laboratory accreditation bodies.

**18.5.2 Moisture and water activity**
Whereas field fungi are generally hygrophiles, not capable of growth at $a_w$
values below around 0.90, a number of food storage and foodborne moulds are xerotrophs or true xerophiles. The normally accepted definition of a xerophile is an organism possessing the ability to grow at water activity 0.85 or below under at least one set of conditions. Although one mould (Xeromyces bisporus) can grow at $a_w$ values as low as 0.61, a large number of xerophiles and xerotrophs (e.g. Chrysosporium spp. and Wallemia sebi) have their minimum $a_w$ for growth in the range 0.70–0.75. Precise values are not easy to give as there is much species to species, and indeed strain to strain, variation, as well as the influence of the growth substrate and environmental conditions. In general, moulds respond to water activity irrespective of the solute (humectant) used, although two true halophiles (Polypaecilum pisce and Basipetospora halophila) are associated with spoilage of salted dried fish, being able to grow, for example, in the presence of saturated salt ($a_w$ 0.747). Wallemia sebi is another xerophile associated with salt fish but it is not a halophile, being able to grow on sugar concentrates such as condensed milk. This latter mould is difficult to observe in some foods as its small colonies and dull brown colour (it is sometimes known as ‘dun mould’) make it nearly invisible when growing on baked goods such as some cakes.

18.5.3 Growth and survival at high temperatures

In manufactured foods, high-temperature storage is uncommon, whereas many stored crops may be subject to very high ambient temperatures. Most of the storage moulds are thermotolerant rather than truly thermophilic and are species of Aspergillus or its teleomorphs such as Eurotium; these are considered in Chapter 17. Upper temperature limits for growth in the species considered here are highly variable, being as low as near 28–30°C in some species of Cladosporium but more usually in the range 35–38°C. Equally, heat resistance is normally restricted to Penicillium, Aspergillus and related teleomorphs and genera. However a heat-resistant anamorphic form has been described in Fusarium oxysporum, capable of surviving temperatures near boiling point, although thermal death characteristics are not published. It is very difficult to compare thermal inactivation characteristics for moulds because of the variety of methods used by various workers, the effect of different substrates, strain to strain variation, non-linear survivor curves caused by small resistant populations and the tendency for heat resistance to increase as the spores become older. Where data are available, it appears that a number of the common moulds described here typically have $D$ values (decimal or 90% reduction) of around 1–5 minutes at temperatures between 60 and 65°C. However Xeromyces bisporus produces ascospores with $D$ values of 2.3 minutes at 82.2°C. Also, where quoted, $z$ values (the number of Celsius degrees ($^\circ C$) needed to change the inactivation time ten-fold) are often of the order 5–7$^\circ C$. Solutes such as sugars and occasionally salt are protective, so that heat resistance increases as solute concentration increases.
Heat resistance is also affected by the pH value and type of acid present, although this is not predictable and varies from species to species. This is particularly significant in fruit processing where different organic acids and weak acid preservatives may be present. In some species heat sensitivity is increased by acids such as citric, fumaric, benzoic and sorbic acids, whereas malic and tartaric acids may be protective. However, there is large species-to-species and acid-to-acid variation, so heat processes generally need to be assessed empirically.

18.5.4 Growth and survival at low temperatures
At temperatures below the limit for growth, moulds are often able to survive dormant for extended periods. The lower limit for growth of individual moulds is not always known with certainty because there are widely differing estimates in the literature. However, in practical terms, the moulds described here often have temperature minima near 0–5°C, although mould growth has been known on deep-chilled carcase meats at temperatures of –5 to –6°C, the moulds causing black spots and whiskers (Cladosporium spp., Thamnidium elegans and Penicillium hirsutum being involved), so a lower limit of –7 to –8°C is likely. At such temperatures growth is exceedingly slow (Pitt and Hocking, 1997), a small colony taking as long as 4 months to appear. In practice, therefore, lower temperatures should not permit microbial growth. The widely used freezer temperature of –18°C or lower is not, in fact, a scientific necessity but is merely a conversion from 0 °Fahrenheit. Rapid cooling to –18°C or lower has the practical benefit of allowing smaller ice crystals to form and hence less damage to food structure. It is, however, less likely to damage or kill microorganisms present in the food.

18.5.5 Preservatives, acids and disinfectants

Preservatives
Some species of mould and yeast are commonly able to resist food preservatives. These are generally species of Penicillium and related genera, and of yeast, that are outside the scope of this chapter. For any mould where preservative resistance is suspected, a convenient isolation technique is to inoculate on a general purpose medium such as malt extract agar containing 0.5% acetic acid and incubate at 25°C for 5–7 days. Most other moulds are susceptible to the common preservatives (sorbate, benzoate and propionate) within the legally permitted concentrations and especially at lower pH values, although published data (excluding Penicillium and Aspergillus) are inconsistent and limited in extent. It is clear, however, that environmental hygiene is of paramount importance in food businesses using preservatives because some species can build adaptive resistance. Where preserved debris is allowed to accumulate, a number of species in the environment can develop resistance
to the common preservatives present, eventually acquiring resistance to the quantities used in the product and causing spoilage within shelf-life.

**Acids**

Although the organic acids commonly found in food (e.g. acetic, citric, lactic, malic and tartaric acids) possess useful antibacterial properties, they generally cannot inhibit mould growth completely although growth of some species may be delayed. Depending on other compositional factors, the lower pH limit for most palatable food is near 2.5, at which value some fungal growth (and indeed mycotoxin production) is actually enhanced (Samson *et al.*, 2002). However, these acids are still useful because they increase the antimicrobial effect of weak acid preservatives by encouraging their undissociated (antimicrobial) form.

**Disinfectants**

Apart from the nearly universal antimicrobial activity of chlorine, relatively little is known about the mechanisms of fungal inactivation or resistance (Russell *et al.*, 1999). Disinfectants showing significant antifungal activity include halogenated phenols, parabens and glutaraldehyde, although the former are rarely used in food businesses because of their tainting properties. Perhaps the most useful intervention remains the traditional activity of thorough cleaning followed by heating with water or steam to above 70°C. This rarely fails if recontamination does not occur. However, there are occasional problems with the use of a terminal sanitiser (see Section 18.8.4).

Alternative antimicrobial interventions are effectively the same as those proposed for bacterial control, including use of peroxide and ozone, modified atmospheres, UV and ionising radiation, ultra high pressure and combination techniques. Comparative studies are limited but effectiveness for moulds would be expected to lie between that for vegetative bacteria and for bacterial spores.

**18.6 Isolation methods**

**18.6.1 Isolation by direct plating**

For particulate foods, a direct plating technique is most appropriate, rather than a dilution plating technique. This is because many irrelevant organisms may be present on the surface of the product and in the associated dust but may be unable or unlikely to grow under the storage conditions. Such products include cereals, rice, pulses, nuts and fried fruit. Organisms of interest would be those that have invaded the interior of the product and may have affected germinability or have grown sufficiently to produce mycotoxins. An exception to this is where the entire food material and its accompanying dust will be processed together, for example where sacks of grain will be milled into flour. In these cases, the total load is more relevant than the specific
invasion, although invasion resulting in mycotoxin production remains a possibility.

Various methods are used, but the basic principles are the same. A particulate sample is immersed in a solution of hypochlorite (1000–2000 ppm) containing a wetting agent such as 0.05% Tween 80 for 1–2 minutes and the hypochlorite is removed by rinsing in sterile water. Particles of food are plated on a suitable medium, typically DG18 agar, or even moist sterile filter paper, and incubated without inversion (as is always the case in food mycology) for 7–10 days at 25 °C. Results are recorded as percentage of particles showing mould growth on the surface of the particle or on the surrounding agar. Unsterilised particles are used as a control. When particles have been invaded, typically growth is observed from a high percentage of particles and usually invasion is with the same type of mould (often species of *Eurotium*).

### 18.6.2 Isolation from dilution plates

Isolation from dilution plates is highly appropriate for examining the total fungal load of dried foods, such as flour, cocoa powder, herbs and spices, as an indicator of production hygiene and of correct moisture control. It parallels the Aerobic Colony Count for bacteria, as a non-specific hygiene indicator. Two major caveats apply.

First, the method detects the species producing the highest number of spores (e.g. *Penicillium* spp.) and not necessarily species (such as *Cladosporium* spp.) that may produce comparatively fewer spores but actually may cause far more damage to the product. Choice of growth medium is also significant. Foods with $a_w$ values above 0.95 should normally be plated on dichloran rose bengal chloramphenicol agar (DRBC). Below $a_w$ 0.95, dichloran 18% glycerol agar should be used, to encourage xerotrophic moulds that might be potential spoilage agents. Trace element solution (or tap water) must always be incorporated in these media to induce correct growth and colony colour. These media also inhibit the spreading of most of vigorous mould types, such as members of the Mucorales (*Mucor* and *Rhizopus*), because they contain dichloran and/or rose bengal, and enable counting by restricting overgrowth of plates. Media such as acidified malt extract agar, potato dextrose agar, chloramphenicol plate count agar, tryptone glucose agar and saboraud dextrose agar are generally considered unsatisfactory for counting (Samson *et al.*, 1992) and should be avoided.

Second, in moist foods where growth of mycelium might be occurring, even if not visible, the colony count is inappropriate and should not be used. An example would be the development of mould on a block of cheese. If visible, the rejection criterion would be ‘mouldy’. Even if only one mould was visible, the cheese would be unfit. Investigation should centre, not on ‘how many moulds?’ but ‘why did it become mouldy?’ If colony counts were used then the result might be:
• variable (a few hundred) colonies, if the mould was sampled but contained only hyphae – the count would depend on the degree of maceration of the sample;
• several million, if the mould was producing spores;
• ‘not detected’ if the laboratory did not capture the mould in the sample.

Whatever the count, the cheese would be unfit for sale and the count would be entirely irrelevant, merely a function of the method used.

18.7 Isolation from the air

In many food production environments where the main spoilage agents are moulds, air movement can be the cause of either contamination of high-risk (ready to eat) materials, or re-contamination from ingredients (such as starches) if air movement between low-risk and high-risk areas is not controlled. Other factors can involve roller doors or strip curtains that are, respectively, left open or tied back for the convenience of those using them, but allow air, containing mould spores, to move in unintended directions. This also applies to mesh screens over doors and windows, used for example in jam factories, to permit ventilation but exclude insects. These may permit unintended airflow directions and result in product contamination.

There are various sampling devices that enable measured volumes of air to be sampled and collected either on adhesive tape for direct examination, or impacted on agar for incubation and growth. While giving a degree of quantification, these devices are limited by the number of samples that can be taken. Scientifically cruder, but nevertheless effective, is making air exposure plates (also known as ‘settle plates’). These are sometimes (correctly) criticised for being potentially inaccurate, but are convenient in businesses lacking the more complex equipment. Mould spores are of varying size and density, some are hydrophilic but some are hydrophobic and so may not adhere to the agar. Others may possess electrostatic charges that repel them from an agar surface. Nevertheless, many plates may be conveniently set in different places for a standard length of time (often 1 hour, but times can be adjusted so that a reasonable number can be counted on the plates, typically 10–100 colonies) and they give an indication of where the most contamination is to be found. The actual numbers are less significant than trends observed over a period of time. Choice of growth media would depend on the water activity of the food in question. For foods with water activity above 0.95 then dichloran rose bengal chloramphenicol agar would be most appropriate, although this should not be exposed to bright sunlight for long periods of time as it is photosensitive and becomes more inhibitory after exposure to bright light. Where the food has a water activity below 0.95 or where bright light is a problem, then dichloran 18% glycerol agar would be most appropriate. Plates should be incubated at 25°C for 5 days before colonies are counted.
When using air exposure plates many species of mould may be isolated, depending on the prevailing climate (Aspergilli are more common in hot climates, Penicillia are more common in temperate climates). Quite often moulds will grow without producing spores. These may be species of *Fusarium*, a genus that often does not sporulate well in culture, or of Basidiomycetes (mushrooms and toadstools) that need specific substrates such as dead wood or a living plant, in order to produce their sporing bodies. Here, while numbers may indicate the quantity of air contamination, identification is not feasible unless the specific host for the fungus is known, and is anyway unnecessary since the majority of significant foodborne moulds do sporulate in culture and non-sporing isolates are therefore irrelevant most of the time. The moulds most useful as markers are Dematiaceae, typically species of *Cladosporium*. These form dark green, velvety colonies, with a blackish reverse often with a ‘pearly’ sheen. While they only spoil a limited range of foods (butter and spreads, cheese and meats and some bakery products), they give an excellent indication of where the main air movements occur and are easily recognisable without the need for preparing slides and using microscopy, which is usually essential in food mycology. They can be considered as the equivalent, in food mycology, of indicator bacteria such as Enterobacteriaceae or coliforms that are widely used to confirm that correct manufacturing practices are in place but are not necessarily characterised further.

Air management has, in recent years, become an essential feature of mould control in food businesses, as well as the use of appropriate mould isolation media containing essential micronutrients.

### 18.8 Implications for control in main foods affected

The chief trends are based on the continued reduction in preservation and reliance on hygiene and, generally, chill conditions to produce foods that are acceptable to modern nutritional requirements but that also have shelf-lives long enough to permit infrequent shopping. Many of these changes, coupled with preventative Hazard Analysis Critical Control Point (HACCP) systems, control bacteriological problems satisfactorily. Moulds (especially psychrotrophic species) are, however, able to take advantage of the new opportunities provided and gain or re-gain dominance in spoilage. Many examples exist.

#### 18.8.1 Bakeries

Eliminating propionate preservatives has permitted the reappearance of moulds that were well known in earlier decades, such as *Chrysonilia sitophila* and the chalk moulds *Hyphopichia burtonii* and *Endomyces fibuliger*. 
18.8.2 Soft drink producers
Removal of benzoate preservatives has resulted in large-scale spoilage by moulds that can utilise the remaining preservative (potassium sorbate) to produce the metabolite trans-1,3-pentadiene, which imparts a ‘kerosene-like’ taint to products. Most of the organisms known to do this are Penicillia or related species.

18.8.3 Jam and preserve manufacturers
Manufacturers of jams and preserves are increasingly being asked to lower the sugar levels in their products. While there may be nutritional benefits, from the point of view of mould spoilage, the normal spoilage organisms (which traditionally were non-toxigenic moulds such as Eurotium spp.) are able to be replaced by less xerophilic species such as Aspergillus flavus and may introduce the possibility of introduction of the hazard of mycotoxicosis.

18.8.4 Bottled mineral waters
These are traditionally considered stable because of the low level of organic carbon present. However, in still waters there is an increasing incidence of visual spoilage by moulds. Where investigated this has been traced to residual pipe-cleaning chemicals such as peracetic acid (stabilised with acetic acid) which, at ppm levels, has supplied enough carbon to permit spoilage in product bottled early in a run. Acetate could be detected in non-spoilt bottles but was not detected in spoilt bottles. Many of the moulds have been Penicillia (especially Penicillium brevicompactum) but others were Dematiaceae, commonly Cladosporium spp. and, of more concern, Exophiala spp. a potential human pathogen.

18.9 Future trends
In the food industry there are various changes and trends occurring, for better or worse. Some of these are associated with necessary improvements based on nutritional advice and knowledge, others are more worrying as they involve decisions made by management personnel who often do not have technical knowledge. Numerous examples exist of these recent developments, but notorious examples include the decision to exclude sodium benzoate from soft drinks because of possible issues with symptoms of hyperactivity in children. Unfortunately, the elimination of sodium benzoate makes soft drinks that are not pasteurised ‘in pack’ susceptible to mould spoilage, as some potential spoilage species are able to degrade potassium sorbate as described above.

Other types of change are, however, sometimes made. Marketing departments, for example, have been known to increase a product size using
an incentive such as ‘20% extra free this month’ without informing production staff that a larger product needed more cooking, resulting in an under-processed product being released and followed by mould spoilage within shelf-life.

Increasingly, changes that affect product stability are being made without technical intervention, and it is not easy to see how this process can be reversed.

On a more positive note, some of the modern microbial control strategies (see Section 18.5.5) may well be employed in the control of moulds, as alternatives to the use of undesired preservatives. Additionally the use of natural antimicrobials such as essential oils, both as preservatives and as processing aids (e.g. in combination with heating processes), is receiving considerable attention. Two properties of these antimicrobials need to be overcome. Firstly, many are strongly flavoured and, while effective, are likely to be unpalatable at effective concentrations in some foods. Secondly, there are frequently moulds with a specific capacity to overcome these antimicrobial effects. An example of the latter is Botrytis aclada, a pathogen of Allium spp. (the onion family) that is resistant to the powerful natural antimicrobials that the Allium family produces.

Some novel controls are already quite widespread. The natural antifungal antibiotic natamycin is now in use in several countries for controlling mould on a number of food types, especially cheese and fermented meats, and its use is likely to be extended. However, there is already one mould (Penicillium discolor) that is resistant to natamycin.

In fact, almost all of the procedures necessary to control moulds in food already exist, and the modern control methods should be seen as bonus opportunities. Good agricultural and storage practices remain fundamental. In manufacturing, the basics will always apply: clean environment and equipment, decontaminated by heat (or controlled dryness in some industries such as baking); process control, either by heat process (before or after packing), or by formulation; hygienic or aseptic packaging and prevention of re-contamination, especially by control of air movement in high-risk areas. Where formulation or process modifications are made, it is inevitable that stability or shelf-life will occasionally be affected and this information must be passed along the food chain to the consumer.

18.10 Sources of further information

Although there is a large body of mycological literature, a few key texts contain all the information needed for most investigations. These texts also direct readers to the specialist literature on important organisms and key topics.


18.11 References


Part V

Spoilage bacteria
19

Pseudomonas and related genera

C. H. Liao, US Department of Agriculture, USA

19.1 Introduction

The bacterial genus *Pseudomonas* comprises a group of Gram-negative and non-spore-forming rods, which are mostly aerobic and motile by polar flagella. In the 3rd edition of the *Berger’s Manual of Systematic Bacteriology*, more than 40 species were included in the genus (Palleroni, 1984). Owing to the phenotypic heterogeneity among these species, the classification of *Pseudomonas* species has been subject to a number of changes during the last few decades. In 1973, Palleroni and collaborators proposed subdividing the genus into five rRNA similarity groups based on DNA:DNA hybridization studies. Because of the phylogenetic diversity revealed, members of the five distinct rRNA similarity groups have now been assigned to more than ten new genera (Kersters *et al.*, 1996). For example, non-fluorescent animal and plant pathogens in rRNA similarity group II were assigned to two genera designated *Burkholderia* (Yabunchi *et al.*, 1992) and *Ralstonia* (Yabunchi *et al.*, 1995). Additionally, non-fluorescent plant pathogens in rRNA similarity group III were assigned to a genus named *Acidovorax* (Willems *et al.*, 1990). Despite the taxonomic changes proposed for members of rRNA similarity groups II to IV, fluorescent pseudomonads in rRNA similarity group I, including *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. chlororaphis*, *P. cichorii*, *P. viridiflava* and *P. syringae*, were retained in the genus *Pseudomonas* (sensu stricto) (Moore *et al.*, 1996).

Mention of trade names or commercial products in this chapter is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.
Members of the genus *Pseudomonas (sensu stricto)* are fluorescent, motile and nutritionally versatile. They can be found abundantly in soils, water and many other habitats. The pseudomonads are globally active in aerobic decomposition and biodegradation, and hence play a key role in the balance of nature and in the economy of human affairs. Some of them are closely associated with plants and animals as pathogens or saprophytic epiphytes. Four major species in the genus, *P. fluorescens*, *P. viridiflava*, *P. fragi* and *P. lundensis*, often in combination with *Shewanella putrefaciens* and *Xanthomonas campestris*, are responsible for a large proportion of food spoilage. Pectolytic strains of *P. fluorescens*, *P. viridiflava* and *X. campestris* are associated with spoilage (or soft rot) of fresh fruits and vegetables. Proteolytic and lipolytic *P. fluorescens*, *P. fragi*, *P. lundensis* and *S. putrefaciens* are associated with spoilage of animal-derived foods including meat, poultry, milk and fish. The spoilage caused by these bacteria is indicated by a slimy or mushy appearance, production of off-odors, and partial or complete degradation of plant or animal tissues.

As to be discussed in this book, food spoilage can be caused by diverse types of organisms including bacteria, fungi and yeasts. This chapter will focus on six major species of bacteria in the genera of *Pseudomonas*, *Xanthomonas* and *Shewanella* as mentioned above. The topics to be discussed include: (a) the description and classification of these bacteria and methods of detection and enumeration, (b) the extrinsic and intrinsic factors affecting their survival and growth on foods, and (c) the mechanism of spoilage caused by these bacteria and methods of control.

### 19.2 Description of *Pseudomonas*, *Xanthomonas* and *Shewanella* species associated with spoilage of plant- and animal-derived foods

The characteristics of six major species of *Pseudomonas* species that are most often associated with fresh and spoiled foods are summarized in Table 19.1. Four of them, namely *P. fluorescens*, *P. lundensis*, *P. fragi* and *P. viridiflava*, have been shown to play a key role in spoilage of both plant- and animal-derived foods and will be described in detail below. *Xanthomonas* and *Shewanella* are two genera closely related to *Pseudomonas* taxonomically or historically. The involvement of these two *Pseudomonas*-related genera in food spoilage will also be discussed.

#### 19.2.1 Characteristics of fluorescent pseudomonads associated with spoilage of fresh produce

Oxidase-positive *P. fluorescens* represents a very heterogeneous group and can be subdivided into more than five biotypes or biovars based on the studies conducted by Stanier *et al.* (1966) and Palleroni (1984). A few selected
Table 19.1  Differentiation of major species of *Pseudomonas* associated with fresh and spoiled foods*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. aeruginosa</em></th>
<th><em>P. fluorescens</em></th>
<th><em>P. fragi</em></th>
<th><em>P. lundensis</em></th>
<th><em>P. putida</em></th>
<th><em>P. chlororaphis</em></th>
<th><em>P. viridiflava</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusible fluorescent pigment</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diffusible non-fluorescent pigment</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Levan formation</td>
<td>–</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase reaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dihydrolase activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pectolytic activity</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth at 41 °C</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth at 4 °C</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Saccharate</td>
<td>–</td>
<td>+/-</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Trehalose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>meso-Inositol</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Creatine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DL-Carnitine</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mucate</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* +, 80% or more strains positive; –, 80% or more strains negative; +/-, positive or negative depending on clusters.

Compiled from Holt *et al.* (1994); Molin & Ternström (1982); Molin *et al.* (1986); Shaw & Latty (1982); Ternström *et al.* (1993); Tryfinopoulou *et al.* (2002).
characteristics useful for differentiation among these five biovars are summarized in Table 19.2. *P. fluorescens* belonging to various biovars have been found associated with fresh produce, including spinach (Babic *et al.*, 1996), lettuce (Bolin *et al.*, 1977; Magnuson *et al.*, 1990; King *et al.*, 1991), cabbage (Geeson, 1979; Brocklehurst and Lund, 1981), potato (Sands and Hankin, 1975; Cuppels and Kelman, 1980), tomatoes (Bartz, 1980), endive (Nyuyen-the and Prunier, 1989; Carlin *et al.*, 1995; Bennik *et al.*, 1996), salad vegetables (Brocklehurst *et al.*, 1987) and baby carrot (Liao and Fett, 2001). Biovar II (biotype B) is the most common biovar isolated from fresh and rotted produce (Table 19.3) and from root rhizospheres (Stenström *et al.*, 1990).

Pectolytic strains of *P. fluorescens* biovar II are often referred to as *P. marginalis* by plant pathologists and are well known for their ability to cause plant diseases in the field and to cause soft rot of fresh produce after harvest (Lelliott *et al.*, 1966). Although biovar II is considered the primary pseudomonad responsible for soft rot of fresh produce, other biovars of *P. fluorescens* are also involved (Table 19.3). For example, studies in our laboratory (Liao and Wells, 1987a) have shown that over 40% of post-harvest rot of fresh produce can be caused by oxidase-negative *P. viridiflava* and at least three *P. fluorescens* biovars (I, II and IV). *P. viridiflava* is also a field pathogen (Braun-Kiewnick and Sands, 2001), which is closely related

Table 19.2  Differentiation of five *Pseudomonas fluorescens* biovars*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>bv. I</th>
<th>bv. II</th>
<th>bv. III</th>
<th>bv. IV</th>
<th>bv. V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diffusible, non-fluorescent pigment</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Levan formation</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pectolytic activity</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin liquefication</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>v</td>
</tr>
<tr>
<td>Butyrate</td>
<td>–</td>
<td>v</td>
<td>v</td>
<td>+</td>
<td>v</td>
</tr>
<tr>
<td>Adonitol</td>
<td>+</td>
<td>–</td>
<td>v</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Ethanol</td>
<td>–</td>
<td>+</td>
<td>v</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>v</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>–</td>
<td>v</td>
<td>v</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Erythritol</td>
<td>v</td>
<td>v</td>
<td>+</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>L(+)-Tartrate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>v</td>
</tr>
</tbody>
</table>

* +, 80% or more strains positive; –, 80% or more strains negative; v, between 21 and 79% of strains positive
to *P. syringae*, one of the most important plant pathogens in the genus *Pseudomonas*.

In addition to pectolytic *P. fluorescens* and *P. viridiflava*, other species of *Pseudomonas* are also known to cause spoilage in specific types of produce. For example, an outbreak of spoilage of onion bulbs was found to be caused by *P. aeruginosa*, the type species of the genus *Pseudomonas* (Cother, 1976). In addition, an outbreak of mushroom blight was found to be caused by *P. tolaasii* (Wong and Preece, 1979). *P. tolaasii* is very similar to *P. fluorescens* but can be readily distinguished from it since *P. tolaasii* exhibits a positive reaction in ice nucleation and a negative reaction in levan and arginine dihydrogenase tests (Braun-Kiewnick and Sands, 2001). *P. cichorii* is another fluorescent pseudomonad known to be associated with spoilage in specific types of produce such as lettuce (Ceponis, 1974). *P. cichorii* is negative for arginine dihydrogenase but positive for oxidase.

The spoilage of fresh produce can also be caused by other genera of bacteria related to *Pseudomonas* taxonomically or ecologically. For example, *Burkholderia cepacia* has been shown to cause sour skin of onion bulb (Yabunchi et al., 1992) and *B. gladioli* to cause gladiolus corm rot (Hildebrand et al., 1973) and mushroom soft rot (Gill and Tsuneda, 1997). Additionally, pectolytic *Erwinia*, in particular *E. carotovora* subsp. *carotovora*, are considered the most common and the most destructive soft-rotting organisms associated with spoilage of fresh produce in the field or stored at ambient temperatures (Lund, 1983). Pectolytic pseudomonads are generally considered the primary cause of the spoilage of fresh produce stored at refrigeration temperatures.

### Table 19.3 Association of various biovars of *Pseudomonas fluorescens* with fresh produce and plant rhizospheres

<table>
<thead>
<tr>
<th>Sources</th>
<th><em>P. fluorescens</em> bv. identified</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotted fruits and vegetables</td>
<td>biovars II, &amp; V</td>
<td>Liao and Wells (1987a)</td>
</tr>
<tr>
<td>Potato tubers</td>
<td>biovars II &amp; V</td>
<td>Cuppels and Kelman (1980)</td>
</tr>
<tr>
<td>Potato tubers, rhizosphere, and leaf tissue</td>
<td>biovar II</td>
<td>Sands and Hankin (1975)</td>
</tr>
<tr>
<td>Potato seed tubers</td>
<td>biovars I, II, IV &amp; V</td>
<td>Sampson and Hayward (1971)</td>
</tr>
<tr>
<td>Fresh-cut lettuce</td>
<td>biovars I, II, III &amp; V</td>
<td>Magnuson et al. (1990)</td>
</tr>
<tr>
<td>Fresh-cut spinach</td>
<td>biovars I, III &amp; V</td>
<td>Babic et al. (1996)</td>
</tr>
<tr>
<td>Rotted celery and cabbage stored at 1°C</td>
<td>biovars II &amp; V</td>
<td>Brocklehurst and Lund (1981)</td>
</tr>
<tr>
<td>Read-to-use endive stored at 10°C for 10 days</td>
<td>biovars I, II, III &amp; V</td>
<td>Nguyen-the and Prunier (1989)</td>
</tr>
<tr>
<td>Tomato roots</td>
<td>biovars I, II, IV &amp; VI*</td>
<td>Stenström et al. (1990)</td>
</tr>
</tbody>
</table>

* See Barrett et al. (1986) for a description of biovar VI, which is closely related to biovars I and II.
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(Brocklehurst and Lund, 1981; Liao and Wells, 1987a). These pseudomonads are psychrotrophic and capable of growing and inducing soft rot of fresh produce at 10°C or below. Pectolytic *Erwinia* species, however, do not grow and induce soft rot of fresh produce stored at refrigeration temperature.

19.2.2 Characteristics of *Xanthomonas campestris* associated with spoilage of fresh produce

*Xanthomonas* is one of more than ten genera now included in the family Pseudomonadaceae. *Xanthomonas* species can be readily distinguished from pseudomonads based on their mucoid and yellow-pigmented phenotype and their requirement of special nutrients for growth. More than 11 species and over 100 pathovars within the genus *Xanthomonas* have been identified (Schaad et al., 2001). Among all *Xanthomonas* species are plant pathogens capable of causing diseases in the form of canker, necrosis or gall. Only a few strains of pectolytic *Xanthomonas* have so far been found associated with spoilage of fresh produce. The xanthomonads isolated from rotted tomatoes, bell pepper, cucumber, and papaya have been identified as *X. campestris* (Liao and Wells, 1987b). Although these xanthomonads are capable of causing soft rot of bell pepper under laboratory conditions (Liao and Wells, 1987b), they possibly attack plants only in the presence of a more aggressive pathogen such as *P. fluorescens* or *E. carotovora* under natural conditions (Nguyen-the and Carlin, 1994).

19.2.3 Characteristics of pseudomonads associated with spoilage of animal food products

Phenotypic and molecular characterization of the psychrotrophs isolated from fresh and spoiled meat (Molin and Ternström, 1982; Shaw and Latty, 1982; Banks and Board, 1983), lamb (Prieto et al., 1992), bovine milk (Shelley et al., 1987; Ternström et al., 1993), goat milk (Cox and MacRae, 1989), cheese (Gennari and Dragott, 1992), fish (Stenström and Molin, 1990) and poultry (Arnaut-Rollier et al., 1999) revealed the presence of three major species of *Pseudomonas* (*P. fragi*, *P. fluorescens* and *P. lundensis*). These three species can be differentiated based on a few physiological tests and their ability or inability to utilize hydroxyl-L-proline, D-mannitol, mucate, and D-quinate, as carbon or energy sources as summarized in Table 19.1.

*Pseudomonas fluorescens*

Unlike the well-defined *P. aeruginosa*, strains of *P. fluorescens* associated with plants or animals are extremely heterogeneous in terms of their phenotypic and molecular characteristics. Taxonomic analysis of *P. fluorescens* strains isolated from fresh and spoiled meat (Shaw and Latty, 1982) indicated that they did not match with the five biovar classification scheme reported by Stanier et al. (1966) and Palleroni (1984). Subsequent analyses of the
Pseudomonas and related genera

Pseudomonads isolated from fish fillets (Molin et al., 1982) and pork sausage (Banks and Board, 1983) confirmed the inadequacy of using the existing five-biovar scheme for classification of the pseudomonads associated with proteinaceous or muscle foods. The internal division of P. fluorescens strains originated from animal-derived foods into five biovars thus needs to be further revised. The inadequacy of this classification scheme results mainly from the fact that pseudomonads associated with fresh and spoiled animal food products including P. fragi and P. lundensis were not included in the study of Stanier et al. (1966). A recent study of the pseudomonads associated with plants and animals indicated that each P. fluorescens biovar may be elevated to a separate species (Barrett et al., 1986). In fact, a well-defined and tightly linked cluster within P. fluorescens biovar V was identified and designated as a new species ‘P. lundensis’ (Molin et al., 1986; Barrett et al., 1986).

Pseudomonas fluorescens biovars I and III, often in combination with P. fragi, and P. lundensis, constitute a dominant component of the microflora associated with various types of foods including milk (Ternström et al., 1993; Wiedmann et al., 2000; Gunasekera et al., 2003; Dogan and Boor, 2003), meat (Molin and Ternström, 1982; Shaw and Latty, 1982, 1984, 1986; Borch et al., 1996), poultry (Barnes and Impey, 1968; McMeekin and Patterson, 1975; McMeekin, 1977; Russell, 1998; Arnaut-Rollier et al., 1999), and fish (García-López et al., 2004). Non-fluorescent and non-motile P. fragi account for up to 50% and P. lundensis for up to 40% of the total pseudomonads isolated. In addition to P. fluorescens, P. fragi and P. lundensis, occurrence of other pseudomonads such as P. aeruginosa, P. putida and P. aureofaciens on fresh and spoiled foods is not uncommon (Table 19.4).

Pseudomonas fragi
As a member of the genus Pseudomonas (sensu stricto) and a member of rRNA similarity group I, the wild-type strains of P. fragi are non-fluorescent and non-motile (García-López et al., 2004). However, atypical P. fragi strains that are fluorescent have been isolated from raw milk (Wiedmann et al., 2000) and spoiled fish (Molin and Ternström, 1986; García-López et al., 2004). Nevertheless, the vast majority of P. fragi strains so far examined are flagellated, although the motility of these flagellated strains is not readily detectable.

Pseudomonas fragi constitutes a regular and major component of the native microflora associated with fresh and spoiled foods. It accounts for 61% of the meat pseudomonads examined by Molin and Ternström (1982) and 76–79% of the pork pseudomonads examined by Shaw and Latty (1982, 1984). This pseudomonad is characterized by its ability to produce ‘fruity off-odor’ and to form an acid ring in litmus milk. The classification of this organism is largely based on the numerical analysis of phenotypic and molecular properties. One of the most comprehensive studies on the taxonomy of this organism was reported by Molin and Ternström (1986). They examined a
### Table 19.4  Association of major *Pseudomonas* species with animal-derived foods

<table>
<thead>
<tr>
<th>Food types</th>
<th><em>Pseudomonas</em> spp. identified</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw &amp; pasteurized milk</td>
<td><em>P. fluorescens</em> biovars I &amp; III (70%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Ternström <em>et al.</em> (1993)</td>
</tr>
<tr>
<td></td>
<td><em>P. fragi</em> (20%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. lundensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. fragi</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td></td>
</tr>
<tr>
<td>Spoiled fish</td>
<td><em>P. fragi</em> (&gt;30%)</td>
<td>Stenström &amp; Molin (1990)</td>
</tr>
<tr>
<td></td>
<td><em>P. lundensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em> biovar III</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td></td>
</tr>
<tr>
<td>Sea bream fish</td>
<td><em>P. lundensis</em> (40%)</td>
<td>Tryfinopoulou <em>et al.</em> (2002)</td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. fragi</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td></td>
</tr>
<tr>
<td>Spoiled meat</td>
<td><em>P. fragi</em> (&gt;50%)</td>
<td>Molin &amp; Ternström (1982)</td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em> biovars I, II &amp; III</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. aureofaciens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td></td>
</tr>
<tr>
<td>Spoiled beef, pork, lamb</td>
<td><em>P. fragi</em> (&gt;70%)</td>
<td>Shaw &amp; Latty (1984)</td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em> biovars I &amp; III</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td></td>
</tr>
<tr>
<td>Spoiled meat, soil, water</td>
<td><em>P. fragi</em> (clusters A, B1, B2 &amp; B3) (&gt;50%)</td>
<td>Molin &amp; Ternström (1986)</td>
</tr>
<tr>
<td></td>
<td><em>P. lundensis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em> biovars I, II, III, &amp; IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. aureofaciens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td>Spoiled poultry</td>
<td><em>P. fragi</em></td>
<td>Russell <em>et al.</em> (1995)</td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. lundensis</em></td>
<td></td>
</tr>
</tbody>
</table>

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A total of 305 pseudomonad isolates from meat, fish and other environments and later divided them into ten major clusters. The ‘*P. fragi* cluster’ consisting of 131 isolates was named collectively as ‘*P. fragi* complex’. This complex could be further divided into four subgroups designated A, B1, B2 and B3. Subgroup A representing the type strain of this species was referred to as *P. fragi* (*sensu stricto*). Members of subgroup B3 are fluorescent and very similar to a tightly-linked cluster within biovar V of *P. fluorescens* previously identified by Barrett *et al.* (1986).

*Pseudomonas fragi* strains have been found in many different types of foods, including milk (Cox and MacRae, 1989; Ternström *et al.*, 1993;
Pseudomonas and related genera

Gunasekera et al., 2003; Dogan and Boor, 2003), fish (Shewan et al., 1960; Shaw and Shewan, 1968; Stenström and Molin, 1990; Gennari et al., 1999), poultry (Barnes and Imprey, 1968; García-López et al., 2004; McMeekin, 1977; McMeekin and Patterson, 1975) and meats (García-López et al., 2004; Molin and Ternström, 1982; Shaw and Latty, 1982, 1984). A large proportion of these pseudomonads are capable of producing extracellular proteases, lipases, exopolysaccharide slime, and ‘fruity off-odors’.

Pseudomonas fragi is believed to play an important role in the spoilage of milk and dairy products (Tryfinopoulou et al., 2002). It often co-exists with other psychrotrophs such as P. fluorescens, P. lundensis, S. putrefaciens or/and Psychrobacter immobilis on fresh and spoiled foods (Table 19.4). During the study of the microflora associated with milk, the same taxa of P. fluorescens have been recovered from both raw and pasteurized milk (Ternström et al., 1993). The Pseudomonas contaminants in pasteurized milk possibly originate from the recontamination of the finished product with raw milk.

Pseudomonas lundensis

Phenotypic and molecular characterization of the pseudomonads isolated from fresh and spoiled meat (Molin and Ternström, 1986), fish (Tryfinopoulou et al., 2002), poultry (Arnaut-Rollier et al., 1999) and milk (Ternström et al., 1993) revealed the presence of a distinct species of Pseudomonas now designated ‘P. lundensis’. The wild-type strains of P. lundensis are fluorescent and motile. As a member of the genus Pseudomonas (sensu stricto) and a member of rRNA similarity group I, P. lundensis is indistinguishable from a tight cluster within biovar V of P. fluorescens previously identified by Molin and Ternström (1982). P. lundensis is also closely related to P. fragi subgroup B3 identified earlier by Molin and Ternström (1986). However, P. lundensis strains can be differentiated from other pseudomonads based on their ability or inability to denitrify, to form levan, and to utilize trehalose, creatine, D-mannitol and mucate (Table 19.1). In addition, P. lundensis can be distinguished from P. fragi based on the inability of the later to produce fluorescent pigment and to display motility. Association of P. lundensis and other pseudomonads with milk (Ternström et al., 1993), fish (Stenström and Molin, 1990; Tryfinopoulou et al., 2002), meat (Molin and Ternström, 1986) and poultry (Russell et al., 1995) is summarized in Table 19.4.

19.2.4 Characteristics of Shewanella putrefaciens associated with spoilage of chilled fish and meat

The genus Shewanella comprises more than 20 species and has been found in a wide range of environments including fishery foods, oil field wastes, and interfaces of marine and fresh water. The type species of this genus now designated ‘Shewanella putrefaciens’ was first isolated from tainted butter and identified as an active spoilage agent. S. putrefaciens is a Gram-negative straight or curved rod, which exhibits a positive reaction in the oxidase test
and is motile by polar flagella. As a non-fermentative rod, *S. putrefaciens* often forms pink colonies on agar media and produces volatile off-odors when grown under anaerobic condition (MacDonell and Colwell, 1985; Jørgensen and Huss, 1989).

*Shewanella putrefaciens* was first identified as ‘Achromobacter putrefaciens’ and later reclassified as ‘Pseudomonas putrefaciens’ by Shewan et al. (1960). The G+C content of *P. putrefaciens* DNA was estimated to be in the range of 43–48 mole %, which was much lower than the typical G+C content of *Pseudomonas* DNAs (58–71 mole %) being determined. Primarily based on this finding, Lee and Shewan (1977) proposed transferring this bacterium from the genus *Pseudomonas* to *Alteromonas* and renaming it as *Alteromonas putrefaciens*. Later, Van Landschoot and De Ley (1983) analyzed the 5S RNA sequences of the type species of *Alteromonas* and *A. putrefaciens* and found that they were distantly related. A new genus ‘Shewanella’ and a new name ‘*S. putrefaciens*’ for this bacterium was proposed (MacDonell and Colwell, 1985) and is still used today.

*Shewanella putrefaciens* strains can be found in a wide variety of foods including meat (Molin and Ternström, 1982), fish (Shewan et al., 1960; Lee and Shewan, 1977; Stenström and Molin, 1990), and also in other environments including oil fields (Semple and Westlake, 1987), surfaces of processing equipments (Bagge et al., 2001) and clinical specimens (Riley et al., 1972; Jorens et al., 2004). They are considered the primary cause of spoilage of chill-stored foods such as sardine fish (Gram and Huss, 1996; Gennari et al., 1999) and meat (Molin and Ternström, 1982). *S. putrefaciens* strains isolated from fresh and spoiling foods are characterized by their ability to produce H2S and volatile off-odors due to compounds such as trimethylamines (TMA).

### 19.3 Detection and enumeration of *Pseudomonas*, *Xanthomonas* and *Shewanella* species

#### 19.3.1 Use of selective agar media

*For isolation of Pseudomonas*

Selective agar media useful for detection and enumeration of *Pseudomonas* spp. are summarized in Table 19.5. The selective agents commonly used to inhibit the growth of non-*Pseudomonas* flora present in water, soil and foods include cetrimide, dyes (fuchsin, crystal violet or fucidin), and antibiotics (erythromycin, chloramphenicol, nalidixic acid, cycloheximide, novobiocin or penicillin). These antimicrobials are usually added at a concentration just sufficient to suppress the growth of non-*Pseudomonas* flora. King’s medium B and medium A (King et al., 1954) commercially available as *Pseudomonas* agar F and P (Difco), respectively, were commonly used as diagnostic media for detection of strains that produce fluorescent pigment (fluorescin) such as
Table 19.5  Selective agar media for *Pseudomonas* spp.

<table>
<thead>
<tr>
<th>Media</th>
<th>Selective agents</th>
<th>Mode of action or targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>King A</td>
<td>Magnesium and sulfate ions</td>
<td>Promote the production of pyocyanin (non-fluorescent, blue pigment) by <em>P. aeruginosa</em></td>
<td>King <em>et al.</em> (1954)</td>
</tr>
<tr>
<td>King B</td>
<td>Phosphate ions</td>
<td>Promote the production of fluorescein (green fluorescent pigment) by <em>Pseudomonas</em> spp.</td>
<td>King <em>et al.</em> (1954)</td>
</tr>
<tr>
<td>Irgansan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>King A + Irgasan&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Suppress Gram-positive and is not active against <em>Pseudomonas</em>, is useful for isolation of <em>P. aeruginosa</em></td>
<td>Available as <em>Pseudomonas</em> Isolation Agar from Difco Lab.</td>
</tr>
<tr>
<td>MGV</td>
<td>Erythromycin + chloramphenicol</td>
<td>Is selective for isolation of <em>Pseudomonas</em> spp.</td>
<td>Masourovsky <em>et al.</em> (1963)</td>
</tr>
<tr>
<td>C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Cetrimide</td>
<td>For isolation of <em>P. aeruginosa</em></td>
<td>Brown and Lowbury (1965)</td>
</tr>
<tr>
<td>CN&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Cetrimide + nalidixic acid</td>
<td>For isolation of <em>P. aeruginosa</em> and others</td>
<td>Goto and Enomoto (1985)</td>
</tr>
<tr>
<td>CFC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cetrimide + fucidin + cephaloridine</td>
<td>For isolation of <em>Pseudomonas</em> spp.</td>
<td>Mead and Adams (1977)</td>
</tr>
<tr>
<td>NC</td>
<td>Nitrofluration + crystal violet</td>
<td>Inhibit Gram-positive bacteria</td>
<td>Krueger and Sheihk (1987)</td>
</tr>
<tr>
<td>MGSP</td>
<td>Modified glutamate-starch-phenol red medium + irgansan</td>
<td>Selective for <em>Pseudomonas</em> spp. in general</td>
<td>Flint and Hartley (1996)</td>
</tr>
<tr>
<td>NPC</td>
<td>Novobiocin + penicillin + cycloheximide</td>
<td>Inhibit fungi, Gram-positive and non-<em>Pseudomonas</em> Gram-negative bacteria</td>
<td>Sands and Rovira (1970)</td>
</tr>
<tr>
<td>FPA</td>
<td>NPC base (above) + pectate</td>
<td>For isolation of pectolytic fluorescent <em>Pseudomonas</em> spp.</td>
<td>Sands <em>et al.</em> (1972)</td>
</tr>
<tr>
<td>ACC</td>
<td>Ampicillin + chloramphenicol + cycloheximide</td>
<td>For isolation of <em>Pseudomonas</em> spp. in water</td>
<td>Simon and Ridge (1974)</td>
</tr>
<tr>
<td>FCTNN</td>
<td>Fuchsin + cycloheximide + Triphenyl tetrazolium chloride + nitrofuratoin + nalidixic acid</td>
<td>For isolation of pseudomonads in natural inhabitats</td>
<td>Grant and Holt (1977)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Irgasan: a trademark of Ciba-Geigy Inc.

<sup>b</sup>Available as Pseudosel (Difco/BD Sys., Sparks, MD, USA), Citrimide Agar (Oxoid Ltd., Hampshire, UK), or Centrimide Agar Base (Sigma Chem., St. Louis, MO, USA).

<sup>c</sup>Selective agents (cetrimide and nalidixic acid): available as C-N Supplement from Oxoid Ltd.

<sup>d</sup>Selective agents (cetrimide, fucidin and cephaloridine): available as C-F-C Supplement from Oxoid Ltd. or as C.F.C. Supplement from Sigma Chem.
P. fluorescens or strains that produce non-fluorescent pigment (pyocyanin) such as P. aeruginosa. King’s media B and A and other Pseudomonas base media (Oxoid) can be supplemented with cetrimide, Irgasan® or nalidixic acid to improve the detection of P. fluorescens, P. aeruginosa and other pseudomonads in water, soil, foods and clinical specimens.

Pseudomonas CFC agar containing cetrimide, fucidin and cephaloridin (Mead and Adams, 1977) is a selective medium commonly used for isolation of psychrotrophic pseudomonads from salad vegetables (Brocklehurst and Lund, 1981), milk (Flint and Hartley, 1996), meat (Shaw and Latty, 1984), fish (Tryfinopoulou et al., 2001) and poultry (Mead, 1985). Use of Pseudomonas CFC agar prevents the growth of the vast majority of non-Pseudomonas flora, including Enterobacteriaceae and Gram-positive Bacillus and Lactobacillus spp., and shows minimal effect on the growth of spoilage-related pseudomonads, including P. fluorescens, P. fragi, P. lundensis, P. aerugionos, and P. putida.

Application of selective agars for isolation of pseudomonads is a technique commonly used to assess the hygiene of food processing facilities and microbiological quality of foods. However, the shortcomings associated with the use of this technique cannot be ignored, which include insufficient selectivity of the media and failure to recover viable but non-culturable (VBNC) or sublethally injured pseudomonads. Although occurrence of VBNC pseudomonads directly related to spoilage has not been thoroughly investigated, presence of injured bacteria in pasteurized milk or on acid-washed beef carcasses has been reported (Hart and Kite, 1977). Injured P. fluorescens cells, as indicated by their inability to resuscitate and grow on selective agar media, can be induced by exposing the bacteria to heating at 36°C for 2h (Gray et al., 1973).

For isolation of Xanthomonas Xanthomonas strains capable of causing spoilage of salad vegetables and fruits can grow on pectate agar media (discussed below) commonly used for isolation of soft-rotting Pseudomonas and Erwinia. Xanthomonas strains, however, can be readily differentiated from pseudomonads based on their ability to produce yellow-pigmented xanthomonadin and mucoid xantham gum. Like other xanthomonads, soft-rotting Xanthomonas are unable to grow in the minimum medium without the addition of organic supplements. The xanthomonads are sensitive to those antimicrobials incorporated in Pseudomonas selective agars, for example triphenyl tetrazolium chloride and others (Table19.5). For isolation of plant-pathogenic Xanthomonas, a number of antimicrobials such as cycloheximide methyl green and vancomycin are frequently used. A number of selective agar media for isolation of a specific species or pathovars of Xanthomonas are available (Schaad et al., 2001). It has not been determined if these selective agars are suitable for isolation of soft-rotting strains of xanthomonads.
For isolation of Shewanella
Members of the genus *Shewanella* (family Vibrionaceae) are widespread in nature and can be found in fresh and spoiling foods, oil field wastes, cold water, sediments of the deep sea, and clinical specimens. As the type species of the genus, *S. putrefaciens* has been found associated with spoilage of many different types of foods including meat, poultry, milk and fish. *S. putrefaciens* produces hydrogen sulfide (H$_2$S) when grown on thiosulfate or polysulfide (Skjerdal *et al*., 2004). This bacterium can also use nitrate sulfur and iron oxide as electron acceptors for respiratory growth. However, *S. putrefaciens* is unable to grow by glycolysis or fermentation and is unable to utilize lactate, pyruvate, formate and amino acids as sole carbon source. These metabolic traits can serve as diagnostic means for detection of this organism. In addition, production of H$_2$S has become a valuable trait for detection of sulfide-producing bacteria on fish (Skjerdal *et al*., 2004). The rate of H$_2$S production by *S. putrefaciens* on chill-stored fish can be used as a parameter to measure the growth of spoilage bacteria and to predict the shelf-life of chill-stored fish (Tryfinopoulou *et al*., 2001; Malle *et al*., 1998).

For isolation of pectolytic or proteolytic bacteria
Pectolytic strains of *P. fluorescens* and *P. viridiflava* are believed to be the primary cause of spoilage of fresh produce stored in air and at refrigeration temperature. They are responsible for over 40% of postharvest rot of fresh produce at wholesale and retail markets (Liao and Wells, 1987a). Several selective media for isolation of pectolytic bacteria including pseudomonads, xanthomonads, erwinias and bacilli have been described (Schaad *et al*., 2001). The CVP (crystal violet-pectate) medium is by far the most common among the pectate media developed for isolation of pectolytic organisms (Cuppels and Kelman, 1980; Liao and Wells, 1987b). *Pseudomonas* CFC medium supplemented with pectate is also useful for isolation of pectolytic pseudomonads from soils or rhizospheres (Sands *et al*., 1972). The *Pseudomonas* base medium supplemented with selective antimicrobials and gelatin (or skim milk) is useful for detection of proteolytic pseudomonads associated with plant diseases (Schaad *et al*., 2001) and with food spoilage.

19.3.2 PCR, ribotyping, rRNA sequencing and impediometry
A number of DNA-based PCR (polymerase chain reaction) and hybridization methods for detection of spoilage pseudomonads and *S. putrefaciens* have recently been investigated and reviewed (van der Vossen and Hofstra, 1996). The oligonucleotide probes for detection of pseudomonads associated with milk (Gunasekera *et al*., 2003), fish (Gutiérrez *et al*., 1997, 1998; García-López *et al*., 2004), meat (Gutiérrez *et al*., 1998) and environmental samples (Widmer *et al*., 1998) have been reported. The oligonucleotide primers for detection of *S. putrefaciens* associated with spoiled foods or with iron/manganese dissimilation (Wallner *et al*., 1996; DiChristina and DeLong,
19.3.3 Carbohydrate utilization patterns, fatty acid profiles and monoclonal antibodies

Physiological characterization and carbohydrate assimilation tests are a classical and standard procedure for identification of pseudomonads. The method and determinative scheme originally described by Stanier et al. (1966) and later modified by Molin and Ternström (1986) and Shaw and Latty (1984) may be followed. The tests required can be performed either manually or using the automated identification kits available commercially. The Biolog GN MicroPlate® and API 20 NE Systems (The bioMérieux Industry, Hazelwood, MO) can be used for identification of *P. fluorescens*, *P. lundensis* and *P. fragi*. 

Unfortunately, attempts to monitor the changes in the number of pseudomonads in raw milk by the impedimetry method have met with limited success (Schulenburg and Bergann, 1998).
associated with meat (Grimont et al., 1996), poultry (Arnaut-Rollier et al., 1999), milk (Wiedmann et al., 2000; Dogan and Boor, 2003) and fish (García-López et al., 2004). The reproducibility of carbohydrate assimilation tests was estimated to be 95.6% (Molin and Ternström, 1986).

The identity of pseudomonads associated with food spoilage can also be determined by analyzing the cellular fatty acid compositions (Stead, 1992). An automated system for identification of the fatty acid composition of bacteria by using the high-resolution gas chromatography is commercially available (MIDI, Microbial ID Inc., Newark, DE). The unique fatty acid profiling preserved for each specific group of bacteria has been successfully used to identify the pseudomonads from chill-stored chicken (Sundheim et al., 1998) and red meat (Vancanneyt et al., 1996).

The outer membrane proteins of Pseudomonas can be used as a target protein for detection of pseudomonads. The lipoprotein I and the gene coding for this protein can be used to differentiate fluorescent and non-fluorescent pseudomonads (De Vos et al., 1993). The outer membrane protein OprF and the antibody against this protein can be used to detect member pseudomonads in rRNA similarity group I (Kragelund et al., 1996). Polyclonal antibodies against outer membrane protein F and live cells of P. fluorescens can also be used to detect pseudomonads in refrigerated meat (González et al., 1994, 1996). Monoclonal antibodies against a specific outer membrane protein of Pseudomonas have been developed and used to detect pseudomonads in refrigerated meat by indirect ELISA (Gutiérrez et al., 1997).

19.4   Factors affecting the survival and growth of Pseudomonas and Shewanella

The survival and growth of spoilage-specific bacteria on foods can be affected by several factors including temperature, pH, water activity, modified atmosphere and competing microflora. The effect of each individual factor on the fate of spoilage bacteria on foods or in vitro will be discussed in more detail below. To determine the combined effect of these factors on bacterial growth and spoilage development, several mathematical models (for examples, Neumeyer et al., 1997; Braun and Fehlhaber, 2003; Braun and Sutherland, 2003) have been proposed.

19.4.1 Temperature

Pseudomonas and Shewanella species associated with spoilage of refrigerated foods are psychrotrophic and capable of forming colonies at 0–7 °C (Brocklehurst and Lund, 1981; Shewan et al., 1960). Pectolytic P. fluorescens and P. viridiflava associated with spoilage of fresh produce are able to grow on fresh produce that are normally stored at 10 °C or below. The mesophilic pseudomonads often associated with plants or animals including P. aeruginosa
and *P. corrugata* do not grow at 10°C or below but do grow at 41°C. In contrast, the psychrotrophic pseudomonads and *S. putrefaciens* associated with spoilage of refrigerated foods are sensitive to higher temperatures and unable to grow at temperatures exceeding 37°C.

### 19.4.2 Atmospheric composition

Growth and survival of spoilage-specific microbes are greatly affected by the gaseous composition of the atmosphere surrounding foods (Ibe and Grogan, 1983; Babic and Watada, 1996; Bennik *et al.*, 1996; Hao and Brackett, 1993). High concentrations of CO$_2$ (up to 10%) inhibit the growth of *P. fluorescens* and *P. fragi* on red meats (Gill and Tan, 1980), chicken carcasses (Gill *et al.*, 1990), fish fillets (Molin *et al.*, 1982), and also inhibit the growth of *S. putrefaciens* on fishery products (Gram and Huss, 1996). Packing of spinach leaves in the bags containing a high concentration of CO$_2$ or a low concentration of O$_2$ also reduced the number of pseudomonads by 10- to 100-fold as compared to those packed in air (Babic and Watada, 1996). Nguyen-the and Carlin (1994) showed that the inhibitory effect of a high concentration of CO$_2$ on the growth of mesophilic bacteria on chicory leaves was observed only when the packages were stored at 2 to 6°C, but not at 10°C. Berrang *et al.* (1990) also noticed that the inhibitory effect of high concentrations of CO$_2$ on the growth of pseudomonads on broccoli was observed at 4°C but not at 10°C.

### 19.4.3 Water activity

Water activity ($a_w$) is another important factor limiting the survival and growth of spoilage and pathogenic bacteria on fresh foods or in the environment. Food-associated bacteria are in general more sensitive to low $a_w$ than to high $a_w$. The pseudomonads and *S. putrefaciens* are more often found on the surfaces of fresh meat, fish and vegetables with high $a_w$ (0.99 or higher). Pectolytic pseudomonads associated with produce spoilage have been shown to survive in pure distilled water for at least 15 years at room temperature (Liao and Shollenberger, 2003). However, pseudomonads and shewanellae showed no sign of growth at $a_w$ 0.91. The minimal $a_w$ value required for their growth is in the range of 0.95–0.97, which is dependent on the types of foods tested or the type of salts or sugars used as solutes in the medium. For example, *P. fluorescens* grew at a relatively lower $a_w$ if the culture medium $a_w$ was adjusted with glycerol than with sucrose or NaCl (Prior, 1978). The shelf-life of fresh-cut lettuce can be extended by reducing the moisture by centrifugation to delay the growth of spoilage organisms (Garg *et al.*, 1990). Even though spoilage pseudomonads are unable to grow at $a_w$ values lower than 0.95, they are able to survive on dry seeds ($a_w < 0.70$) for at least 4 years (Liao and Fett, 2001).
19.4.4 pH
The pHs of a vast majority of foods are within the range (5–7) suitable for the growth of spoilage and pathogenic bacteria. The minimum pH for the growth of P. fragi and S. putrefaciens was estimated to be 5.0 and 5.3, respectively (Jay, 1996). All seven major species of spoilage bacteria mentioned above are sensitive to low pH. For example, S. putrefaciens failed to grow in milk that had been acidified to pH 5.3. Because of their sensitivity to acidic pH, S. putrefaciens cells are more likely to cause spoilage in chicken legs (pH 6.4–6.7) (McMeekin, 1975) than in chicken breast (pH 5.7–5.9) (McMeekin, 1977). This observation indicates that a slight difference in the pH of foods has a crucial effect on the growth of bacteria and the development of spoilage. It is also worth noting that the pH minimum for the growth of spoilage Pseudomonas or Shewanella is dependent on the type of acidulant used. The use of citric, hydrochloric or phosphoric acid as acidulents usually permits the growth of spoilage organisms at a relatively lower pH compared with the use of acetic or lactic acid (Juven, 1976).

19.4.5 Native microflora
The growth of spoilage pseudomonads and S. putrefaciens on foods is greatly affected by the presence of antagonistic or beneficial native microflora. Growth of naturally occurring human pathogens, including Salmonella spp. and Listeria monocytogenes, on fresh or fresh-cut produce can also be inhibited or promoted by the presence of native fluorescent pseudomonads (Liao and Sapers, 1999; Liao et al., 2003a). A natural antagonist isolated from fish has been shown to retard the growth of S. putrefaciens by producing iron-chelating siderophores (Gram and Melchiorsen, 1996). The interactions between native and spoilage flora influence the composition and structure of the microbial community on fresh and spoiling foods. Prevalence of acetic or lactic acid bacteria such as Acinetobacter and Gluconobacter in acidic foods also effectively delays the development of spoilage caused by Pseudomonas and Shewanella species.

19.4.6 Antimicrobials
The presence of natural antimicrobials in plant- and animal-derived foods has been known for many years. Organic acids including short-chain fatty acids and fatty acid esters are two common classes of antimicrobials found in plant extracts. These natural compounds exhibit a broad spectrum of antimicrobial activity against pathogenic and spoilage bacteria. Acetic, citric, benzoic and sorbic acids are widely used as additives to suppress the growth of spoilage microorganisms in foods. The isothiocyanate (ITC) present in a variety of crucifer plants also exhibit a broad spectrum of antimicrobial activities (Delaquis and Mazza, 1995). The ITCs are in general more effective against Gram-negative than Gram-positive bacteria. The essential oils from
different types of plants including oregano inhibit the growth of a variety of pathogens including *Escherichia coli* O157:H7, *Listeria monocytogenes* and *P. aeruginosa* (Elgayyar *et al*., 2001). Hydroxycinnamic acid and its derivatives including coumaric, caffeic and chlorogenic acids are naturally present in plants and are also inhibitory against bacteria and fungi (Nguyen-the and Carlin, 1994). Cow and goat milk contain at least two antimicrobials ‘lactoferrin’ and ‘conglutinin’, which are highly inhibitory against *P. fluorescens* (Zapico *et al*., 1995).

19.5 Spoilage mechanisms employed by *Pseudomonas* and *Shewanella*

Meat and poultry begin to emit offensive odors when the bacterial population on the surface reaches $10^7$ cfu/cm$^2$ and exhibit a slimy appearance when the population reaches $10^8$ cfu/cm$^2$ (Whitfield, 1998). The odors are generated mainly from the emission of ethyl/methyl esters, short chain fatty acids or sulfide compounds (Huis in’t Veld, 1996). The slimy appearance results from the accumulation of the exopolysaccharides and the softening of food texture results from the action of degradative enzymes (pectinases, proteases and lipases) produced by spoilage bacteria. Production of these spoilage-related factors (odors, biofilm and depolymerases) is assumed to be mediated by at least three gene regulatory systems in *Pseudomonas* including transcriptional activator (RpoS), two-component regulators (GacS and GacA) and quorum-sensing regulators (LuxR and LuxI). These regulators are thought to act in concert to mediate the production of spoilage-related factors possibly in response to environmental stimuli such as nutritional starvation, extreme pH, or high temperature (see reviews by Miller and Bassler, 2001; Hebb and Haas, 2001; Dodd and Aldsworth, 2002).

19.5.1 Production of pectate lyases by pseudomonads and their role in spoilage of fresh vegetables and fruits

Pectolytic *P. fluorescens* and *P. viridiflava* are widespread in nature and can be found in diverse environments and habitats. They are the predominant component of the native microflora found on the surfaces of fresh fruits and vegetables. These pseudomonads account for up to 40% of the total aerobic counts isolated from various types of produce, including potato (Cuppels and Kelman, 1980), spinach (Babic *et al*., 1996), tomatoes (Brackett, 1988) and baby carrot (Liao and Fett, 2001). They are responsible for 43% of post-harvest rot of fresh produce stored at low temperatures (Liao and Wells, 1987a; Brocklehurst *et al*., 1987; Nguyen-the and Prunier, 1989).

The ability of fluorescent pseudomonads to cause soft rot is mainly due to production of pectin-degrading enzymes for degradation of plant cell walls.
Pseudomonas and related genera (Liao et al., 1988). Unlike the complex pectin-degrading enzyme system demonstrated in soft-rotting Erwinia (Py et al., 1998), pectolytic P. fluorescens and P. viridiflava produce a single pectate lyase (PL) with a pI of 9.7 (Liao, 1989). Only a very few strains of P. fluorescens so far examined produce polygalacturonases. A series of biochemical and molecular genetic studies, including PL enzyme purification and characterization (Liao et al., 1997) and analysis of PL structural and regulatory genes (Liao, 1991; Liao et al., 1993, 1994, 1996), have recently been carried out and reviewed (Liao and Ukuku, 2005). The use of isoelectric focusing electrophoresis and overlay enzyme activity staining (Liao, 1989) indicates that PL is the sole enzyme required for induction of tissue maceration or soft rot by most pectolytic pseudomonads.

19.5.2 Production of proteases and lipases by P. fluorescens and P. fragi and their role in spoilage of animal-derived foods

The ability of P. fluorescens, P. lundensis and P. fragi strains to cause spoilage of proteinaceous foods is in part due to their ability to produce proteases and lipases for degradation of protein or lipid components in meat, milk, poultry and seafood (Odagami et al., 1994). Production of proteases is also required for the growth of P. fluorescens on the surfaces of muscle foods (Fukao and Ohta, 1999). Although production of proteases and lipases has not been demonstrated in all food-related pseudomonads, the spoilage-associated strains appear to be more likely to possess such a characteristic. Production of multiple lipases (Dieckelmann et al., 1998) or multiple proteases (Fairbairn and Law, 1986) has been demonstrated in P. fluorescens strains associated with milk spoilage. A soft-rotting strain of P. fluorescens has been shown to produce a heat-stable protease, which is capable of causing gelation of raw milk but incapable of causing maceration of plant tissues (Liao and McCallus, 1998). A heat-stable protease produced by a psychrotrophic pseudomonad from raw milk is also able to cause spoilage of raw milk (Patel et al., 1983). This metalloprotease has a molecular weight of 40–50 kDa and a D value of 118 min at 70°C (Stepaniak et al., 1982; Fairbairn and Law, 1986).

The spoilage of meat, poultry, milk and fish can be caused by different ribotypes of Pseudomonas. A positive correlation between the spoilage development potential of Pseudomonas ribotype and their ability to produce and secrete proteases and lipases has been established (Wiedmann et al., 2000). Production of proteases and lipases by bacteria is required for degradation of milk casein, butterfat and phospholipids in dairy products. The lipases produced by P. fragi and P. fluorescens are also responsible for the rancid and bitter flavors in raw milk, cheese and other dairy products (Shelley et al., 1987).

19.5.3 Production of off-odors by Pseudomonas and Shewanella

Production of off-odor in spoiling meat and chicken carcasses results mainly
from the growth of *S. putrefaciens*, *P. fluorescens* and *P. fragi* (Hebert *et al*., 1971; McMeekin, 1977; Russell *et al*., 1995). The ‘sweet’ and ‘fruity’ odors detectable by gas chromatography (Miller *et al*., 1973; Freeman *et al*., 1976; Lee *et al*., 1979; Dainty, 1996) are produced by spoilage-specific pseudomonads such as *P. fragi*. As an active spoiler of meat and fish products, *S. putrefaciens* also produces unpleasant odors such as hydrogen sulfide (H₂S) and trimethylamine (TMA) (Hebert *et al*., 1971). Production of TMA by *S. putrefaciens* can be used as an indication for spoilage of cold-stored fish (Debevere *et al*., 2001). Since pseudomonads cannot utilize trimethylamine oxide (TMAO) as a terminal electron acceptor to produce TMA (Whitfield, 1998), production of TMA or sulfur compounds is not associated with the spoilage caused by *Pseudomonas* spp. (Huis in’t Veld, 1996).

### 19.6 Control of *Pseudomonas* and *Shewanella*

An array of chemical, physical and biological measures are available for controlling spoilage caused by *Pseudomonas* and *Shewanella* species on the surfaces of fresh and minimally processed foods. However, none of these treatments can completely eliminate the pseudomonads or shewanellae from the surfaces without affecting the organoleptic quality of food products. Several factors that may limit the effectiveness of the decontamination treatments have been identified and are required to be further investigated. Langsrud *et al*. (2003a, b) showed that, following repeated exposure of raw foods to disinfectants such as quaternary ammonium compounds, food-related *Pseudomonas* spp. gradually acquired resistance to disinfectants. Wirtanen *et al*. (2001a, b) demonstrated that formation of biofilms on stainless steel surfaces increased the tolerance of *P. aeruginosa*, *P. fragi* and *Salmonella Typhimurium* within the biofilms to disinfectants. The significance of biofilm formation in food industry has been reviewed (Kumar and Anand, 1998). Takeuchi *et al*. (2000) also showed that a small portion of *P. fluorescens* that came in contact with cut lettuce became internalized and escaped the disinfection treatments.

#### 19.6.1 Chemical interventions

**Ozone**

Use of ozone for reducing the spoilage microbes on meat, poultry and fish has been previously investigated. Sheldon and Brown (1986) reported that exposure of poultry carcasses to ozonated water killed *P. aeruginosa* and *Enterobacter faecalis* on the surfaces more effectively than exposure of the carcasses to chill water. Moore *et al*. (2000) showed that Gram-negative bacteria are in general more sensitive to ozone than Gram-positive organisms. Kim and Yousef (2000) demonstrated that exposure of *P. fluorescens*, *E. coli*
O157:H7 and *Listeria monocytogenes* to ozone at the concentration of 2.5 ppm for 40 s caused a 5–6 log reduction in bacterial counts. Restaino *et al.* (1995) also showed that exposure of suspended bacteria to ozonated water (0.19 mg/ml) for 5 min caused more than a 5 log decrease in the number of *P. aeruginosa* and *Salmonella Typhimurium*.

**Chlorine/chlorine dioxide**

*Pseudomonas* spp. and *S. putrefaciens* are highly sensitive to chlorine (Cl₂) and chlorine dioxide (ClO₂). The bactericidal activity of chlorinated compounds results largely from the formation of hypochlorous acid when Cl₂ or a hypochlorite salt (either sodium or calcium) is dissolved in water at pH 6.0–7.5. For surface decontamination of fresh foods, chlorinated compounds are usually applied at a concentration of 50–200 ppm and for a minimal contact time of 1 to 2 min to achieve approximately 2 log reductions in the population of bacteria (Hwang and Beuchat, 1995; Beuchat *et al.*, 1998). ClO₂ is also effective for killing spoilage and pathogenic bacteria. It has been approved for washing of raw fruits and vegetables at a concentration not exceeding 5 ppm and for sanitization of processing equipment at a concentration not exceeding 200 ppm. Treatment of shredded lettuce and cabbage with 5 ppm of ClO₂ can reduce the number of *L. monocytogenes* by more than 90% (Beuchat, 1998).

**Hydrogen peroxide**

H₂O₂ is generally recognized as safe (GRAS) and has been approved for application as a bleaching or antimicrobial agent in food processing. The potential of H₂O₂ as a surface decontaminant for fresh produce has been evaluated. Immersion of squash or cantaloupe slices in 5% H₂O₂ for 1 min has been shown to reduce the number of fluorescent *Pseudomonas* on the surfaces of fruits by 90% (Sapers and Simmons, 1998). The number of native bacteria on the surfaces of prunes can also be reduced to an undetectable level following exposure of the fruits to vaporized H₂O₂ for 10 min. Treatment of fresh-cut apple or bell pepper with a mixture of H₂O₂ and acetic acid can reduce the number of *Salmonella* spp. and *P. fluorescens* by almost 4 logs (Liao *et al.*, 2003b). The effectiveness of using H₂O₂ as a surface disinfectant for chicken carcasses has been demonstrated (Lillard and Thomson, 1983). However, H₂O₂ is not suitable for washing of animal carcasses because the interaction between H₂O₂ and catalases causes the discoloration or swelling of meat or poultry (Lillard and Thomson, 1983).

**Organic acid**

Organic acids including lactic, acetic, citric, malic, benzoic and sorbic acids are naturally present in plants or accumulated as fermentation products. These organic acids are GRAS and have been widely used as preservatives or surface disinfectants (Ouattara *et al.*, 1997). Sodium benzoate is usually added at the concentration of 0.1% to inhibit the growth of molds, yeasts and
spoilage *Pseudomonas*. Potassium sorbate is usually added at the concentration of less than 0.2% to prevent the growth of spoilage microbes (Robach and Sofos, 1982). Spraying animal carcasses with 6% lactic or 3% acetic acid has been found effective in reducing the number of spoilage pseudomonads and human-pathogenic bacteria on the surfaces of beef and chicken carcasses (Van der Marel *et al*., 1988; Dickson, 1992). Washing apple and bell pepper slices with a mixture of acetic acid and H$_2$O$_2$ reduced the number of spoilage bacteria (*Erwinia* and *Pseudomonas*) and *Salmonella* by almost 4 logs (Liao *et al*., 2003b; Liao and Shollenberger, 2004).

### 19.6.2 Physical interventions

**Modified atmospheres**
The storage of food in atmospheres containing increased amounts of CO$_2$ up to 10%, often referred to as ‘modified atmosphere’ (MA) storage, is an effective method for reducing the spoilage and extending the shelf-life of fresh and minimally processed foods. The principle of MA storage is to replace the air surrounding the foodstuff with a mixture of CO$_2$, O$_2$ and N$_2$. CO$_2$ is used to inhibit the growth of aerobic bacteria and molds and N$_2$ used to inhibit the oxidation of fats. O$_2$ is used primarily to prevent the growth of anaerobic bacteria and to maintain the organoleptic properties of foods. The effect of MA storage on the growth of foodborne microorganisms and on the development of spoilage has been reviewed (Gram and Huss, 1996; Sivertsvik *et al*., 2002). In general, the storage of foods under high concentrations of CO$_2$ or low concentrations of O$_2$ reduces the respiration rate and aerobic contaminants on the surfaces of foods (Brackett, 1987, 1988; Gill and Tan, 1980).

Application of high concentration of CO$_2$ (>10%) can inhibit the growth of aerobic microbes and extend the shelf-life of animal-derived food products (Tryfinopoulou *et al*., 2002). However, it is important to store the foods at 10°C or below to maximize the effect of increased concentrations of CO$_2$. Vacuum and shrink-wrap packaging can also be used to retard the growth of aerobic pseudomonads (Brackett, 1988; Enfores and Molin, 1980). For foodstuffs that are stored in vacuum or under high concentrations of CO$_2$, CO$_2$-tolerant psychrotrophs such as *Photobacterium phosphoreum* or lactic acid bacteria may replace *S. putrefaciens* or other spoilage-related bacteria such as *P. fluorescens*, *P. fragi* and *P. lundensis* as a dominant component in spoiled meat, poultry or fish (Gram *et al*., 1987; Dalgaard *et al*., 1993).

**Irradiation and other non-thermal technologies**
Application of ionizing irradiation ($^{60}$Co or $^{137}$Cs) for decontamination of red meat was approved for use by the US Food and Drug Administration (FDA) in 1997. The minimum irradiation of 1.5 kGy as permitted by FDA is effective in reducing the number of *S. putrefaciens* on the surfaces of beef, pork, turkey, and poultry (Thayer and Boyd, 1996). Irradiation of beef steaks
with 1.5 kGy of gamma or electron beam caused a 4–5 log decrease in the number of *P. fluorescens* on beef steaks (Chung et al., 2000). Prakash et al. (2000) showed that exposure to 1.0 kGy gamma irradiation reduced the number of inoculated bacteria on diced celery to an undetectable level and extended the shelf-life of treated products from 7 to 29 days if stored at 5°C. The $D_{10}$ values for *P. fluorescens* and *P. aeruginosa* were determined to be in the range of 0.5 to 1.0 kGy depending on the suspending medium and irradiation temperature (Isotron Inc., 2005).

In addition to irradiation, several other nonthermal intervention technologies have recently been evaluated for the potential of replacing the conventional thermal treatments. For example, processing of skim milk by pulsed electric field (PEF) can reduce the number of spoilage bacteria such as *P. fluorescens* by 0.3 to 3.0 logs (Michalac et al., 2003). Application of high hydrostatic pressure is another promising nonthermal technology for pasteurization of foods. García-Graells et al. (2003) showed a synergistic effect of the application of high-pressure and the lactoperoxidase system on the killing of spoilage pseudomonads and pathogenic bacteria in milk.

### 19.6.3 Biological interventions

Because of the safety risk concerned, numerous efforts have been made to limit or restrict the use of chemicals for washing or preservation of foods. The biological approach using microbial antagonists or essential oils from plants to control spoilage of foods has become a subject of extensive investigation. Growth of *P. fragi* in ground beef can be inhibited by the application of lactic acid bacteriae (LAB) such as *Lactobacillus* and *Pediococcus* (Gilliland and Speck, 1975; Reddy et al., 1975; Kannappan and Manja, 2004). Similarly, growth of *Pseudomonas* species on poultry was greatly reduced in the presence of a selected strain of LAB (Raccach et al., 1979). In addition to LAB, other types of bacterial antagonists that are active against spoilage and pathogenic bacteria, including Enterobacteriaceae (Enomoto, 2004), fluorescent pseudomonads (Gram, 1993) and yeasts (Janisiewicz et al., 1999; Liao and Fett, 2001), have been isolated and characterized. The mechanism of biocontrol could be due to one or more of several factors, including lowering of the pH below the level required for the growth of targeted organism, production of antimicrobial compounds, or competition for nutrients and growth requirements. In addition to biological agents, plant-derived antimicrobials such as isothiocyanates (Delaquis and Mazza, 1995; Lin et al., 2000) have been evaluated for the potential of controlling spoilage and pathogenic bacteria in packaged foods.

### 19.7 Conclusions and future trends

The spoilage or soft rot of fresh produce can be caused by pectolytic *P.*
Food spoilage microorganisms

*fluorescens, P. viridiflava* and *X. campestris*. The spoilage of animal-derived foods such as meat, poultry, milk and fish is caused primarily by *P. fluorescens*, *P. lundensis*, *P. fragi* and *S. putrefaciens*. *P. fluorescens* represents a heterogeneous group of bacteria consisting of at least five biovars (I–V) and each *P. fluorescens* biovar may be elevated to a distinct species. Within biovar V of *P. fluorescens*, two tightly linked clusters have now been identified as two separate species ‘P. lundensis’ and ‘P. fragi’.

The ability of *Pseudomonas* and *Shewanella* species to cause spoilage is at least in part due to their ability to produce an array of extracellular pectinases, proteases, lipases and exopolysaccharide slimes. Growth of bacteria and production of degradative enzymes are greatly affected by temperature, pH, water activity, atmosphere and native microflora. The combined effect of these factors on spoilage development may be predicted by several proposed mathematical models. Bacterial growth and spoilage development are thought to be mediated by three regulatory gene systems, e.g, RpoS (transcriptional activator), GacS/GacA (two-component regulators), and LuxR/LuxI (quorum-sensing regulators).

The control measures currently available for *Pseudomonas* and *Shewanella* are not sufficiently effective to reduce the number of bacteria to an acceptable level. Three factors may limit the efficacy of sanitization treatments for fresh produce, including acquisition of resistance to sanitizers, formation of biofilms, and bacterial attachment and internalization. Further investigation of these factors may lead to the development of more effective methods for control. Additionally, the use of biocide rotation and new nonthermal technologies such as PEF may provide an effective approach for inactivation of organisms on fresh foods and should be further investigated.

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19.9 References


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20.1 Introduction

A common feature associated with lactic acid bacterium (LAB) spoilage is that the growth of rapidly growing aerobic spoilage organisms is limited by extrinsic or intrinsic factors. Food packaging resulting in low oxygen content, such as a vacuum or modified atmosphere, low pH and low temperature are the most common factors selecting LAB as the main spoilage-causing bacterial group. Some LAB are also sugar and salt tolerant: excessive growth of carbon dioxide producing LAB (Etchells et al., 1975) during brined cucumber manufacture can result in bloater formation, and sugar tolerant (up to 15% sucrose) LAB can cause serious spoilage problems in raw sugar material (Tilbury, 1975).

Table 20.1 gives an overview on the types of spoilage observed in various food products and some information on the substrates and spoilage products of the LAB and the species involved.

20.1.1 Souring, changes in odour and taste

Atypical flavours, such as cheesy, sour, acid and sometimes liver-like, are usually the first changes associated with LAB spoilage in raw meat (Pierson et al., 1970; Eagan and Shay, 1982; Eagan, 1983; Schillinger and Lücke, 1987). These changes have been noted, together with atypical aromas and off-odours. Similar changes also affect cooked meat products spoiled by LAB (Allen and Foster, 1960; Reuter, 1970c; Eagan et al., 1980; Borch and Nerbrink, 1989; Korkeala et al., 1985, 1987, 1989). Odour and taste changes may naturally be accompanied with other spoilage changes, such as gas formation. When the LAB population had reached about $10^7$ cfu/g on the
Table 20.1  Spoilage of different food substrates by lactic acid bacteria

<table>
<thead>
<tr>
<th>Type of food</th>
<th>Food product</th>
<th>Parameters and antimicrobial processing</th>
<th>Type of spoilage (sensory)</th>
<th>Spoilage substrate</th>
<th>Spoilage product</th>
<th>Spoilage organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat (fresh and packaged)</td>
<td>Turkey breast</td>
<td>pH &gt; 4.5; $a_w &gt; 0.95$</td>
<td>Slime</td>
<td>Sucrose</td>
<td>Dextran (EPS)</td>
<td>Leuconostoc</td>
<td>Samelis et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Vacuum packaged meats</td>
<td>pH &gt; 4.5; $a_w &gt; 0.95$; nitrite; heat processing</td>
<td>Greening</td>
<td>$H_2O_2$ + nitroso- hemochromes</td>
<td>Porphyrin</td>
<td>Weissella viridescens</td>
<td>Niven and Evans (1957)</td>
</tr>
<tr>
<td></td>
<td>Vacuum packaged meats</td>
<td>pH &gt; 4.5; $a_w &gt; 0.95$</td>
<td>Greening</td>
<td>$H_2S$ + muscle pigment</td>
<td>Cysteine</td>
<td>Sulphmyoglobin</td>
<td>Lactobacillus sakei</td>
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<td></td>
<td>MAP marinated broiler meat</td>
<td>pH about 6; $a_w &gt; 0.95$</td>
<td>Gaseous spoilage</td>
<td>Glucose</td>
<td>Gas (CO$_2$)</td>
<td>Lactobacillus sakei</td>
<td>Shay and Eagan (1981)</td>
</tr>
<tr>
<td>Cooked meat products</td>
<td>Vacuum-packaged bologna sausage</td>
<td>pH &gt; 4.5</td>
<td>Greening</td>
<td>$H_2O_2$ + di- nitrosyl- hemochrome</td>
<td>Cholemyoglobin or oxidised porphyrins</td>
<td>Leuconostoc gascomitatum, Lactobacillus oligofermentans Carnobacterium viridans</td>
<td>Björkroth et al. (2000), Koort et al. (2005), Holley et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Vacuum-packaged ham</td>
<td>pH about 6; $a_w &gt; 0.95$</td>
<td>Sour off-odour</td>
<td>Glucose</td>
<td>Lactic and acetic acids</td>
<td>Leuconostoc carnosum</td>
<td>Björkroth and Korkeala (1997b), Björkroth et al. (1998), Korkeala et al. (1988), Mäkelä et al. (1992)</td>
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<tr>
<td></td>
<td>Vacuum-packaged meat products</td>
<td>pH about 6; $a_w &gt; 0.95$</td>
<td>Ropy-slime</td>
<td>Carbohydrate apparently but not sucrose</td>
<td>Glucose-galactose polymer (EPS)</td>
<td>Lactobacillus sakei</td>
<td>Mäkelä et al. (1992)</td>
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<tr>
<td>Type of food</td>
<td>Food product</td>
<td>Parameters and antimicrobial processing</td>
<td>Type of spoilage (sensory)</td>
<td>Spoilage substrate</td>
<td>Spoilage product</td>
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<td>Citrate</td>
<td>CO$_2$</td>
<td>LAB</td>
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<tr>
<td>Fish products</td>
<td>Herring</td>
<td>pH $&lt; 4.5$; $a_w &gt; 0.95$</td>
<td>Gaseous spoilage</td>
<td>Amino acids</td>
<td>Gas (CO$_2$)</td>
<td><strong>Lactobacillus alimentarius</strong></td>
<td>Lyhs et al. (2001)</td>
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<td></td>
<td></td>
<td>pH $&lt; 4.5$; $a_w &gt; 0.95$</td>
<td></td>
<td>Slime and gaseous</td>
<td>Dextran and gas (CO$_2$)</td>
<td><strong>Leuconostoc gasomitatum, Leuconostoc gelidum</strong></td>
<td>Lyhs et al. (2004)</td>
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<td>Acetic acid preserved</td>
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<td>Carbohydrates</td>
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<td>herring</td>
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<tr>
<td>Vegetables</td>
<td>Brined cucumbers</td>
<td>pH $&lt; 4.5$; $a_w &gt; 0.95$</td>
<td>Bloater damage</td>
<td>Glucose</td>
<td>Gas (CO$_2$)</td>
<td><strong>Heterofermentative lactobacilli</strong></td>
<td>Fleming et al. (1973)</td>
</tr>
<tr>
<td></td>
<td>Ketchup</td>
<td>pH $&lt; 4.5$; $a_w &gt; 0.95$</td>
<td>Gaseous spoilage</td>
<td>Glucose</td>
<td>Gas (CO$_2$)</td>
<td><strong>Lactobacillus fructivorans</strong></td>
<td>Björkroth and Korkea (1997a)</td>
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<td></td>
<td>Salad dressings</td>
<td>pH $&lt; 4.5$; $a_w &gt; 0.95$</td>
<td>Gaseous spoilage</td>
<td>Fructose; citric acid</td>
<td>Acidification; gas (CO$_2$); off-flavours</td>
<td><strong>Lactobacillus fructivorans</strong></td>
<td>Charlton et al. (1934)</td>
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<td>Radford and Board (1993)</td>
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<td>Back (1993); Back et al. (1999)</td>
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<tr>
<td>Non-alcoholic</td>
<td>Lemonades and nectars</td>
<td>pH $&lt; 4.5$; $a_w &gt; 0.98$</td>
<td>Souring; off-taste</td>
<td>Sugar</td>
<td>Lactic acid; acetic acid; CO$_2$; diacetyl</td>
<td><strong>Leuc. mesenteroides, Lactobacillus perolens, Lactobacillus paracasei</strong></td>
<td>Drinan et al. (1976), Juven (1976)</td>
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<td>beverages</td>
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<td></td>
<td>Fruit juices</td>
<td>pH $&lt; 4.0$; $a_w &gt; 0.97$</td>
<td>Buttery flavour</td>
<td>Citrate</td>
<td>Diacetyl</td>
<td>Leuconostocs, Lactobacilli</td>
<td>Drinan et al. (1976), Juven (1976)</td>
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Radnor Environments
<table>
<thead>
<tr>
<th>Type of Food</th>
<th>Food product</th>
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<tr>
<td>Wine</td>
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<tr>
<td><strong>Parameters and antimicrobial processing</strong></td>
<td>pH &lt; 4.5; aw &gt; 0.98</td>
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<td></td>
<td><strong>Type of spoilage (sensory)</strong></td>
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<td><strong>Spoilage substrate</strong></td>
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<td><strong>Reference</strong></td>
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<td></td>
<td>pH &lt; 4.5; aw &gt; 0.098</td>
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<td></td>
<td>pH &lt; 4.5; aw &gt; 0.98</td>
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<tr>
<td>Beer</td>
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<tr>
<td><strong>Parameters and antimicrobial processing</strong></td>
<td>pH around 4.5; aw &gt; 0.98; low hops or none</td>
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<td></td>
<td><strong>Type of spoilage (sensory)</strong></td>
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<td><strong>Spoilage organism</strong></td>
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surface of vacuum-packaged cooked sausages, lactic acid concentration (both D and L isomers) started to rise (Korkeala et al., 1990a). Above a level of 3-4 mg lactic acid/g, most of the sausage samples were deemed unfit in sensory evaluation. Simultaneously the pH of the sausages had dropped from 6.3 to below 5.8.

The acidic odours are believed to be caused by the accumulation of end-products formed in LAB metabolism. Short-chain fatty acids, mainly acetic and butyric acids and certain ninhydrine-positive compounds are associated with the sensorial changes produced by LAB on vacuum-packaged meat (Sutherland et al., 1976; Dainty et al., 1979; Dainty, 1982). Some lactobacilli are also able to produce hydrogen sulphide, causing strong sulphur-like off-odours (Shay and Eagan, 1981; Hanna et al., 1983; Lee and Simard, 1984; Eagan et al., 1989).

The appearance of acetic and propionic acids in raw milk subjected to temperature abuse leads to sour/acid odours during the fermentation of lactose by LAB (Shipe et al., 1978). Off-flavours constituting spoilage in cheese may result from an unsuitable starter culture or the predominance of particular strains leading to fermentation imbalance. Excessive casein proteolysis may lead to the formation of bitter oligopeptides. Some strains, mainly Lactococcus lactis, can produce esters that impart a fruity off-flavour or produce 3-methylbutanal, which gives a malty off-flavour to milk products (Morgan, 1970; Shipe et al., 1978). Under certain conditions, a malty flavour has also been detected in spoiled meat (Patterson and Gibbs, 1977).

Lactic acid was present in every case of spoilage in canned, non-fermented vegetables investigated by Ackland et al. (1981), and it has been suggested that this is an indication of microbial spoilage in this type of foods (Ackland and Reeder, 1984). In fresh vegetables, LAB are usually outgrown by moulds. However, if anaerobic conditions develop and the sugar content of the vegetable is high, as in young peas or beans, the spoilage may be due to sourness and off-flavours produced by LAB (Sharpe and Pettipher, 1983).

In fruit juices, tomato juice and citrus products, the growing LAB may produce diacetyl from the citrate present (Drinan et al., 1976), resulting in an unpleasant buttery flavour. Levels as low as 5 ppm diacetyl in concentrated orange juice spoil the product. Diacetyl and acetoin can indicate the contamination of tomato juice by LAB in the early stages of growth (Luster, 1978; Juven and Weisslowicz, 1981). In the case of an acetic acid fish preserve showing slime and gas formation (Lyhs et al., 2004), an off-odour described as buttery, butyrate-like or curd cheese-like was recognized in the sensory evaluation. The butter-like off-odour detected in the spoiled product was likely associated with diacetyl formation. Because the fish muscle is not rich in citrate, another precursor producing pyruvate may have triggered diacetyl formation.

LAB spoilage in wine and alcoholic beverages is manifested by haze, gas and various flavour changes. LAB cause malo-lactic fermentation in wine and cider, where malic acid is metabolized to form carbon dioxide and lactic
acid. If started, this reaction tends to go to completion, halving that part of titratable acidity that was due to the original malic acid. Malo-lactic fermentation raises the pH of the wine and may also cause flavour changes, such as the formation of diacetyl. This reaction is harmful to some wine types but beneficial to others (Goswell, 1986). LAB may also attack citrate, glycerol, tartrate and above all glucose and fructose, producing acetoin, butanediol-2,3, lactic acid, carbon dioxide, acetic acid, succinic acid and acrolein, which may lead to severe sensorial problems. Similar spoilage also affects cider (Whiting, 1975). ‘Mousy’ off-flavours are also produced by LAB in wine and three heterocyclic volatile bases are responsible: 2-acetylterahydropyridine, 2-ethylterahydropyridine and 2-acetyl-1-pyrroline (Lonvauad-Funel, 1999). Beer spoilage is usually characterised by turbidity, accompanied by light acidity and the formation of malodorous compounds, such as diacetyl (Rainbow, 1975, 1981).

20.1.2 Colour changes
The greening of cured, cooked meat products (Niven et al., 1949; Niven and Evans, 1957; Lörincz and Incze, 1961) is supposed to be due to hydrogen peroxide produced by LAB. Greening has also been observed in vacuum or nitrogen packaged meat loaves, frankfurters and pork liver paté (Simard et al., 1983; Lee et al., 1984; Madden, 1989). Hydrogen peroxide reacts with nitric oxide haemochromogen and nitric oxide myoglobin in meat to produce greenish oxidised porphyrin. Weissella viridescens has especially been associated with the greening of meat products (Niven and Evans, 1957). Light greening is also observed with meat spoilage caused by hydrogen sulphide producing lactobacilli such as some Lactobacillus sakei strains (Shay and Eagan, 1981; Hanna et al., 1983; Lee and Simard, 1984; Eagan et al., 1989). Pigment production, such as pink and orange pigments, can occasionally cause spoilage in cheese (Sharpe and Pettipher, 1983).

20.1.3 Gas formation, bloater formation and protein swell (decarboxylation)
Unwanted production of carbon dioxide can cause many problems of open texture or blowing curd or cheese. Many species are known to be associated with this problem (Sharpe and Pettipher, 1983). Increased CO₂ concentration in vacuum meat packages has been attributed to metabolic by-product of heterofermentative lactobacilli and leuconostocs (Korkeala and Lindroth, 1987; Korkeala et al., 1987; Ahvenainen et al., 1990; von Holy et al., 1991). The packages become loose due to the gas formation. In modern, modified atmosphere packaged marinated broiler meat products, rapid spoilage caused by heterofermentative species Leuconostoc gasicomitatum and Lactobacillus oligofermentans has been detected (Björkroth et al., 2000; Koort et al., 2005). These species grow very rapidly in marinated products apparently
because the carbohydrates and possible other ingredients used in marinades are promoting their growth. Since these products are devoid of pentoses and neither of the species has been able to decarboxylate amino acids typically present in meat products, the CO₂ production has been considered to result from glucose utilization through heterofermentative pathway (Murros, 2004; Koort et al., 2005). Spoilage LAB are not associated with formation of cadaverine and putrescine as the Gram-negative meat spoilage organisms are.

Canned vegetables may be spoiled by LAB forming carbon dioxide blowing the cans (Sharpe and Pettipher, 1983). Blowing of cans may also affect sour fruit products (Juven, 1976). In salad dressings, the formation of carbon dioxide caused by *Lactobacillus fructivorans* leads to bubble-filled dressing and/or blowing of the containers (Charlton et al., 1934; Kurzman et al., 1971; Smittle and Flowers, 1982; Meyer et al., 1989).

Bloater damage in brined cucumbers results from an increase in gas pressure inside the cucumbers during fermentation. The gas pressure is due to the combined effects of nitrogen trapped inside the cucumbers and carbon dioxide (Fleming and Pharr, 1980). Carbon dioxide originates from the cucumber tissue, or the activity of gas-forming microorganisms in the brine. Heterofermentative LAB produce carbon dioxide in glucose fermentation and some LAB may either cause malic acid degradation to lactic acid and carbon dioxide, or produce carbon dioxide by other pathways (Fleming et al., 1973; McFeeters et al., 1982, 1984).

‘Protein swell’ is an unusual type of LAB spoilage, because it raises the pH of the spoiled product. Normally in case of a clear LAB spoilage, the product pH decreases due to lactic acid formation. This type of LAB spoilage was reported first by Meyer (1956a, b) in canned fish marinades. He called it ‘protein swell’ and distinguished it from the ‘carbohydrate swell’ where the increased acidity and CO₂ formation result from heterofermentative utilisation of glucose. In ‘protein swell’, proteins are decomposed by proteolytic enzyme action and the subsequent decarboxylation of amino acids leads to enhanced CO₂ production. Therefore, the LAB having an effect on gas production in ‘protein swell’ may also possess homofermentative glucose metabolism. Decrease in acidity related to ‘protein swell’ has been attributed to production of ammonia by bacterial deamination of amino acids. This spoilage type usually affects canned fish products but it has been reported to affect anchovy-stuffed olives (Harmon et al., 1987). Harmon et al. (1987) found *Lactobacillus brevis* as the main spoilage organism in the anchovy-stuffed olives. Since this species was not proteolytic, it was thought that autolytic enzymes persisting due to inadequate processing released the amino acids that were further utilised by *Lactobacillus brevis*.

In the case of raw fish products, proteolysis has been considered to be due to the action of proteases from the muscle of fish and therefore the bacterial species involved in the spoilage process must not necessarily be proteolytic. Since the carbon dioxide formed is due to the amino acid decarboxylation
reaction, homofermentative LAB may also be involved. Lyhs et al. (2001) detected *Lactobacillus alimentarius* in herring products showing bulging due to gas formation. This species does not produce carbon dioxide through its glucose metabolism and since a slight pH rise was detected, ‘protein swell’ was suspected. In cases of tryptophane, histidine, tyrosine, phenylalanine, ornithine and lysine decarboxylation, formation of biogenic amines happens which should be kept in mind. Usually formation of biogenic amines is related to fermented foods or family Scombridae fishes possessing high amounts of histidine which is decarboxylated as histamine in ‘scomboid fish poisoning’. The activity of unwanted non-starter bacteria in fermented foods may result in formation of biogenic amines.

### 20.1.4 Exopolysaccharide formation
Exopolysaccharide production is essential in the production of some types of food, such as the Finnish ‘ropy’ fermented milk product ‘viili’. In meat products, cider, wine and in the sugar cane industry, exopolysaccharide production can cause severe problems. *Lactobacillus sakei* and *Leuconostoc citreum* have been found to cause ‘ropy spoilage’ of vacuum-packaged cooked meat products because of the formation of glucose–galactose-containing polymer (Korkeala et al., 1988; Mäkelä et al., 1992). Heterofermentative lactobacilli (Dueñas et al., 1995) or *Pediococcus* (Llaubères et al., 1990; Lonvaud-Funel et al., 1993; Manca de Nadra and Strasser de Saad, 1995; Fernandez et al., 1996) have been associated with the ropy spoilage of wine and/or cider. *Leuconostoc*, mainly *Leuconostoc mesenteroides* and to a lesser extent *Weissella confusa*, *Lactobacillus plantarum* and *Lactobacillus casei* may produce dextran from sucrose, thus reducing the gain of sucrose in the sugar industry (Tilbury, 1975). *Leuconostocs*, *Leuconostoc gasicomitatum* and *Leuconostoc gelidum*, were also the predominating LAB causing slime and gas formation in an acetic acid fish preserve (Lyhs et al., 2004).

### 20.2 Characteristics and conditions for lactic acid bacterium growth/death

#### 20.2.1 General aspects related to spoilage LAB and their growth requirements
The ‘true’ LAB are a heterogeneous group, but have in common that they are catalase negative (with some strains able to produce a haem-dependent catalase), non-spore-forming, strictly fermentative, facultative aerobic, and producing lactic acid as a major product of glucose fermentation. Some strains may produce a haem-independent catalase, called pseudocatalase, which may cause confusion in their identification. Within the phylum *Firmicutes*, i.e. Gram-positive organisms, they belong to the clostridial branch, with less than 55 mol% G+C content in the DNA. Phenotypic differentiation has
traditionally been on the basis of their cell morphology, by their metabolism (homo- or heterofermentation) and the type of products of primary metabolism (e.g. the lactic acid isomer), by the spectrum of fermented sugars, and also using features such as ability to grow at particular temperatures, resistance to extreme factors such low and high pH values, and elevated salt concentrations, and also using chemotaxonomic features such as the chemical composition of the cell wall and particularly of the interpeptide bridge of the peptidoglycan (Stiles and Holzapfel, 1997). Differentiating among the major genera is still generally possible on the basis of physiological features, with perhaps *Weissella* as the major exception, being a genus with both rod and coccus-shaped species, showing physiological ‘relationships’ both with the genus *Leuconostoc* and the heterofermentative lactobacilli.

With respect to their growth and ability to cause spoilage, LAB are an intriguing bacterial group. They are generally considered to be fastidious in respect of their growth requirements and differ from Gram-negatives which are able to synthesize essential growth factors. Lactic acid bacteria require purines, pyrimidines, vitamins and several amino acids in order to grow. They are generally detected in association with rich, carbohydrate-containing sources. Genetic diversity and habitat variation are considerably wide within LAB. Therefore, no general limits for pH, $a_w$, temperature, or other parameters exist for the LAB, and the growth-limiting circumstances vary depending on the species in question. Some species are, however, able to grow under quite adverse circumstances. Comprising both spoilage and technologically beneficial bacteria, the role of a particular LAB strain may be determined by the specific situation and product. *Lactobacillus sakei*, for example, is an important starter culture for the production of fermented meat products, while, on the other hand, it may dominate the spoilage association of vacuum-packaged processed meat products (Mäkelä *et al.*, 1992; Schillinger and Holzapfel, 2003).

Most LAB prefer an initial pH of 6 to 7 for growth. However, many lactobacilli and *Oenococcus oeni* are acidophilic and tolerate low pH conditions. *Oenococcus oeni* grows well at pH 4.2 to 4.8 (Garvie, 1967). Among the *Lactobacillus* species, *Lactobacillus hilgardii* shows preferable growth within a pH range of 4.5 to 5.5. *Lactobacillus suebicicus* isolated from fermented apple and pear mashes (Kleynmans *et al.*, 1989) and *Lactobacillus acetotolerans* from rice vinegar (Entani *et al.*, 1986) can even grow at pH 2.8 and pH 3.3 respectively, while the lowest pH values for growth of *Lactobacillus sakei*, *Lactobacillus brevis* and *Lactobacillus plantarum* have been stated as 3.0, 3.16 and 3.34, respectively (although no information on the medium/substrate has been given; Jay *et al.*, 2005). *Lactobacillus acetotolerans* is resistant to acetic acid concentrations up to 11% at pH 5.0 (Entani *et al.*, 1986). A *Lactobacillus fructivorans* ketchup spoilage strain was able to grow in MRS (de Man–Rogosa–Sharpe) broth at pH 3.5 and it also showed growth in MRS broth containing 15% (w/w) ethanol or 8% of NaCl (Björkroth and Korkeala, 1997a). The pH of the spoiled ketchup was 3.3. On the other hand, carnobacteria do not tolerate acidic circumstances but a neutral to high pH from 6.8 to 9.0
favours the growth and alkaline pH ranging from 8.0 to 9.0 has been suggested
to serve in selective purposes differentiating carnobacteria from other LAB.
Tetragenococci, enterococci and some pediococci are also known for growth
ability at alkaline pH (Axelsson, 2004).

In addition, LAB include halophiles. Enterococci and some pediococci,
lactobacilli and leuconostocs possess growth in 6.5% NaCl. Tetragenococci
even grow at 18–24% NaCl (Axelsson, 2004; Holzapfel et al., 2005). Some
Weissella and Pediococcus species grow in up to 10% NaCl (a_w 0.92–0.94),
and Weissella halotolerans was reported to grow very weakly in 14% NaCl,
corresponding to an a_w value below 0.90 (Hammes and Vogel, 1995).
*Carnobacterium viridans* tolerates 26.4% NaCl and can survive in saturated
brine for long periods at 4 °C (Holley et al., 2002). *Tetragenococcus halophilus*
and *T. muriaticus* are the predominating halophilic LAB species in high-salt
fermented foods, particularly fish products. It has been shown that the high
saline concentrations and low pH of these food substrates have significant
impact on the growth, lactic acid production and pH reduction ability of
these species (Kobayashi et al., 2004). A major physiological difference
between the two species is in the ability of *T. halophilus* to grow in medium
not supplemented with NaCl in contrast to *T. muriaticus* (Kobayashi et al.,
2000). All *T. halophilus* strains appear to tolerate 26% NaCl while reaching
an optimum growth rate at pH 7.5 (Gürtler et al., 1998). Histamine production
at NaCl concentrations >10% has been found to be a typical characteristic of
*T. muriaticus*, while its ability to form histamine at low acidity around pH
5.8, and under O_2_-limiting conditions, optimal NaCl concentration (5–7%)
and glucose >1% has also been reported (Kimura et al., 2001).

As can be expected from those LAB associated with alcoholic beverages,
e.g. *Oenococcus oeni*, *Lactobacillus fructivorans* and some pediococci, they
tolerate the ethanol levels typical for these beverages, ranging from 10 to
13% vol%, and with an initial malic acid concentration ranging from 12 to
20 g/l (Dittrich, 1993). Strains of *Lactobacillus plantarum* and *Oenococcus
oeni* from wine were reported to grow at pH 3.2 and in the presence of 13%
ethanol at 18 °C, while also showing high resistance to lyophilisation (G-
Alegria et al., 2004).

LAB are quite variable in their growth temperature spectrum. They are
generally mesophiles, showing best growth at temperatures between 20 and
40 °C, and with a maximum around 50 °C, although a few lactobacilli (e.g.
*Lactobacillus delbrueckii* subsp. *delbrueckii*), may even grow at 55 °C. On
the other hand, the LAB comprise many psychrotrophic species able to grow
at refrigerated temperatures, even at subzero temperatures (Korkeala et al.,
1990b). Carnobacteria as well as some lactobacilli and leuconostocs are well
adapted to low temperatures and many of them grow well at temperatures
close to 0 °C. Packaging and cold storage has changed the order of significance
of the predominating specific spoilage organisms from aerobic Gram-negative
bacteria to psychrotrophic spoilage LAB especially in meat products.
Depending on intrinsic nutrient availability and further hurdles applied in
processing, microbial populations consisting of different proportions of heterofermentative and homofermentative LAB usually predominate in cold-stored packaged meat products as shown in Chapter 9.

Depending on the substrate and environment, LAB may survive conditions, e.g. within a food, for long time periods. Strains resembling *Lactobacillus casei* were isolated from the interior of a cheese found in a capsized sailing ship after 105 years (Minor *et al.*, 1970).

The heat resistance of LAB is influenced by (food) substrate, and also by factors such as the growth phase and former history of the strain. In acid foods (pH < 4.0) the $D_{65^\circ C}$ value for most LAB ranges from 0.5 to 1.0 (Krämer, 1997). Typical of most Gram-positive bacteria, the LAB are more heat resistant than Gram-negative bacteria associated with foods. Within the LAB, higher resistance to thermal destruction has been observed for enterococci associated with meat products (Franz and von Holy, 1994). At $a_w > 0.95$ and pH of 7, $D_{60^\circ C}$, $D_{65^\circ C}$ and $D_{70^\circ C}$ values of 7–15 min, 1.6–2.3 min and 0.3 min, respectively, has been reported for *Enterococcus faecium*; the corresponding values reported for *E. faecalis* were 5–20, 1.6–2.3 and 0.3 min (Mossel *et al.*, 1995). Generally, the pediococci are more resistant to heat than other LAB, and among the pediococci, *Pediococcus pentosaceus* and *Pediococcus acidilactici* strains, associated with dairy products, show elevated resistance (Wallhäusser, 1988). An exception may be exemplified by *Weissella viridescens*, a typical spoilage bacterium of processed meat products, for which $D_{65^\circ C}$ values of 20–30 min under ‘optimal’ conditions of $a_w > 0.95$ and pH 7.0, have been reported (Mossel *et al.*, 1995).

Relatively sparse information is available on the radiation resistance of LAB. Strains of *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus alimentarius* and *Lactobacillus farciminis* have been isolated from radurised meat, and their radiation resistance (in $\gamma D_{10}$ values) was found to range from 0.28 to 0.88 kGy (Niemand and Holzapfel, 1984). In another study, the radiation resistance of strains isolated from radurised meat was found to be generally higher than that of authentic strains, of which some isolated strains showed $\gamma D_{10}$ values of $> 1.0$ kGy. Moreover, a hitherto unexplained phenomenon was observed for several *L. sakei* strains, isolated from radurised meat, which showed higher $\gamma D_{10}$ values in the log phase compared with the stationary phase (Hastings *et al.*, 1986). This was not observed for any authentic strains, including *L. sakei* DSM 20017. For vegetative cells of *Sporolactobacillus inulinus*, the average resistance to $\gamma$ irradiation was comparable to other vegetative bacterial cells, ranging from 0.350 to 0.525 kGy. The average $\gamma D_{10}$ value for its endospores was 2.5 kGy (Botha and Holzapfel, 1988).

### 20.2.2 Meat and meat products
Vacuum or modified atmosphere packaging of meat and meat products creates the most important meat-related LAB spoilage issue. LAB form the
predominant spoilage microbiota in vacuum or modified atmosphere packaged meat (Pierson et al., 1970; Sutherland et al., 1975; Dainty et al., 1979; Schillinger and Lücke, 1987). These packaging technologies prevent the growth of Gram-negative, aerobic bacteria. Since lactobacilli and other LAB are resistant to the inhibition of nitrite and smoke, and are able to grow in relatively high sodium chloride concentrations (Castellani and Niven, 1955; Dodds and Collins-Thompson, 1984; Korkeala et al., 1992), they are also able to grow in vacuum or modified atmosphere packaged cooked meat products (for reviews see Korkeala and Björkroth, 1997; Borch et al., 1996). These LAB tolerate nitrite concentrations of 50 to 100 ppm, typically used in such cooked meat products (Korkeala et al., 1992). Metabolic activity of LAB produces the typical spoilage changes, making products eventually unfit for human consumption. Sour flavour and off-odour, formation of CO₂ resulting in bulging of packages, slime formation and discolorations are typical sensory changes involved in LAB meat spoilage. Spoilage changes are usually delayed until the stationary growth phase of LAB (Korkeala et al., 1989; Reuter, 1970c), and a product is expected to retain good sensorial quality for several days. In comparison to aerobic spoilage rate, LAB spoilage is much slower and therefore prolonged product shelf life is obtained by anaerobic packaging of meat.

There are differences between the ability of various LAB species and strains to spoil meat products (Borch et al., 1996; Korkeala and Björkroth, 1997; Björkroth et al., 1998). On the basis of LAB enumeration, it cannot be determined, if a sample contains species/strains later associated with the spoilage of a product. In the study of Björkroth et al. (2005) dealing with LAB spoilage in modified atmosphere packaged marinated raw broiler legs, a clear change from the initial contaminants to the spoilage-causing LAB was detected during the storage at 6°C. The results showed that enterococci (35.7% of the initial LAB population) dominated in the fresh product whereas carnobacteria (59.7%) predominated the spoilage LAB population. Only about 19% of the initial LAB contaminants were carnobacteria and no enterococci were detected in the spoiled products. In general, when the initial LAB population was compared with the spoilage LAB, a shift from homofermentative cocci towards carnobacteria, Lactobacillus sakei/curvatus and heterofermentative rods were observed in this marinated product.

Marinating of meats has a dramatic selective influence on the spoilage LAB populations (Björkroth, 2005). Owing to the buffering capacity of meat, the acidic marinades are rapidly neutralized and the carbohydrates present are utilised by spoilage LAB. Leuconostoc gasicomitatum (Björkroth et al., 2000; Susiluoto et al., 2003) and Lactobacillus oligofermentans (Koort et al., 2005) have been two novel LAB, typically predominating in spoiled, marinated modified atmosphere packaged poultry meat products together with carnobacteria.

Selective effect of smoking on the LAB in turkey breasts packaged under vacuum was documented by Samelis et al. (2000). If the product was smoked
Lactic acid bacteria predominated, whereas in unsmoked product the population was more diverse, consisting in addition to *Lactobacillus sakei* of leuconostocs and *Weissella viridescens*. Even within one product category such as meat products, it should be kept in mind that different products harbour different spoilage LAB species. Therefore, if spoilage is modelled, strains chosen for the modelling should be carefully selected. In addition, some clones of a species are more potent at causing spoilage than others. This was shown in the case of *Leuconostoc carnosum* spoiling vacuum-packaged cooked ham (Björkroth and Korkeala, 1997a; Björkroth et al., 1998).

Species reported to dominate in meat and meat products include the genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Weissella*, *Pediococcus* and *Enterococcus*. Using modern taxonomic methods, it has become evident that the major spoilage group in vacuum-packaged meat products, previously assigned as atypical streptobacteria (Reuter, 1970a,b, 1975), consists of *Lactobacillus sakei* and *Lactobacillus curvatus* strains (Mäkelä et al., 1992; Vogel et al., 1993; Dykes and von Holy, 1994; Klein et al., 1996; Torriani et al., 1996). Identification of these two species has been controversial and they were both divided into two subspecies (Klein et al., 1996; Torriani et al., 1996). However, recently *L. curvatus* subsp. *melibiosus* was shown to be a later synonym of *Lactobacillus sakei* subsp. *carnosus* (Koort et al., 2004). Currently, only *L. sakei* is divided between two subspecies: *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus*. Lately, two novel lactobacilli, *Lactobacillus algidus* (Kato et al., 2000) and *Lactobacillus fuchuensis* (Sakala et al., 2002), have been described in association with meat products.

20.2.3 Fish products

As in meat products, LAB play a role as spoilage bacteria in vacuum packaged lightly preserved fish products. ‘Gravad’ (sugar-salted) Scandinavian fish products, hot and cold-smoked fish have been mainly studied. Fish differs from meat generally in respects of its pH and carbohydrate contents having an effect on the LAB population. The most important intrinsic factor related to fish flesh is the very high post-mortem pH (> 6.0) and the low carbohydrate content (< 0.5 %) in the muscle tissue (Gram and Huss, 1996). Carnobacteria have been reported to predominate in vacuum packaged, hot-smoked rainbow trout (Zorn et al., 1993) at the end of storage period (8 °C). In vacuum packaged cold-smoked products, LAB together with varying proportions of other bacteria have been detected. Truelstrup Hansen (1995) concluded that in vacuum packaged cold-smoked salmon at the time of sensorial rejection three bacterial groups (predominated by LAB) LAB, Enterobacteriaceae and *Photobacterium* were present. In ‘gravad’ products, carnobacteria (Leisner, 1992; Leisner et al., 1994) or *Lactobacillus sakei* has been reported as predominating (Jeppesen and Huss, 1993). Lyhs et al. (1998) identified 76% of the spoilage population in vacuum-packaged, sodium nitrite or potassium nitrate treated cold-smoked rainbow trout as LAB, leuconostocs, *Lactobacillus sakei* and *Lactobacillus curvatus* being the predominating species.
In marinated herring and other fish products, LAB spoilage occurs mainly in incorrectly stored marinades or in retail containers where the acetic acid and salt concentrations are decreased to 2 and 3% respectively, to give a more palatable taste. Acetic acid-tolerant strains of *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus delbrueckii* ssp. *leichmannii*, some facultatively heterofermentative strains and *Pediococcus pentosaceus* have been associated with spoiled fish products (Meyer, 1964; Sharpe and Pettipher, 1983; Harmon et al., 1987). Lyhs et al. (2001) detected *Lactobacillus alimentarius* in herring products showing bulging due to gas formation. This species does not produce carbon dioxide through its glucose metabolism and since a slight pH rise was detected, ‘protein swell’ was suspected.

### 20.2.4 Salad dressings, juices and products with a low pH
LAB spoilage is also common in acidic products, such as fruit juices, citrus products, tomato juice and vinegar preserves. The growth of LAB in fruit and tomato juices is favoured by high acid and sugar contents and by some additional stimulatory factors present (Amachi, 1975; Yoshizumi, 1975). The low pH of fruit juices between 3.0 and 3.7 and the high sugar content (50–150 g of fermentable sugars glucose, fructose and sucrose per litre, although generally a protein content of < 1%) (Jay et al., 2005) allows growth of some lactobacilli and leuconostocs, especially in ‘moderately’ acid orange and apple juices with pH ranges around 3.3 to 4.5. *Lactobacillus brevis* spoilage in a citrus product with a pH lower than 3.5 has been described (Juven, 1976). Juice pH has significant effects on the growth and survival of specific microorganisms in citrus juices. A rise in the pH from 3.4 to 4.0 increases the growth rate of *Lactobacillus brevis* and *Leuconostoc mesenteroides* ssp. *mesenteroides* by 200% in single strength orange juice (Rushing et al., 1956).

LAB are responsible for about 25% of the spoilage of mayonnaise, salad dressing and vegetable preserves manufactured by acetic acid preserving (Sharpe and Pettipher, 1983). These products have a pH in the range of 2.9 to 4.4, contain acetic acid 0.29 to 2.0%, salt 4 to 10%, and have low water activity of about 0.92 (Smittle and Flowers, 1982; Sharpe and Pettipher, 1983; Meyer et al., 1989). The organisms able to grow in these products must be able to tolerate high concentrations and combinations of acetic acid and salt. *Lactobacillus fructivorans* is a common cause of the spoilage in these types of product, *Lactobacillus brevis*, *Lactobacillus buchneri* and *Lactobacillus plantarum* being occasionally isolated (Kurtzman et al., 1971; Smittle and Flowers, 1982; Sharpe and Pettipher, 1983; Meyer et al., 1989). *Lactobacillus fructivorans* has also been associated with spoilage of ketchup (Björkroth and Korkeala, 1997a).
20.2.5 Fermented vegetables
Fermentation of vegetables can be achieved either by adding a known starter culture or by encouraging the growth of indigenous LAB. Indigenous fermentation is typically used in sauerkraut, olive and brined cucumber manufacture (Stamer, 1975; Etchells et al., 1975). The predominance of certain unwanted LAB may prevent the normal sequence of events necessary for the successful fermentation process. Disturbance of the fermentation process can result in many different types of spoilage problems (Carr et al., 1975; Fleming, 1982; Sharpe and Pettipher, 1983).

20.2.6 Alcoholic beverages
LAB are the only bacteria that will grow in properly stored wine (Kunkee and Goswell, 1977). Ethanol content (11–14%), low pH (3.2–3.8) and added sulphur dioxide do not prevent the growth of some lactobacilli, *Oenococcus oeni* and pediococci (Goswell, 1986). These species may cause the malolactic fermentation of wine, which may be either beneficial or detrimental depending on the wine type (Lafon-Lafourcade, 1975; Goswell, 1986). When during wine-making heterofermentative LAB such as *Oenococcus oeni* produce acetic and lactic acids (in addition to ethanol and CO₂) from hexoses not totally fermented by yeasts during alcoholic fermentation, volatile acidity of wine increases and if it exceeds the limit of 1 g/l volatile acidity, the wine is unmarketable (Lonvaud-Funel, 1999). In particular, wines with residual (unfermented) sugars from moderate regions may be spoiled by formation of acetic acid (‘vinegar’ off-taste) by heterofermentative LAB, simultaneously linked to the production of mannitol from fructose, mainly by strains of *Lactobacillus brevis*. Under controlled conditions, lactic acid formation from malic acid fermentation may enhance desired sensory changes towards ‘mild’ wines; however, excessive lactic acid production, also by fermentation of residual sugars, may result in an undesirable acid taste. In addition, detrimental sensory changes are also associated with diacetyl, especially by pediococci, while the formation of lactic acid ethyl ester in quantities normally exceeding 80 mg/l may occur simultaneously (Dittrich, 1993; Bartowsky and Henschke, 2004). Moreover, ‘slimy’ or ‘ropy’ wine defects may be caused by different LAB, but predominantly by *Pediococcus damnosus* strains, producing 1,2-β-glucan even from minute quantities of glucose. *Leuconostoc mesenteroides* ssp. *dextranicum*, some strains of *Oenococcus oeni* and also some heterofermentative lactobacilli may also be associated with sliminess and ropiness of wines (Dittrich, 1993). As in wine, malo-lactic fermentation in cider manufacture may be favoured.

LAB isolated from breweries are always potential spoilage organisms: owing to their ability to resist alcohol and antibacterial substances of hops they can be particularly troublesome (Simpson and Fernandez, 1992; Fernandez and Simpson, 1995). *Lactobacillus brevis*, *Pediococcus damnosus* and *Pediococcus clausenii*, represent the most important beer-spoilage bacteria (Back 1981, 1994a,b; Holzapfel et al., 2005).
LAB may also compete with yeast in whisky manufacture, and by lowering the pH inhibit the residual amylase of the yeast (Dolan, 1979). This leads to incomplete fermentation, decreasing the alcohol yield up to 10%, if not corrected.

20.3 Specific detection, identification and enumeration methods

The methodology currently used for detection and enumeration of spoilage LAB comprises a number of different approaches including culture-dependent and independent methods. There is the classical methodology based on the cultivation of the organisms on appropriate media followed by the identification of the strains isolated from the agar plates on the one hand and methods which are not dependent on the cultivation of the spoilage organisms on the other hand.

20.3.1 Culture-dependent methods

Enumeration

The traditional method to examine a food for the presence of a specific microbiota and to detect the organisms responsible for the spoilage of the food product uses plating methodology on appropriate media. The food sample is macerated or homogenised using a stomacher or a waring blender (or a similar instrument), appropriate dilutions of the sample are prepared and these dilutions are plated onto selective or semi-selective media followed by isolation and identification of the colonies. The number of the spoilage-associated LAB is normally estimated by enumeration of the colonies.

A number of limitations of this methodology may lead to failure to detect the organisms responsible for the sensory defects, to misidentification or to underestimation of the spoilage bacteria. The results depend on several factors such as the correct choice of the media for the organisms expected to contribute to the spoilage, on the physiological state of these bacteria, and the identification method used. One of the problems associated with the enumeration of bacteria on agar plates results from the fact that sublethally injured bacteria will not grow on selective media and may therefore not be detected. Moreover in the food environment there is the viable but non-cultur able (VBNC) phenomenon that means that some spoilage bacteria may have entered a non-cultivable state. These bacteria are known to be still metabolically active and viable (and produce sensory defects) but will not produce colonies on media that normally support their growth (Fleet, 1999). In foods, many adverse conditions such a nutrient depletion, low temperature and other stresses can induce this VBNC state (Fleet, 1999). These cells are, however, able to resume their health state. In wine, a part of the acetic acid bacteria and LAB population
obviously survive in a VBNC state (Millet and Lonvaud-Funel, 2000). Sulphur
dioxide strongly affects the viability and culturable state of wine LAB. Millet
and Lonvaud-Funel (2000) observed that at least at the day after sulphiting,
the LAB entered a VBNC state and were no longer cultivable on nutrient
plates while they retained some metabolic activity as shown by direct
epifluorescence technique (DEFT).

In order to determine the number of LAB present in a spoiled food product,
numerous media have been proposed including MRS (deMan et al., 1960)
and Rogosa agar (Rogosa et al., 1951). The most commonly used medium is
MRS agar which complies with the German DIN-Norm 10109 and the
International Standard ISO 13721 (1995) for the enumeration of LAB counts
from meat and meat products and is also recommended for dairy products
and other foods. MRS medium may be modified by the addition of 0.1%
cysteine hydrochloride and 0.02% potassium sorbate to enhance the growth
of LAB and prevent the growth of yeasts (Dykes et al., 1995). In addition the
pH may be changed to 5.7 and anaerobic incubation is preferentially used.
The latter medium was successfully used for the examination of the LAB
spoilage microbiota of vacuum-packaged, smoked Vienna sausages (von
Holy et al., 1991, 1992). Other modifications of MRS agar are used to
specifically detect carnobacteria which may be involved in spoilage of
unprocessed, vacuum-packaged meat, poultry and seafood. *Carnobacterium
viridans* was found to be responsible for green discoloration of refrigerated
vacuum-packaged bologna sausage (Holley et al., 2002).

For the selective detection of carnobacteria CTAS (cresol red thallium
acetate sucrose) agar (Holzapfel, 1992) and EBRER agar (Millière and
Lefèbre, 1994) were developed. CTSI (cresol red thallium sucrose inulin)
agar is a modification of CTAS agar (Wasney et al., 2001) and was also
proposed as a selective medium for enumeration of carnobacteria. An alternative
to MRS agar is NAP (nitrite actidione polymyxin) agar (Davidson and Cronin,
1973), originally developed for meat LAB. This agar was used in studies on
the spoilage association of chilled, slightly preserved shrimps stored under
modified atmosphere (Dalgaard et al., 2003) and vacuum-packaged cold-
smoked salmon (Paludan-Müller et al., 1998). It allowed the detection of
lactobacilli, enterococci and carnobacteria, the latter being identified as forming
the dominant parts of the spoilage LAB associated with both products. Tomato
juice is known to stimulate the growth of some LAB and tomato juice agar
(TJA) was found to be superior to MRS for the detection of LAB associated
with marinades containing tomato purée (Susiluoto et al., 2003).

Media designed for the enumeration of wine spoilage LAB have to consider
the specific nutritional requirements of these slow-growing organisms adapted
to the wine substrate such as *Oenococcus oenos*. The media suggested for
detection of wine LAB include various modifications of MRS agar or other
basal media, incorporating tomato juice, apple juice, malic acid, pantothenic
acid and cysteine. Tomato juice medium (Yoshizumi, 1975) and M 5 agar
(Zúniga et al., 1993) are media recommended for detection and isolation of
lactobacilli from wine. In a study on the prevalence of spoilage LAB in Italian table wines, lactobacilli, pediococci and leuconostocs were isolated from MRS acidified to pH 5.6 containing 20% apple juice and 100 mg/l cycloheximide (Beneduce et al., 2004). Acidic grape agar supplemented with ethanol (17%) and actidione (40 mg/l) was used by Dicks et al. (1995) to detect *Oenococcus oenos* in South African fortified wines, where this bacterium probably is the major spoilage organism.

In the brewery industry, other media are used for the enumeration of beer spoilage LAB. Many of these media are based upon the use of beer as the basic component in addition to complex nutrient ingredients, minerals and growth factors (Jespersen and Jakobsen, 1996). The ingredients in the beer included inhibit the growth of most other bacteria than beer spoilage organisms (Jespersen and Jakobsen, 1996). In addition selective agents such as actidione, cycloheximide or sorbic acid are used to prevent the growth of yeasts and sodium azide, polymyxin B or thallous acetate to inhibit Gram-negative bacteria (Schillinger and Holzapfel, 2003).

So-called ‘indirect’ methods for rapid enumeration of LAB in foods have been applied in some situations with rather moderate success, probably due to the need for standardisation, varations in the composition of the food substrate, and the inclusion of controls for reference purposes. Such methods include impedance/conductance determination, as was reported by Lanzanova et al. (1993) and Wawerla et al. (1998). DEFT as a further ‘non-culture’ method has been used for the rapid detection of LAB in wine (Couto and Hogg, 1999).

**Identification**

After cultivation of the suspected spoilage bacteria on the appropriate agar plates, their identity still has to be examined. In most cases, colonies will be picked from the plates and after subculturing of the strains, several procedures for identification may be applied. Alternatively the identity of the colonies may be determined by using colony hybridisation techniques. Selecting of the isolates from a dilution series associated with an analysis of a spoiled food plays a dramatic role to the outcome of the analysis. If characterisation of the predominant LAB population is wanted, only colonies obtained from the highest dilutions should be picked for species identification purposes.

**Phenotypic methods**

The classical identification of LAB is based on morphological, physiological and biochemical characteristics. Most identification keys use features such as cell morphology, growth at certain temperatures, production of gas from glucose, and the type of lactic acid isomer produced from glucose in addition to assimilation or fermentation patterns of a number of carbohydrates and nitrogen sources. Miniaturised phenotypic fingerprinting systems are available such as the Biolog system or API CH 50 (Biomérieux) using a battery of dehydrated reagents and the addition of a standardised inoculum to create a
phenotypic fingerprint that is compared with the database provided by the manufacturer. The results of these automated systems have to be carefully interpreted because of the limitations of these systems. The quality of the results depends greatly on the reliability of the database. Generally, these phenotypic methods suffer from a relatively poor reproducibility and a low taxonomic resolution that often does not allow differentiation between related organisms (Axelsson, 2004; Temmermann et al., 2004).

Whole cell protein analysis is a more reliable technique for identification of LAB. The comparison of whole-cell protein patterns obtained by highly standardised SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis) helped to identify wine spoilage LAB (Patarata et al., 1994), non-starter LAB from Italian ewe cheeses (De Angelis et al., 2001) and LAB from spoilage associations of cooked and brined shrimps (Dalgaard et al., 2003).

Fourier transform infrared (FTIR) spectroscopy could be a method for rapid identification of spoilage LAB. Its applicability for discrimination of Lactobacillus species from breweries and LAB used by the dairy industry was reported (Curk et al., 1994; Amiel et al., 2000).

DNA-based methods
Various DNA-based techniques mainly developed during the last two decades are applicable for the detection and identification of spoilage LAB. One of the main advantages of these methods is their independence of variations in the growth conditions of the microorganisms (Temmermann et al., 2004). These identification methods include sequencing of 16S or 23S rDNA regions polymerase chain reaction, (PCR) assays utilising genus or species specific primers and several DNA fingerprinting techniques either relying on patterns of PCR-amplified DNA fragments or on restriction analyses of total DNA or amplicons resulting from a selective PCR reaction (restriction fragment length polymorphism RFLP).

16S rDNA sequencing is a frequently applied method to identify an unknown isolate. Barney et al. (2001) differentiated Pediococcus isolates from a brewery by sequencing their 16S rRNA genes in addition to another molecular typing method. Another example is the application of 16S rDNA sequencing for the specific detection of Lactobacillus brevis known as the most frequent beer spoiler in the brewing industry (DiMichele and Lewis, 1993). However, it should be kept in mind that there are no unanimously agreed taxonomic criteria on similarity levels of 16S rRNA genes in association with the bacterial species status. Many LAB species share high 16S rRNA gene sequence similarities. In the case of leuconostocs, this can be elucidated with Leuconostoc gascomitatum associated with poultry meat spoilage. The Leuconostoc gascomitatum strain 18812 possesses 99.0% 16S rRNA gene similarity with the type strain of Leuconostoc gelidum, even their DNA–DNA reassociation level was as low as 33.4% (Björkroth et al., 2000).
PCR methodology using species- or genus-specific primers can successfully be applied to discriminate LAB involved in spoilage. Targets of these primers are usually 16S rDNA, 23S rDNA or 16S/23S rRNA spacer regions. Thus, species-specific primers from the 16S–23S rDNA intergenic spacer regions were designed for a number of lactobacilli including *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus alimentarius* and *Lactobacillus farciminis* (Berthier and Ehrlich, 1998; Rachman et al., 2003). Recently, PCR primers with enhanced specificity were designed to differentiate between beer-spoilage producing strains of *Lactobacillus paracollinoides* from the genetically very similar *Lactobacillus collinoides* without beer spoilage capacity (Suzuki et al., 2004). Yasui et al. (1997) developed a species-specific PCR for the rapid detection of *Lactobacillus lindneri* often associated with beer spoilage. The authors report that starting from a small colony on an agar plate, the whole procedure (DNA extraction, PCR and electrophoresis) would not take more than 5 hours. Yost and Nattress (2000) suggested the use of genus-specific primers for *Carnobacterium* and *Leuconostoc* in combination with species-specific primers for *Lactobacillus curvatus* and *Lactobacillus sakei* in several multiplex reactions to identify LAB isolates associated with meat spoilage. Some of their primers were also used by Beneduce et al. (2004) for the molecular characterisation of LAB involved in wine spoilage. A similar approach was followed by Macián et al. (2004). They designed genus-specific, group-specific and species-specific primer pairs for the simultaneous detection of *Carnobacterium* and *Leuconostoc* in meat products and the identification of non-motile *Carnobacterium* species. In artificially inoculated meat samples, the presence of *Leuconostoc* and *Carnobacterium* was demonstrated at a level of $10^3$–$10^4$ cfu/g by the use of a multiplex PCR (Macián et al., 2004). In order to overcome problems associated with the poor resolution and detection sensitivity of DNA fragments in agarose gel electrophoresis, a combination of the multiplex PCR with capillary gel electrophoresis and laser-induced fluorescence detection of amplification products was proposed (Garcia-Canas et al., 2004).

Ribotyping was found to be a useful tool for identifying meat spoilage LAB (Björkroth and Korkeala, 1996, 1997b; Björkroth et al., 1998, 2000), LAB associated with spoiled fish products (Lyhs et al., 2004) and brewery *Pediococcus* isolates (Barney et al., 2001). It is based on the restriction endonuclease digestion of chromosomal DNA, followed by Southern hybridisation to probes for sequences in the highly conserved regions of the rRNA operon. The resulting rRNA restriction patterns are used for identification of the unknown isolates. An automated ribotyping system (Qualicon RiboPrinter from Du Pont) was developed which requires only 8 hours to produce reliable results from isolated colonies. In the study of Björkroth and Korkeala (1997b) on the LAB contamination routes of vacuum-packaged sliced cooked whole-meat products, the different ribotypes obtained were successfully used to distinguish spoilage strains among LAB samples from different sites of product manufacture. By using numerical analysis of *Hind*III ribopatterns, *Leuconostoc*
carnosum was found to be the Leuconostoc species specifically associated with spoilage of vacuum-packaged sliced cooked ham (Björkroth et al., 1998). In a similar study, characterisation of LAB isolates from modified atmosphere packaged marinated broiler meat by ribotyping resulted in the identification of Leuconostoc gasicomitatum as the dominating spoilage organism (Susiluoto et al., 2003).

During investigations on spoiled fish products, ribotyping enabled the identification of Lactobacillus alimentarius as the spoilage organism in marinated herring (Lyhs et al., 2001) and of Lactobacillus sakei, Lactobacillus curvatus and Carnobacterium piscicola (maltaromaticum) as the major species associated with spoiled vacuum-packaged gravad rainbow trout (Lyhs et al., 2002). Slime-producing Leuconostoc strains from an acetic acid herring preserve could be attributed to the species Leuconostoc gelidum and Leuconostoc gasicomitatum by the comparison of HindIII 16 and 23S RFLP patterns (Lyhs et al., 2004). Other types of RFLP such as ARDRA and AFLP may also be applied for the identification of spoilage LAB. 16S-ARDRA was used for identification of LAB from grape must and wine (Rodas et al., 2003) and AFLP for characterisation of Carnobacterium strains from meat and seafood (Laursen et al., 2005). Kabadjova et al. (2002) developed a strategy for identification of Carnobacterium isolates based on RFLP of PCR-amplified 16S–23S ribosomal intergenic spacer regions.

Rapid amplification of polymorphic DNA (RAPD)-PCR is a frequently used method for molecular typing of LAB and for genomic diversity studies as it is rapid and easy to execute. It is based on the amplification of random DNA segments with single small primers of arbitrary nucleotide sequences under low stringency conditions. Examples for its application to discriminate lactobacilli and other LAB possibly involved in food spoilage are the characterisation of microbial populations contributing to spoilage of vacuum packaged beef (Yost and Nattress, 2002), of LAB from fermented sausages (Andrighetto et al., 2001), and of Lactobacillus plantarum strains involved in wine spoilage (Spano et al., 2002). Repetitive element PCR (REP-PCR) is a related approach using amplification of fragments by PCR with oligonucleotides specific for simple repetitive DNA sequences. This technique was demonstrated to be useful for the discrimination of closely related Lactobacillus species such as L. plantarum, L. paraplantarum and L. pentosus (Kostinek et al., 2005).

Another approach is the use of group or species-specific rRNA targeted oligonucleotide probes for the direct detection of LAB by colony hybridisation. Lonvaud-Funel et al. (1991) used colony hybridisation with non-isotopic probes for specific enumeration of LAB in fermenting grape must and wine, and Nissen and Dainty (1995) applied rRNA probes for colony blot hybridisations to identify meat lactobacilli.

20.3.2 Methods applicable without previous cultivation

The fastest culture-independent approach for the genus-, species- or strain-
specific detection of LAB in a food matrix is the use of specific primers for a PCR reaction with bacterial DNA extracted from the sample (Temmerman et al., 2004). This method allows the detection of certain spoilage organisms that might be expected in a food during processing without the need for time-consuming cultivation procedures. For example, for the quality control of the wine-making process it is important to know if glucan-producing *Pediococcus damnosus* strains are present, since they may cause unacceptable viscosity. Gindreau et al. (2001) designed specific primers from the sequence of a plasmid involved in glucan production and used them to specifically detect such ropy *Pediococcus* strains. Species-specific primers for various meat LAB were also developed to directly identify these organisms in low-acid sausages (Aymerich et al., 2003).

Another culture-independent approach involves *in situ* hybridisation techniques where synthetically designed oligonucleotides bind to specific target sequences (mostly 16S rDNA) in bacterial DNA. The most commonly applied method is the fluorescent *in situ* hybridisation (FISH) especially for the detection of bacteria in liquids (beverages) such as wine. It allows the direct identification and quantification of LAB by microscopy without previous cultivation within a few hours (Blasco et al., 2003). The bacterial cells of the sample are fixed and immobilised on microscopic slides or membrane filters, are permeabilised for the fluorescently labelled oligonucleotide, *in situ* hybridised with the probe and then counted under the epifluorescent microscope. Several authors (Sohier and Lonvaud-Funel, 1998; Blasco et al., 2003) demonstrated the application of this rapid method for the identification of LAB species related to wine spoilage. *In situ* hybridisation may also be coupled with flow cytometry, as shown by Connil et al. (1998).

Another rapid method using epifluorescence microscopy is DEFT involving capturing the bacterial cells on the surface of membrane filters and staining with a fluorochrome. It has found applications in the dairy and wine-making industry.

Denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient gel electrophoresis (TGGE) may also be used as culture-independent techniques to detect and analyse the LAB spoilage association of foods. In these methods, a variable region of the 16S rRNA gene is amplified by PCR and DNA fragments of the same size are separated by their denaturing profile on an acrylamide gel. The resulting banding patterns can be digitally captured and normalised using reference patterns, allowing the identification of band positions though comparison of those present in a database of well-characterised type and reference strains (Temmerman et al., 2004). This approach was followed in a study on the identification of lactobacilli involved in the sour dough fermentation process (Meroth et al., 2003) and in investigations of the microflora of cheeses (Ercolini et al., 2004; Ogier et al., 2002). A PCR-DGGE including identification of the band patterns by sequencing was applied to analyse the LAB association of different spoiled meat products in a meat processing plant (Takahashi et al., 2004).
20.4 Implications for control in foods affected

Numerous methods are available to control spoilage by LAB in foods and thus extend the shelf-life of the product. Their application, of course, depends on the type of food to be preserved. For each product, the measures of choice have to be determined individually. In many foods, e.g. meat products, hygienic improvements during the manufacturing process can solve the spoilage problem. For instance, recontamination of cooked meat products during packaging and slicing has to be avoided by the consistent application of clean room technology and of hygienic working methods with the aim of physically handling the product as little as possible. Problems with dextran formation by leuconostocs may be avoided by not using sucrose (precursor of dextran) in the recipe of the product. However, this does not have an effect on *Lactobacillus sakei* forming ropy slime in vacuum-packaged meat products. The formation of ropy slime by *Lactobacillus sakei* was overcome in Finnish meat industry when the handling and packaging of fermented products was separated from the handling of cooked products in early 1990s. In other products, e.g. cheeses, the selection of highly competitive starter cultures may prevent problems associated with the production of off-flavours, CO₂ production and bitterness resulting from excessive proteolysis of casein.

To control food spoilage, several new preservation technologies including non-thermal decontamination by high hydrostatic pressure (HHP) and pulsed electric fields are available and may be increasingly used. They have been developed mainly because of increased consumer demand for tasty, nutritious, natural and easy-to-handle food products (Devlieghere et al., 2004). These novel preservation technologies also include the application of the concept of active packaging, bioprotective cultures and natural antimicrobial compounds such as essential oils and other phytopreservatives, enzymes and bacteriocins. All preservation treatments may also be used in combination to make use of synergistic or additive effects.

The active packaging concept involves the incorporation of naturally occurring antimicrobials such as nisin or lysozyme in food packaging materials in order to control the growth of undesirable organisms on the surface of foods. These antimicrobial packaging films can be prepared by incorporating the antimicrobial agent either into the packaging material by coating the active compound onto the surface of the packaging film or by adding a sachet into the package from which the antimicrobial compound is released during further storage (Devlieghere et al., 2004). Potential applications include vacuum packaged products as an intensive contact between the active material and the food product is essential for the antimicrobial efficacy. Scanell et al. (2000) developed active food packaging materials using the immobilised bacteriocins nisin and lacticin 3147, and this antimicrobial packaging resulted in a reduction of the population of LAB in sliced cheese and ham stored in modified atmosphere packaging at refrigeration temperatures, thus extending the shelf-life. Another example is the use of antimicrobial films, designed to slowly release acetic or propionic acid, to improve the
preservation of vacuum packaged processed meats during refrigerated storage (Quattara et al., 2000).

Numerous studies have demonstrated that bacteriocin-producing LAB can successfully be applied in foods to inhibit *Listeria monocytogenes* and other food pathogens (Bredholt et al., 1999; Budde et al., 2003; for reviews see Rodgers, 2001; O’Sullivan et al., 2002; Holzapfel et al., 2003). These bioprotective cultures may also be of value in controlling spoilage LAB as most bacteriocins produced by them are also effective against related organisms. However, spoilage LAB populations in many foods consist of several species, of which some may possess resistance to the bacteriocin used. Therefore, cooked products possessing more limited species diversity may be more suitable for these applications. Inhibition of LAB associated with spoilage by bacteriocin-producing cultures was observed in various foods including vacuum-packaged beef (Leisner et al., 1996), cooked meat products (Vermeiren et al., 2004), dairy products (O’Sullivan et al., 2002), and vegetable fermentations such as olive fermentation (Ruiz-Barba et al., 1994). A bacteriocin-producing strain of *Leuconostoc gelidum* delayed the spoilage caused by a sulphide-producing *Lactobacillus sakei* on vacuum packaged beef for up to 8 weeks of storage (Leisner et al., 1996). In cheese production, control of non-starter lactic acid bacteria (NSLAB) is considered essential to guarantee a consistent quality of the end-product in terms of flavour. The adventitious LAB population can cause defects in cheese including formation of calcium lactate crystals, off-flavour development and slit formation (O’Sullivan et al., 2002). It was shown that cheddar cheeses manufactured with bioprotective bacteriocin-producing *Lactococcus lactis* strains contained markedly lower NSLAB levels in the ripening period, thereby reducing the risk of off-flavour development (Ryan et al., 1996; O’Sullivan et al., 2003).

Essential oils such as carvacol, thymol and eugenol have been shown to exert antibacterial activity against spoilage and pathogenic bacteria. Significant antibacterial effects are achieved with concentrations between 0.5 and 20 µg l⁻¹ in foods (Burt, 2004). Up to now, however, few studies aimed at specifically inhibiting spoilage LAB in foods have been conducted. Tsigarida et al. (2000) observed a reduction of the initial LAB population after addition of 0.8% oregano essential oil to beef fillets.

Lysozyme was investigated among others as a potential preservative for alcoholic beverages. The application of lysozyme to grape or wine after completion of the malo-lactic fermentation delayed the spoilage by LAB (Gerbaux et al., 1997). In order to enhance the effectiveness of lysozyme, combinations of this antimicrobial compound with nisin or nisin and EDTA (ethylenediamine tetraacetic acid) were used in meat inoculation experiments, and those combinations were found to be inhibitory to the proliferation of spoilage LAB on pork loins and ham and bologna sausages respectively (Gill and Holley, 2000; Nattress and Baker, 2003).

Nisin alone was reported to be able to inhibit the growth of spoilage LAB inoculated into vacuum-packaged pasteurised bologna-type sausages stored
at 8 °C (Davies et al., 1999). Another application of nisin to prevent spoilage caused by LAB without affecting yeasts could be in the brewing and wine industry. The addition of nisin might be an effective measure to protect wines against later bacterial spoilage by LAB, in particular after malo-lactic fermentation. Nisin at a level as low as 100 IU ml−1, alone, or in combination with nisin-resistant starter cultures, was shown to be effective in controlling the malo-lactic fermentation in wines (Daeschel et al., 1991).

Several investigations have shown that chitosan also has some potential as a natural preservative to control spoilage LAB in certain foods. For instance, growth of LAB was retarded in a mayonnaise-based shrimp salad prepared with chitosan added and stored at 5 °C (Roller and Covill, 2000), and the dipping of pork sausages in chitosan solutions resulted in a reduction of the LAB and in an increased shelf-life of these products (Sagoo et al., 2002).

20.5 Future trends

Food spoilage is an extremely complex process involving various interactions between the microorganisms and the substrate. Therefore, the role of the LAB has to be evaluated carefully as it is highly dependent on the particular food substrate in question, and its condition in terms of intrinsic (ripeness and composition, aw, pH, maturity, age, endogenous enzymatic activities, etc.) and extrinsic (atmosphere, temperature, etc.) factors. Their metabolism may result in both beneficial and harmful changes depending on the type of food. A Lactobacillus strain with a strong spoilage potential, e.g. for cooked meat products, because of its excessive souring capacity may be an excellent starter culture for the production of fermented sausages. The decarboxylation of malic acid to lactic acid by Oenococcus oenos or other LAB is regarded beneficial to acid wines, but detrimental to low acid wines where it must be controlled to avoid spoilage, also associated with secondary fermentation after bottling. Despite this knowledge on the dual nature of the behaviour of LAB in foods, more information is needed on their role in complex spoilage associations.

In many food ecosystems, the exact contribution of several groups of LAB to the spoilage process is not yet understood. For instance, carnobacteria and enterococci were shown to dominate the spoilage association of cooked and brined shrimps (Dalgaard et al., 2003). However, it is not known how these LAB genera contribute to the spoilage of the product. Another example is the limited information on the role of carnobacteria in the spoilage of vacuum-packaged cold-smoked salmon. In inoculation studies, strains of Carnobacterium maltaromaticum (formerly C. piscicola) did not accelerate the spoilage process of packed cold-smoked salmon although this species is most commonly being isolated from spoiled salmon (Paludan-Müller et al., 1998).
Biodiversity studies may be helpful to increase knowledge on the specific role of certain LAB in the food ecosystem and to understand why this organism is predominant in that situation. Comparison of various strains of the same species may reveal specific characteristics of the spoilage strain that aid in growth niche occupation. This information would also facilitate the evaluation of the spoilage potential of strains that might be used as bioprotective cultures. There is a discrepancy between the ability of *Carnobacterium divergens* and *Carnobacterium maltaromaticum* to dominate a large range of meat and seafood products and their documented ability to spoil a limited number of these products (Laursen *et al*., 2005). Similarly, the evaluation of *Leuconostoc carnosum* strains in terms of their spoilage potential is difficult. *Leuconostoc carnosum* was identified as the specific spoilage organism in vacuum packaged cooked ham (Björkroth *et al*., 1998) and on the other hand, a strain of this species was suggested as bioprotective culture to be applied in vacuum packaged cooked meat products (Jacobsen *et al*., 2003). A comprehensive study on *Leuconostoc carnosum* strains in terms of their spoilage potential is difficult. *Leuconostoc carnosum* was identified as the specific spoilage organism in vacuum packaged cooked ham (Björkroth *et al*., 1998) and on the other hand, a strain of this species was suggested as bioprotective culture to be applied in vacuum packaged cooked meat products (Jacobsen *et al*., 2003). A comprehensive study on *Leuconostoc carnosum* strains isolated from a meat processing plant revealed that only one pulsed field gel electrophoresis (PFGE) type of *Leuconostoc carnosum* was associated with spoilage of cooked ham despite a tremendous diversity of strains of this species (25 different PFGE types) detected in the plant and despite the fact that the spoilage strain was not overwhelmingly present in the production environment (Björkroth *et al*., 1998).

In the future, focus of research will be directed to the development and optimisation of rapid and reliable techniques for detection of LAB involved in spoilage with a special emphasis on methods applicable in routine laboratories and for the industry. For instance, for the microbiological quality control of breweries and in the wine-making industry, it is important to be able to detect small numbers of spoilage LAB at an early stage to enable the application of corrective measures before manifestation of the spoilage phenomena. Molecular methods not requiring time-consuming culture and colony isolation steps are available for these purposes. Species-specific primers or probes can be designed which are very fast tools for detecting the target organisms. For the wine-making industry, these methods are indispensable for the early and specific detection of ropy *Pediococcus damnosus* strains in wine, which may initially be present in small numbers among other non-spoilage pediococci (Gindreau *et al*., 2001). Similarly, low numbers of *Lactobacillus* strains with beer-spoilage capacity can be identified among other LAB in beer by the use of appropriate specific primers.

In the future, new nucleic acid-based technologies will be evaluated for routine analyses of spoiled food samples. The rapid evolution in total genome sequencing offers new possibilities for fast and accurate identification and detection, when implemented in microarray technology (Temmermann *et al*., 2004). However, the genomic projects have mainly produced whole genome sequences of LAB associated with dairy or other fermentations, whereas the spoilage LAB have been less studied. The genomic sequences of
LAB have shown that an extensive unexpected variation exists and many novel metabolic functions have been discovered. With the help of the genomic data, studies targeting the metabolism of spoilage-associated LAB may in the future provide new tools useful in restricting the growth of these organisms in various foods.

### 20.6 Sources of further information and advice

For further information on food spoilage associated with LAB, the reader is referred to *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, third edition, edited by Dworkin *et al.* (2003). The chapters on *Lactobacillus* and *Carnobacterium* by Hammes and Hertl (2003), ‘*Leuconostoc and Weissella*’ by Björkroth and Holzapfel (2003), and ‘*Pediococcus and Tetragenococcus*’ by Holzapfel *et al.* (2005) describe spoilage-associated LAB of the various types of foods. Reviews on spoilage of vacuum or modified atmosphere packaged cooked meat products or marinated meat products were given by Korkeala and Björkroth (1997), Borch *et al.* (1996) and Björkroth (2005). Information on the taxonomy of the LAB involved in spoilage can be obtained from ‘The genera of lactic acid bacteria’ edited by Wood and Holzapfel (1995).

Chapter 8 (‘Culture media for lactic acid bacteria’) in the *Handbook of Culture Media for Food Microbiology* edited by Corry *et al.* (2003) might be consulted for further information on selective media for LAB. Modern methods applied to the detection of LAB were summarised in a review by Temmermann *et al.* (2004). The application of protective cultures was reviewed by Rodgers (2001), O’Sullivan *et al.* (2002) and Holzapfel *et al.* (2003). General information on bacteriocins including nisin can be obtained in the review from Jack *et al.* (1995) and the textbook *Bacteriocins of Lactic Acid Bacteria* edited by DeVuyst and Vandamme (1994).

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Lactic acid bacteria


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21

Spore-forming bacteria
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21.1 Introduction

Spore-forming bacteria are a diverse group of microorganisms and a number of these are important causes of food spoilage. These organisms are commonly associated with spoilage of heat-treated foods because of the spore’s ability to survive the high temperatures commonly used to preserve foods. A number of different prokaryotic organisms have developed non-dividing resting stages that have little or no metabolism and this allows them to remain dormant for extremely long periods of time. Important features of these dormant states are an increased resistance to various environmental ‘stresses’, such as heat, that would normally kill the progenitor vegetative cells and also an ability to survive long periods of nutrient deprivation. Once suitable environmental conditions for growth have been restored, the resting form is able to return to the vegetative state and resume multiplication and normal metabolism.

One of the resting forms that is part of the life cycle in some bacterial species is the endospore, so-called because it is formed within an existing cell after asymmetric division. Of all the resting forms that are known (including exospires from Actinomycetes and methylotrophic bacteria, myxospores from myxobacteria, cysts from Azotobacter and Bdellovibrio), the endospore formers are the largest group and they also contain the most dormant resting forms. Consequently, they are often the main target for foods that are designed to have long shelf-lives at ambient temperatures, such as canned products or products that are heat-treated in-line and then aseptically filled. This chapter is restricted to those structures (referred to here as endospores or spores) produced intracellularly by Bacillales (see below) which differ from their
mother cell in both structure and chemical composition, containing dipicolinic acid and having little or no metabolism and are more resistant than vegetative cells to extreme environmental conditions.

21.1.1 Historical aspects
For some time, endospore formation was thought to be restricted to two Gram-positive genera, the aerobic Bacillus and anaerobic Clostridium. In the original classification (Bergey et al., 1923), which is still in use today (Slepecky, 1972), all rod-shaped, spore-forming bacteria were classified as belonging to one family Bacillaceae, in the order Eubacteriales. Most of the knowledge about the physiology, sporulation, germination, ecology and response to environmental stresses (some of which are commonly used in food processing) of endospore-forming bacteria has been derived from these two groups of organisms. Bacterial endospores were first reported in 1876 independently by Cohn (1876) and slightly later by Koch (1876). The work by Cohn focused on heat sterilisation of organic material and included the finding that Bacillus subtilis spores could survive 100°C without losing their viability, while that of Koch concentrated on the aetiology of anthrax.

Since these discoveries, a wider variety of endospore-forming bacteria have been described. Anaerobic spore-formers were isolated and described by Kitasato in 1889, who was the first to isolate tetanus bacillus and by Van Ermengem (1896), who was the first to isolate C. botulinum. These early reports of toxin-producing endospore-forming bacteria were followed by the first report of Clostridium pasteurianum by Winogradsky (1902) and reports of new Bacillus species (such as B. macerans, B. larvae and B. thuringiensis) soon after this (see Keynan and Sandler, 1983, for more detail). In 1915, Hammer first isolated B. coagulans from spoiled canned milk. Adamson (1919) was the first to describe butyric acid fermentation by Clostridium butyricum and the first of the thermophilic spore-formers, named B. stearothermophilus was first described by Donk in 1920. The significance of thermophilic spoilage was identified by Barlow (1913) in his studies on canned corn. During this time and up to the 1930s, the leading cause of spoilage in canned goods was probably thermophilic spoilage. Following work on a series of investigations in canning factories, refined sugar was identified as a likely source of thermophilic spoilage organisms (Cameron, 1930), leading to the setting of standards for these raw materials and similar ingredients such as starch, flour and spices (Denny 1981).

In the 1940s, fermentation by anaerobic spore-formers in acid canned products was extensively studied by Townsend (1939) and Spiegelberg (1940a,b) and various options for the processing of these products were identified by Bowen et al. (1954a,b) and later by York et al. (1975). The major causes of microbial spoilage of canned foods today are underprocessing (thermal process set is insufficient to destroy the organisms likely to spoil
the product) and post-process leakage of cans. Problems also occur, although less frequently, due to failure in retorting (failure to achieve the thermal process set) and also because of spoilage occurring prior to heat processing.

‘Bone taint’ spoilage or deep-tissue spoilage of beef and cured hams was a significant cause of spoilage in the early part of the 20th century and was associated with \( \text{C. putrefaciens} \), first described as \( \text{Bacillus putrefaciens} \) by McBryde (1911). Better control in the processing of these products has led to the incidence of this type of spoilage being largely reduced.

Although spoilage incidents (referring to cases where spoiled products are in the marketplace) may be seen to result only in commercial losses, which can be considerable, they are often indicative of faults in processing, container integrity or hygiene standards and, as such, these failures can also impact on safety. It is often the case that a spoilage incident is characterised by the presence and growth of harmless organisms detected in containers of foods that have been examined, but it can be difficult, and sometimes impossible, to exclude the possibility that an unopened container made from the same affected batch contains pathogenic microorganisms. For this reason, spoilage can be an indicator of a potentially much more serious incident.

21.1.2 Types of spore-forming spoilage bacteria

Traditionally, spore-forming bacteria that are associated with spoilage tend to be grouped according to their growth characteristics. For example, reports describing thermophilic spoilage (Denny, 1981; Ashton, 1981; Ito, 1981; Speck, 1981; Thompson, 1981) refer to four groups of heat-resistant bacteria that are important in the spoilage of canned foods. Thermophilic spoilage bacteria are distinguished on the basis of their preference for growth at elevated temperatures (optimum growth temperature >45°C). That said, use of the term ‘thermophile’ is inconsistent and there may be strains of organisms, such as \( \text{B. coagulans} \), normally regarded as ‘thermophilic’, that can grow at temperatures below 30°C and have optimum temperatures for growth less than 45°C. As such, these strains should be regarded as mesophilic strains. For this reason, strains of the same species may be classified as thermophilic or mesophilic, depending on their optimum growth temperature. Cameron and Esty (1926) classified organisms growing at 55°C but not at 37°C as obligate thermophiles and those growing at 55°C and 37°C as facultative thermophiles. This definition is generally accepted by food microbiologists and is followed here. \( \text{B. coagulans} \) is typically described as ‘facultative thermophilic’ (Thompson, 1981). The four thermophilic or facultative-thermophilic groups commonly referred to are non-hydrogen sulphide (H₂S) producing anaerobes (e.g. \( \text{Thermoanaerobacterium thermosaccharolyticum} \), formerly \( \text{Clostridium thermosaccharolyticum} \)), aciduric flat-sour aerobic bacteria (e.g. \( \text{B. coagulans} \)), thermophilic flat-sour (non-aciduric) aerobic bacteria (e.g. \( \text{Geobacillus stearothermophilus} \), formerly \( \text{B. stearothermophilus} \)), and thermophilic sulphide (or H₂S-producing) anaerobes (e.g. \( \text{Desulfotomaculum nigrificans} \)). A fifth
group of facultative thermophilic facultative anaerobes (e.g. *B. subtilis*) is also associated with thermophilic spoilage.

Mesophilic (organisms with an optimum growth lying in the range 15–45 °C) spore-forming bacteria also cause spoilage of foods and these include anaerobic spore-formers commonly referred to as proteolytic or putrefactive anaerobes (e.g. *C. sporogenes*, *C. putrificum*, *C. putrefaciens*), acid-tolerant butyric anaerobes (e.g. *C. butyricum* and *C. pasteurianum*), facultative anaerobes (e.g. *B. circulans*, *Paenibacillus macerans*, *Brevibacillus laterosporus*) and acidophilic facultative anaerobes (e.g. *Alicyclobacillus acidoterrestris*).

There are a small number of cold-growing, or psychrotolerant (either psychrotrophic or psychrophilic), spore-formers that can cause spoilage of chilled foods and these include anaerobes (*C. estertheticum*, *C. laramiense*, *C. algidicarnis*, *C. frigidicarnis*, *C. gasigenes*) and facultative anaerobes (*B. circulans*, *B. mycoides*). Psychrotrophs are those organisms capable of growth at low temperatures (e.g. 0–5 °C), with an optimum growth temperature >15 °C and maximum temperature for growth >20 °C. Psychrophiles, as the name implies, are cold-loving organisms capable of relatively rapid growth at low temperatures, having optimum growth temperatures less than 15 °C and an upper growth limit of about 20 °C. The different groups of spore-forming spoilage bacteria are shown in Table 21.1, together with the types of foods affected and typical spoilage effects observed.

### 21.1.3 Taxonomy of spore-forming spoilage bacteria

Foodborne bacteria have generally been defined on the basis of microscopic and phenotypic properties, together with biochemical reactions. With the advent of new methods that allow genotypic characterisation of microorganisms, e.g. through comparison of 16S rRNA sequences and DNA–DNA hybridisations, there has been a revolution in the taxonomic status of a number of foodborne bacteria. This has resulted recently in many changes, most notably for some species of the genus *Bacillus*, where eight new genera have been described in recent years. Of these eight new genera, four contain organisms that are associated with food spoilage. These four genera are *Alicyclobacillus*, *Brevibacillus*, *Paenibacillus* and *Geobacillus*, described in detail by Wisotzkey *et al.* (1992), Shida *et al.* (1996), Ash *et al.* (1993) and Nazina *et al.* (2001) respectively. The application of more molecular techniques, such as recN gene sequences, in the characterisation of different strains, provides further evidence that the taxonomy of organisms such as *Geobacillus* requires further reassessment of these genera (Ziegler, 2005).

The taxonomy of the clostridia has also come under close scrutiny in recent years and six of the 118 species recognised in 1991 have been assigned to new genera. Only two of these new genera, *Moorella* and *Thermoanaerobacterium*, contain species known to be associated with food spoilage. These reclassifications are described by Collins *et al.* (1994) and Lee *et al.* (1993).
<table>
<thead>
<tr>
<th>Spoilage group</th>
<th>Spoilage organisms</th>
<th>Typical foods affected</th>
<th>Spoilage effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermophilic aciduric flat sours</td>
<td><em>B. coagulans</em> (formerly <em>B. thermoacidurans</em>), <em>B. smithii</em></td>
<td>Low pH, canned/hermetically sealed products, e.g. tomatoes (pH 4.2–4.4) Higher pH dairy products, e.g. evaporated milk</td>
<td>Acid production (decrease in pH), off-taste, off-odour, little or no gas production. In evaporated milk, causes firm coagulation</td>
</tr>
<tr>
<td>Thermophilic flat sours</td>
<td><em>G. stearothermophilus</em> (formerly <em>B. stearothermophilus</em>)</td>
<td>High pH canned/hermetically sealed products (most meats, milk, vegetables)</td>
<td>Acid production (decrease in pH) off-taste, off-odour, little or no gas production</td>
</tr>
<tr>
<td>Facultative thermophiles, facultative anaerobes</td>
<td><em>B. subtilis</em></td>
<td>High pH canned/hermetically sealed products. Bread held at high ambient temperatures</td>
<td>Some gas production, sometimes acid production, causes soft swells. Causes ‘rope’ in bread</td>
</tr>
<tr>
<td>Thermophilic non-H₂S producing anaerobes</td>
<td><em>Thermoanaerobacterium thermosaccharolyticum</em> (formerly <em>C. thermosaccharolyticum</em>), <em>C. thermoaceticum</em></td>
<td>Canned/hermetically sealed, high pH or acidic (pH 4.1–4.4) products held at high temperature (30–60 °C) for extended periods, e.g. vending machine soups, vegetables, pet foods</td>
<td>Hard swells, acid production (decreasing pH), gas (H₂, CO₂), cheesy or butyric odours</td>
</tr>
<tr>
<td>Thermophilic H₂S producing anaerobes</td>
<td><em>Desulfitomaculum nigrificans</em> (formerly <em>C. nigrificans</em>)</td>
<td>Canned/hermetically sealed products held at high temperature (&gt;55 °C) for extended periods</td>
<td>H₂S production (‘sulphide stinkers’), blackening</td>
</tr>
<tr>
<td>Mesophilic facultative anaerobes</td>
<td><em>B. coagulans</em>, <em>B. smithii</em>, <em>B. amyloliquefaciens</em>, <em>B. circulans</em>, <em>Brevibacillus laterosporus</em> (formerly <em>B. laterosporus</em>), <em>Brevibacillus brevis</em> (formerly <em>B. brevis</em>), <em>Paenibacillus macerans</em> (formerly <em>B. macerans</em>), <em>B. pumilus</em>, <em>Paenibacillus polymyxa</em> (formerly <em>B. polymyxa</em>), <em>B. betanigrificans</em></td>
<td>Canned/hermetically sealed products Fresh vegetables growing/ held at high ambient temperatures</td>
<td>Hydrolyse starch, some gas-forming types (<em>P. macerans</em>, <em>B. subtilis-pumilus</em> group). Some produce flat-sour spoilage (<em>B. coagulans</em>, <em>P. macerans</em>). Soft rot of fresh vegetables. <em>B. betanigrificans</em> causes black beets</td>
</tr>
<tr>
<td>Spoilage group</td>
<td>Spoilage organisms</td>
<td>Typical foods affected</td>
<td>Spoilage effects</td>
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<tr>
<td>Mesophilic putrefactive anaerobes</td>
<td><em>C. sporogenes</em>, <em>C. parasporogenes</em>, <em>C. putrificum</em></td>
<td>Canned/hermetically sealed products</td>
<td>Putrefaction (proteolysis), off-odours, hard swells</td>
</tr>
<tr>
<td>Mesophilic butyric anaerobes</td>
<td><em>C. butyricum</em>, <em>C. pasteurianum</em>, <em>C. beijerinckii C. tyrobutyricum</em></td>
<td>Low pH canned/hermetically sealed products, e.g. tomatoes, corn, pears Hard cheeses, e.g. Gouda, Emmental</td>
<td>Butyric acid production, reducing pH, hard swells Early blowing of cheeses</td>
</tr>
<tr>
<td>Acidophilic facultative anaerobes</td>
<td><em>A. acidoterrestris</em>, <em>A. acidocaldarius</em>, <em>A. acidiphilus</em></td>
<td>Low pH (e.g. &lt; 3.8) canned/hermetically sealed products, e.g. fruits, fruit juices</td>
<td>‘Medicinal’ taints (e.g. guiacol, 2,6, dibromophenol, 2,6-dichlorophenol)</td>
</tr>
<tr>
<td>Psychrotolerant anaerobes</td>
<td><em>C. estertheticum</em>, <em>C. laramiense</em>, <em>C. algidicarnis</em>, <em>C. frigidicarnis</em>, <em>C. gasigenes</em>, <em>C. aligidixylanolyticum</em>, <em>C. putrefaciens</em></td>
<td>Vacuum packed chilled meat products Brine-cured hams</td>
<td>Gas (H₂) formation, butanol, butanoic acid, esters, swelling packs, sickly odours Souring taints/off-odours</td>
</tr>
<tr>
<td>Psychrotolerant facultative anaerobes</td>
<td><em>B. circulans</em>, <em>B. mycoides</em> <em>P. macerans</em>, <em>B. polymyxa</em>, <em>B. laterosporus</em>, <em>B. lentus</em>, <em>B. sphaericus</em></td>
<td>Vacuum-packed chilled foods Milk</td>
<td>Off-odours, sometimes gas production Sour/sweet curdling/bitter, sometimes gassy</td>
</tr>
</tbody>
</table>
The genus Bacillus

Members of the genus Bacillus are Gram-positive, aerobic or facultatively anaerobic, typically catalase-positive, rod-shaped, motile, endospore-forming bacteria. Recent taxonomic changes have been recently reviewed by Jay (2003) and Fritze (2004). The organisms are ubiquitous and typically occur as saprophytes in soil and water but some species, such as B. cereus, B. anthracis and to a lesser extent B. subtilis, are pathogenic in humans and other mammals. There is evidence (Kramer and Gilbert, 1989; Jackson et al., 1995; te Giffel et al. 1997; Salkinoja-Salonen et al., 1999) that some strains of other species including B. licheniformis, B. thuringiensis, B. pumilus and Brevibacillus (formerly Bacillus) brevis can cause foodborne illness (e.g. gastrointestinal symptoms that are self-limiting) typical of either B. cereus or B. subtilis. Nevertheless, B. subtilis, B. licheniformis, B. pumilus and Br. brevis are commonly regarded as spoilage organisms. Other important spoilage organisms in the genus include B. coagulans, B. smithii and B. circulans.

The genus Bacillus was divided into three broad groups by Drobniewski (1993), depending on the morphology of the spore and sporangium and into five groups by Ash et al. (1991), depending on comparative analysis of small subunit ribosomal RNA sequences. Since then, at least eight different phylogenetic groups have been identified (Shida et al., 1996). Garrity et al. (2003) provide a recent update of the grouping of aerobic endospore forming bacteria within the order Bacillales and this also includes some non-spore-forming organisms, sometimes within the same genus as spore-forming organisms.

Bacillus spp. generally cause spoilage through the action of proteolytic, lipolytic and phospholipase enzymes. B. coagulans is worthy of special mention because of its significance as a spoilage organism, affecting milk, vegetable and fruits (see below). The criteria used to describe B. coagulans (formerly B. thermoacidurans) are described by Smith et al. (1952), However, heterogeneity within the species has led to a number of taxonomic adjustments and a recent detailed study of members of this species using a number of different phenotypic and genotypic methods (De Clerck et al., 2004a). The authors concluded that the intra-species groupings were not consistent throughout all the methods applied but they did constitute a single species and there were no grounds to delineate the species any further into sub-species. The same study provides an emended description of B. coagulans.

In a detailed study of 90 strains of B. coagulans, Nakamura et al. (1988), only 52 were found to belong to B. coagulans sensu stricto and the majority of the other strains were assigned to a new species which they proposed as B. smithii.

The genus Alicyclobacillus

Alicyclobacillus spp. are Gram-positive, rod-shaped, thermophilic and acidophilic spore-forming facultative anaerobic bacteria and are distinguished because of their ability to grow under a wide range of pH conditions, generally
ranging from 2.0 to 6.0 (Darland and Brock, 1971; Pettipher et al., 1997; Walls and Chuyate, 1998; Orr et al., 2000; Yamazaki et al., 2000) and presence of ω-aticyclic fatty acids (e.g. ω-cycloheptane or ω-cyclohexane, or ω-cyclohexyl fatty acids) as the major membrane component. Wisotzkey et al. (1992) proposed that presence of these fatty acids is responsible for the exceptional resistance to low pH and high temperatures and the same study also proposed the name of this new genus. Presence of hopanoids is also used to characterise members of the genus. Spores are formed terminally or sub-terminally with or without swollen sporangia. Colonies on Bacillus acidocaldarius medium (see later) are nonpigmented and creamy white, flat and circular. The optimum temperature for growth is between 42 and 60°C and can range between 12 and 80°C. Members of this genus include A. acidocaldarius, first isolated by Darland and Brock (1971), A. acidoterrestris, A. cycloheptanicus, A. herbarius and A. acidiphilus. The history and general characteristics of these organisms are reviewed by Chang and Kang (2004).

The genera Geobacillus, Paenibacillus, Brevibacillus and Anoxybacillus Members of the genus Geobacillus (described by Nazina et al., 2001) are rod-shaped, catalase positive, motile by means of peritrichous flagellae, facultatively anaerobic and produce terminal ellipsoidal or cylindrical spores in swollen or non-swollen sporangia and stain Gram-positive or negative. Colonies on agar are round, mucous, small and colourless. The optimum temperature for growth ranges from 55 to 75°C, with the minimum ranging from 30 to 45°C and growth only occurs above pH 5.3 (up to about 7.0). The most important species of this genus is G. stearothermophilus. Although these organisms are often described as ‘thermophiles’, this characteristic (ability to grow at 55°C) is not the best to use to describe the species since there are exceptions to this general rule. More detailed characteristics of G. stearothermophilus are described by Ito (1981) and in Bergey’s Manual (Buchanan and Gibbons, 1975). G. stearothermophilus produces the most heat-resistant spores of the aerobic spore-forming bacteria.

The genus Paenibacillus was originally described by Ash et al. (1993) and contains Gram-variable, Gram-positive or Gram-negative, rod-shaped, motile, facultatively anaerobic or aerobic, mostly catalase-positive, urease-negative bacteria that produce ellipsoidal spores formed in swollen sporangia and produce nonpigmented colonies on agar. They do not produce H2S, some strains reduce nitrate to nitrite and acid/sometimes gas are produced from various sugars, including glucose and with odd exceptions cellobiose, galactose, raffinose and salicin. Some species decompose polysaccharides by extracellular enzymes and the major cellular fatty acid is anteiso-C15:0. The G+C content ranges from 45 to 54 mol%. Of the species known to cause food spoilage, P. macerans and P. polymyxa also produce gas. The type species is P. polymyxa. Members of this genus are not easily distinguished from other genera of the Bacillaceae on the basis of phenotypic properties. An emended description of the genus is provided by Shida et al. (1997).
**Brevibacillus** spp. are defined by Shida et al. (1996) as Gram-positive or Gram-variable rods, catalase-positive, oxidase variable, motile by peritrichous flagellae, forming ellipsoidal spores in swollen sporangia, forming yellowish-grey colonies in nutrient agar. All species are strictly aerobic apart from *Br. laterosporus*, which is facultatively anaerobic. The species known to cause food spoilage include *Br. laterosporus* and *Br. brevis*, the type species of this genus.

The genus *Anoxybacillus* was separated from the genus *Bacillus* by Pikuta et al. (2000) and species of this genus are defined as rod-shaped (often with rounded ends), Gram-positive, spore-forming, obligately anaerobic or facultatively anaerobic, catalase variable, thermophilic and alkalophilic or alkalitolerant bacteria. Species of the genus include *A. pushchinensis*, *A. flavithermus* (formerly *Bacillus flavithermus*), *A. gonensis*, *A. ayderensis* and *A. kestanbolensis* and these are often associated with hot springs. The organism of relevance to the food industry is *A. flavithermus*, since it has been isolated from milk powders and gelatin (De Clerck et al., 2004b). The gelatinase activity of *A. flavithermus* raises concerns about the quality of gelatin. It has been suggested that the presence of *A. flavithermus* in milk powders showing elevated thermophile counts indicates factory-derived contamination where growth may occur in the process line (Rückert et al., 2004). The culturing of these organisms is not straightforward, so their presence in foods may go undetected. Their significance in terms of spoilage is unknown, but because of their alkaliphilic properties (pH growth range 8.0–10.5), are unlikely to be a concern in neutral pH and low-pH foods. There are few foods that have alkaline pH values, apart from egg and egg products, and there is no record of these organisms causing problems in these products. These are not considered any further.

**The genus Clostridium**

Members of the genus *Clostridium* are Gram-positive, obligately anaerobic, rod-shaped, spore-forming, usually catalase negative and unable to carry out dissimilatory sulphate reduction. The genus contains over 100 species. The taxonomy of the clostridia has been the subject of much debate because of the heterogeneity of members of the genus and it has been recommended that the taxonomy of the genus undergoes major revision (Collins et al., 1994). This study of the phylogeny of the genus found 19 clusters within the genus. It is also noteworthy that the phylogenetic intermixing of some clostridia with non-spore-forming bacteria challenges the belief that spore-forming ability is an important determinant of relatedness. It is likely that a comprehensive rearrangement of the genus will likely result in a number of new genera being identified. It has been recognised that the genus *Clostridium* should be restricted to the group I organisms defined by Johnson and Francis (1975), with the remaining clostridial species losing their status as members of the genus and requiring reclassification (Collins et al., 1994).
Spoilage by clostridia is caused by saccharolytic or proteolytic activity and is manifested by changes in product pH (caused by organic acid production), gas production and production of foul odours (e.g. through volatile acids). *Clostridium sporogenes*, *Clostridium butyricum* and *Clostridium tyrobutyricum* are common causes of food spoilage and are all of economic concern to the food industry. These three microorganisms are considered non-pathogenic. *Clostridium butyricum* and *C. pasteurianum* are often referred to as butyric anaerobes. The mesophilic pathogenic clostridia, *C. botulinum* and *C. perfringens* will also cause spoilage of foods, e.g. produce gas and have proteolytic activity, whereas psychrotrophic *C. botulinum* does not have proteolytic activity. Other mesophilic clostridia that have been associated with spoilage of foods include *C. pasteurianum*, *C. barati*, *C. bifermentans*, *C. beijerinckii*, *C. pseudotetanicum*, *C. fallax*, *C. sordellii* and *C. felsinium* (Lake et al., 1985; Kokubo et al., 1986; de Jong, 1989; Morton, 1998). There are two reports of *C. barati* producing *C. botulinum* type F toxin and causing infant botulism (Hall et al., 1985; Trethon et al., 1995). Nevertheless, this species is commonly regarded as a spoilage organism and not a pathogen.

In recent years there have been a number of newly identified psychrotolerant clostridia described that have been recognised as the cause of spoilage of chilled, vacuum packed fresh meats and these are sometimes referred to as ‘blown pack’ clostridia (Broda et al., 2002). These clostridia constitute a very diverse, dominantly Gram-positive, low-G+C organisms belonging to the cluster I and XIVa groups described by Collins et al. (1994) and include *C. estertheticum* subsp. *estertheticum* (formerly *C. estertheticum*) (Collins et al., 1992; Spring et al., 2003), *C. estertheticum* subsp. *lariamiense* (formerly *C. lariamiense*) (Spring et al., 2003), *C. algidicarnis* (Lawson et al., 1994), *C. frigidicarnis* (Broda et al., 1999), *C. gasigenes* (Broda et al., 2000a) and *C. algidixylanolyticum* (Broda et al., 2000b). They have some common features such as production of butyrate, acetate, H₂ and CO₂, and are saccharolytic, fermenting carbohydrates such as fructose, cellobiose, mannose, mannitol and trehalose. They can be distinguished into psychrophiles (e.g. *C. estertheticum* subsp. *estertheticum*) with maximum growth temperatures below about 20°C and psychrotrophs (e.g. *C. frigidicarnis*, *C. gasigenes*, *C. algidicarnis*) with maximum growth temperatures between 32 and 40°C. The psychrotolerant clostridia also include organisms such as *C. putrefaciens* which was associated with putrefaction or souring of cured hams about 90 years ago. Differentiation of this organism from other clostridia was reviewed by Ross (1965) and general characteristics of psychrotrophic clostridia are described by Beerens et al. (1965).

The genus *Thermoanaerobacterium*

The genus *Thermoanaerobacterium* contains spore-forming, Gram-positive, saccharolytic, thermophilic, obligately anaerobic bacteria (Lee et al., 1993). These organisms were first described by McClung (1939) and were originally...
assigned to the genus *Clostridium*. The organisms are non-H₂S producers but generate copious amounts of CO₂ and H₂ from a wide variety of carbohydrates, including glucose, lactose and starch. The organisms cannot hydrolyse proteins or reduce nitrates. Vegetative cells are Gram-negative rods and spores are terminal, and swell the sporangium. The organisms can grow at pH values typical of acid and acidified foods. The organisms can grow down to 32°C and have an optimum around 55°C. They can be found widely in soil and typically contaminate root vegetables and mushrooms.

*The genus Desulfotomaculum*

In 1965, Campbell and Postgate reassigned *Clostridium nigrificans* to the species *Desulfotomaculum nigrificans*, because of some major inconsistencies with this organism and other clostridia. The organism is Gram-negative, has very different DNA base composition and has three cytochromes present. The name was devised because of the organism’s ability to reduce sulphur compounds (*desulfo*) and an association with sausage (*tomaculum*). The organism is rod-shaped, with sometimes rounded ends, sometimes lenticulate and swollen, motile with twisting or tumbling motility, peritrichous flagellae, with terminal or sub-terminal oval spores and is an obligate anaerobe.

The organism produces H₂S from cystine, does not reduce nitrate, does not ferment glucose or other carbohydrates and does not liquefy gelatine. Growth can occur down to pH 5.6 but 6.2 is generally considered to be the lower pH limit for growth in foods (Speck, 1981). Although the organism can be ‘trained’ to grow down to 30–37°C, spoilage has not been reported at these temperatures.

*The genus Sporolactobacillus*

*Sporolactobacillus* spp. are Gram-positive, mesophilic, microaerophilic homofermentative, catalase variable, motile, non-nitrate reducing spore-forming bacteria. They are found in soil and root crops but their relevance to foods is currently unclear. The organisms were discovered in 1963 by Kitahara and Suzuki, who thought that these organisms were an intermediate form between *Lactobacillus* and *Clostridium*. These organisms have since been transferred to their own genus (Kitahara and Lai, 1967) and then to the Bacillaceae (Kitahara and Toyota, 1972) and it has been proposed that these are intermediates between Bacillaceae and Lactobacillaceae (Okada *et al.*, 1976). More recently, Suzuki and Yamasato (1994) proposed amendments to the definition to include catalase-positive strains. The type species is *S. inulinus* and the genus also includes *S. racemicus* and *S. laevus*. There is relatively little information about physiology, spore characteristics and habitat of these organisms and they are not reported as causes of spoilage in the food industry but, in theory, they have the potential to cause spoilage problems if the spores survive heat processing of products and may be the cause of unexplained spoilage (Doores, 1983a).
21.2 Foods affected

21.2.1 Foods spoiled by aerobic and facultatively anaerobic spore-formers

Members of the genus *Bacillus* and related species are common causes of spoilage mainly in low-acid foods (pH > 4.6) that have received a low temperature pasteurisation and are then chilled, or are commercially sterilised (e.g. ultra-high temperature or UHT-treated milk), or acid foods that have received a pasteurisation and are stored at ambient temperature. *Bacillus* spp. and related organisms cause spoilage because of their proteolytic, lipolytic, saccharolytic or pectinolytic activity. Raw materials, such as vegetables, meat and milk, are contaminated at source and endospores survive the thermal processes applied.

Psychrotrophic strains of *Bacillus* spp. able to grow at 1–4°C have been reported in pasteurised milk (Coghill and Juffs, 1979) and cream. These organisms produce serious off-flavours such as bitter, putrid, stale, rancid, fruity, yeasty and sour tastes in a wide range of dairy products such as milk, cream and cheese. *Bacillus* spp., such as *B. sphaericus*, *B. subtilis*, *B. lentus* and *B. cereus*, and related organisms (e.g. *Br. laterosporus*, *P. macerans*, *P. polymyxa*) have all been associated with flavour defects. Other defects include sweet curdling in milk and ‘bitty’ cream, caused by *B. cereus* and *B. cereus* var. *mycoides*, due to lecithinase activity on phospholipids, and these determine the shelf-life of pasteurised milk and milk products during extended storage (Frank *et al*., 1993). A review of the thermoduric psychrotrophic *Bacillus* spp. associated with the spoilage of milk and milk products is provided by Meer *et al*., (1991). Currently, the most limiting factor in extending the shelf-life of conventionally pasteurised fluid milk products beyond 14 days is bacterial spoilage by heat-resistant psychrotrophic Gram-positive bacteria, made up mostly by *Paenibacillus* spp. and *Bacillus* spp. (Fromm and Boor, 2004). In a recent study looking at spore contamination of milk and application of various heat treatments (Hanson *et al*., 2005), the majority of isolates recovered were characterised as *B. mycoides*, leading the authors to conclude that this organism might be more significant in causing sweet curdling of milk than previously reported.

The importance of *G. stearothermophilus* and *B. coagulans* in the flat-sour spoilage of evaporated milk was established by Speck (1976) and a later study (Kalogridou-Vassiliadou *et al*., 1989) suggested that this type of spoilage is also caused by *B. subtilis*, *B. licheniformis* and *B. macerans*. Active enzymes thought to be responsible include esterase, esterase lipase, lipase, valine aminopeptidase, phosphoamidase, β-glucuronidase and β-glucosidase (Kalogridou-Vassiliadou, 1992). *Bacillus coagulans* has also been associated with spoilage of evaporated milk, resulting in firm coagulation (hence the name *B. coagulans*) caused by production of high concentrations of lactic acid. There are some examples of *B. sporothermodurans* causing spoilage of UHT milk but these are relatively unusual because of the inability of this
Spore-forming bacteria

organism to produce acids during growth – growth is not normally associated with noticeable spoilage (Pettersson et al., 1996).

Flat-sour spoilage of tomato products was reported much earlier and the first association of *B. coagulans* (named then as *B. thermoacidurans*) with these products was by Berry in 1933. At first, when tomato products were processed in the 1920s, it was assumed that their low pH would only require boiling temperature to achieve sterilisation but outbreaks of spoilage were noticed in 1931 and these incidents prompted Berry to carry out investigations to identify the causative agent. Berry also concluded that *B. coagulans* was possibly also responsible for a similar type of spoilage in asparagus, corn, lima beans and peas. This organism does not produce gas, can grow at low pH and produces acid from a variety of sugars. Consequently, the organism has been responsible for large economic losses since spoilage is not easily detected, even after cans/containers have been opened and its occurrence is not easily predicted. Although spoilage of canned foods from ‘flat-sours’ is no longer thought to be a major problem in developed countries (Damaré et al., 1985), *B. coagulans* continues to be reported as a cause of spoilage in dairy products and low-acid canned foods (Cosentino et al., 1997; Kalogridou-Vassiliadou, 1992; Nakajyo and Ishizu, 1985; Roman-Blanco et al., 1999).

*Geobacillus stearothermophilus* was first identified as a cause of spoilage in canned corn and string beans. The organism has also been associated with spoilage of peas and asparagus and, once present in manufacturing plant, can grow in warm holding tanks, blanchers, warm filler bowls, some of which are difficult to clean (Ito, 1981).

Incidents of gaseous spoilage of canned fruit products by *P. macerans*, *P. polymyxa* and *B. licheniformis* (canned mango pulp) have also been reported (Vaughn et al., 1952; Azizi and Ranganna, 1993). A common spoilage condition of bread, known as ‘rope’, is caused by growth of *Bacillus* spp. such as *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. megaterium* and *B. cereus* (Hansen and Bautista, 2000). Spores of these organisms are commonly present in flour and subsequently in bakery equipment and survive within the centre of baked bread. Rope is characterised by development of fruity or sweet odours, or rotting fruit/bad cheese. As the organisms grow, they impart a sticky texture to the centre which develops to fine threads. Rope is still a major problem in hot countries such as South Africa (Kirschner and von Holy, 1989). *Paenibacillus circulans* and *B. subtilis* are also known to produce non-volatile acidity in wines (Hansen and Bautista, 2000).

The ability of some species, e.g. *B. subtilis*, *P. polymyxa*, and some clostridia, to produce pectinases, such as extracellular endo-pectate lyase enzymes (Nagel and Wilson, 1970; Kurowsky and Dunleavy, 1976; Chesson and Codner, 1978; Rombouts and Pilnick, 1980) enables these organisms to attack plant tissues. This results in softening and the production of odours. Susceptible vegetables include root crops, crucifers, cucurbits, solanaceous vegetables and onions.
The first food linked to spoilage by *Alicyclobacillus* spp. was apple juice (Cerny *et al.*, 1982), in Germany. Since then, these organisms have been the focus of increasing attention in the fruit juice industry and spoilage incidents have been reported more widely in the UK, Australia, Japan and the USA (Walls, 1994; Yamazaki *et al.*, 1996; Pettipher *et al.*, 1997; Walls and Chuyate, 1998). A survey carried out by the National Food Processors Association (Walls and Chuyate, 1998) revealed a relatively large-scale problem, encountered by 35% of respondents involved in fruit juice manufacture. The products affected include apple juice, diced canned tomatoes, orange juice (Matsubara *et al.*, 2002), iced tea (Duong and Jensen, 2000), cranberry-apple beverage (Splittstoesser *et al.*, 1994) and pear juice (Sinigaglia *et al.*, 2003). Spoilage caused by *Alicyclobacillus* spp. is characterised by the formation of ‘taints’ described as ‘medicinal’, ‘antiseptic’, ‘smoky’, ‘phenolic’ or ‘hammy’. The primary metabolite responsible for these taints is guaiacol, but they are also caused by 2,6-dibromophenol and 2,6-dichlorophenol (Jensen and Whitfield, 2003). Spoilage is detected only on opening of the container, so usually remains undetected until consumer complaints are reported.

### 21.2.2 Foods spoiled by anaerobic spore-formers

The range of foods spoiled by anaerobic spore-formers is quite wide. The foods affected range from raw or uncooked foods through to processed foods. A feature of spoilage by anaerobic spore-formers is the low redox potential of the foods affected and elimination or inhibition of competitive microorganisms, either through heat treatment or through intrinsic properties of the food.

Vacuum-packed raw and cooked meats, such as beef, lamb, pork and venison are spoiled by a number of different psychrotolerant clostridia (see Section 21.1.3 above) and this is a relatively recent development since clostridial species were previously thought to play only a minor role in spoilage of meat. These vacuum packed meats are often held for extended periods at low chill temperatures, and psychrotolerant clostridia spoiling these products produce copious amounts of gas and butyl esters. Earlier in the 20th century, deep tissue spoilage of salted hams was very common and this type of spoilage was caused by organisms such as *C. putrefaciens* and *C. algidicarnis*. This resulted in 0.1–0.25% losses of some 3 million cured hams produced annually in the USA at the time. It is interesting that development of chilled storage and modern packaging technologies appears to have created a niche for these psychrotolerant anaerobes.

*Clostridium sporogenes* is responsible for causing spoilage of a number of foods including cheese, condensed milk, cooked meats, canned vegetables and canned fish. It is a common *Clostridium* species found in milk and meat. *C. sporogenes* is proteolytic and lipolytic whereas the other clostridia associated with spoilage are not. There is an example of spoilage of canned mango pulp resulting from the combined growth of *B. licheniformis* and *C. sporogenes*. 
It was proposed that growth of bacilli raised the pH sufficiently to allow growth of *C. sporogenes* present (Azizi and Ranganna, 1993).

*Clostridium butyricum* is responsible for causing spoilage of cheese, condensed milk and acid canned foods (pH ≤ 4.6), in particular tomatoes and other fruits (e.g. pear and apricot juices and canned pineapple), together with *C. pasteurianum* (Spiegelberg, 1936, 1940a; Ikegami et al., 1970; Hernandez and Feria, 1971). These organisms can initiate growth at low pH values (e.g. down to 3.6) but are not as heat resistant as *B. coagulans*. *Clostridium butyricum* is a common species found in milk. As suggested by its name, it produces butyric acid, is strongly saccharolytic and is not proteolytic. The deteriorative changes brought about by clostridia in sweetened condensed milk are described by Bhale et al. (1989). The organism has also been associated with spoilage of wines, giving rise to rancid taint due to production of butyric acid (Hansen and Bautista, 2000).

*Clostridium tyrobutyricum* is a significant economic concern for the dairy industry because it is a major cause of structural and sensory defects in cheeses (the ‘late-blowing’ defect) through production of large quantities of gas (carbon dioxide and hydrogen), butyric acid and acetic acid (Wasserfall and Teuber, 1979). The late-blowing effect, which is a consequence of the outgrowth of *C. tyrobutyricum* spores, occurs most frequently in brine-salted, hard and semi-hard cheeses (e.g. Gouda, Edam, Emmental, Gruyere). This is because of their relatively high pH and moisture content and low interior salt content. Processed cheeses are susceptible to late blowing because spores are not inactivated during processing. Butyric acid levels greater than 200 µg l⁻¹ produce detectable off-flavours which result in downgrading of cheese. In some cases, gas production is sufficient to rupture the entire cheese structure. *C. tyrobutyricum* is considered the main *Clostridium* spp. responsible for the late-blowing defect in cheese. *C. tyrobutyricum* is thought to enter cheese in milk contaminated with bovine faecal matter. Presence in faecal material has been traced to consumption of contaminated silage. For this reason, feeding of silage is banned in Switzerland and Germany if milk is to be used for the manufacture of Emmental cheese.

Raw vegetables such as potatoes suffer from soft rot caused by *C. puniceum* and other clostridial species (Lund and Nicholls, 1970; Lund 1983). A number of morphological types have been observed but there has been relatively little further characterisation. Some strains are thought to cause slimy rot (Campos et al., 1982) and a group of psychrotolerant strains are known to produce pectate lyase.

Thermophilic anaerobes such as *Thermoanaerobacterium thermosaccharolyticum* (formerly *C. thermosaccharolyticum*) and *Desulfotomaculum nigrificans* cause spoilage in low-acid foods. *D. nigrificans* causes sulphide spoilage of high-pH products such as canned sweet corn, peas, mushrooms, mushroom-containing high pH foods and canned baby clams. The H₂S produced in spoiled containers is soluble and therefore does not result in swelling of containers and is therefore not easily detected (Doores,
1983a). Blackening of product, due to H2S production, will sometimes occur. Spoilage due to *D. nigrificans* can develop only when the temperature of the product is able to support growth of the organism, e.g. 55–65 °C, and this type of spoilage is now relatively rare (Brown, 2000) although it has occurred recently (together with *C. thermoaceticum*) in hot-vended canned coffee and ‘Shiruko’ (a soft drink made from red beans and cane sugar) (Matsuda et al., 1982).

*Thermoanaerobacterium thermosaccharolyticum* can also cause spoilage of acid and acidified products such as tomato and other fruit-containing products including those containing starchy (farinaceous) ingredients, such as spaghetti. Other products affected include canned sweet potatoes, pumpkin, green beans, mushrooms, asparagus, vegetable soup and dog food (Ashton, 1981). Growth can occur down to about 32 °C, but spoilage usually occurs in products held for significant periods at 37 °C or above, and can occur in products that are not cooled quickly enough. Hot-vended canned food products (e.g. drinks or soups) are particularly susceptible to this type of spoilage and high numbers can develop in ingredients such as chicken stock, beef extract and yeast hydrolysate (Ashton, 1981). This type of spoilage is not uncommon.

### 21.3 Characteristics and conditions for growth and inactivation

#### 21.3.1 Conditions for growth

The major factors limiting or controlling the growth of spore-forming spoilage bacteria in foods are pH, temperature/time of heating and temperature/time of cooling and storage. The limits of growth for the different groups of spore-forming bacteria involved in food spoilage are summarised in Table 21.2. There is much strain-to-strain variability, even within the same species, and reported limiting conditions will often focus on the more extreme or pessimistic observations made, since product and process design will want to take account of those strains that pose the biggest challenge. Nevertheless, it may be overly conservative to choose very extreme values that are unrealistic, in terms of the challenge that the product is likely to see, so choice of relevant target limits requires careful consideration.

This notwithstanding, there are some examples of growth under unusual and extreme conditions and this serves to demonstrate the versatility of different microorganisms to take advantage of what might be regarded as adverse conditions. For example, the ability of *Alicyclobacillus* spp. to grow at extremely low pH values, as low as pH 2.0 (see Table 21.2) is unique for spore-forming microorganisms, and this poses a challenge for control of these microorganisms, although it appears that the types of product affected by these organisms are limited mostly to fruit products at present. Similarly, the ability of psychrophilic organisms, such as *C. gasigenes*, to grow at
<table>
<thead>
<tr>
<th>Group of organisms</th>
<th>Species</th>
<th>pH</th>
<th>Reference</th>
<th>Temp (°C)</th>
<th>Reference</th>
<th>a_w</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>Thermophilic aciduric flat sours</td>
<td>B. coagulans</td>
<td>4.05(^1)</td>
<td>York et al. (1975)</td>
<td>20</td>
<td>Thompson, (1981)</td>
<td>0.94</td>
<td>Alzamora et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.4(^2)</td>
<td>York et al. (1975)</td>
<td></td>
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</tr>
<tr>
<td>Facultative thermophiles, facultative anaerobes</td>
<td>B. subtilis</td>
<td>4.5</td>
<td>Lindsay et al. (2000)</td>
<td>10</td>
<td>Shapton and Shapton (1991)</td>
<td>0.9</td>
<td>Troller and Christian (1978)</td>
</tr>
<tr>
<td>Mesophilic facultative anaerobes</td>
<td>P. polymyxa</td>
<td>4.0–4.5</td>
<td>Ingram (1969)</td>
<td>5–10</td>
<td>Gibson and Gordon (1974)</td>
<td>0.89–0.91</td>
<td>Alzamora et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>P. macerans</td>
<td>4.0–4.5</td>
<td>Ingram (1969)</td>
<td></td>
<td></td>
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<td>4.5</td>
<td>Lindsay et al. (2000)</td>
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</tr>
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<td></td>
<td>B. pumilus</td>
<td>5.2</td>
<td>Lindsay et al. (2000)</td>
<td>4–7</td>
<td>Meer et al. (1991)</td>
<td>0.95</td>
<td>Troller and Christian (1978)</td>
</tr>
<tr>
<td>Group of organisms</td>
<td>Species</td>
<td>pH</td>
<td>Reference</td>
<td>Temp (°C)</td>
<td>Reference</td>
<td>$a_w$</td>
<td>Reference</td>
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</tr>
<tr>
<td>Mesophilic</td>
<td>C. sporogenes</td>
<td>5.0</td>
<td>Montville et al (1985)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>putrefactive</td>
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<td>C. butyricum</td>
<td>4.0–4.5</td>
<td>Ingram (1969)</td>
<td>10</td>
<td>Jong (1989)</td>
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<tr>
<td>butyric</td>
<td>C. beijerinke</td>
<td>4.7</td>
<td>Lake et al. (1985)</td>
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<tr>
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<td>C. pasteurianun</td>
<td>3.6–4.5</td>
<td>Ingram (1969)</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>Psychrotolerant</td>
<td>C. estertheticum</td>
<td>5.3</td>
<td>Spring et al. (2003)</td>
<td>1</td>
<td>Collins et al. (1992)</td>
<td></td>
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<tr>
<td>anaerobes</td>
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</tr>
<tr>
<td></td>
<td>C. frigidicarnis</td>
<td>4.7</td>
<td>Broda et al. (1999)</td>
<td>3.8</td>
<td>Broda et al. (1999)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>C. gasigenes</td>
<td>5.4</td>
<td>Broda et al. (2000a)</td>
<td>−1.5</td>
<td>Broda et al. (2000a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. putrefaciens</td>
<td></td>
<td></td>
<td>5</td>
<td>Roberts &amp; Derrick (1975)</td>
<td>&lt;0.97</td>
<td>Roberts and Derrick (1975)</td>
</tr>
<tr>
<td>Psychrotolerant</td>
<td>C. algidixylanolyticum</td>
<td>4.7</td>
<td>Broda et al. (2000b)</td>
<td>2.5</td>
<td>Broda et al. (2000b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>facultative</td>
<td>B. cereus</td>
<td></td>
<td>As above</td>
<td></td>
<td>As above</td>
<td></td>
<td>Shehata et al. (1971)</td>
</tr>
<tr>
<td>anaerobes</td>
<td>B. macerans/B subtilis</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

1In unheated food, 2in food heated to 93 °C for 10 min.
temperatures as low as \(-1.5\,^\circ\text{C}\) is remarkable and worthy of special mention. Again, this is an example of unusual growth characteristics posing real problems for a particular commodity type, in this case, vacuum packed chilled meats.

In some foods, inhibition of growth of spoilage spore-forming bacteria is afforded by other factors such as nitrite and other salts in cured meats (Ingram, 1969), propionic, sorbic and acetic acids (and their salts) in bread (see Doores, 1983b, for more information), nisin in soups, liquid egg, crumpets, fruit juice, canned food, meat products, dressings, sauces and cheeses and nitrate in cheeses. The factors known to inhibit growth of bacterial spores and mechanisms by which they act are reviewed by Cook and Pierson (1983). The antimicrobial effects and application of nisin are reviewed by Adams and Smid (2003).

### 21.3.2 Conditions for inactivation

Table 21.3 describes typical reported $D$ (decimal reduction time) values and $z$ values for thermal inactivation, where these are known. The many studies on heat resistance of spore-forming bacteria generally show that growth temperature is related to heat resistance. For example, spores of psychrophilic organisms are much more sensitive to heat than mesophilic strains, which in turn are more sensitive to heat than thermophilic strains. For convenience, organisms are often labelled according to their growth temperature range (see Table 21.4), but in reality, there is a continuum of growth range characteristics for different strains. For example, although *G. stearothermophilus* is regarded as a thermophilic species, there are strains capable of growth at temperatures as low as \(30\,^\circ\text{C}\) or below. Therefore, although there are some general ‘rules’ that can be used to predict the heat resistance of different strains, depending on growth temperature characteristics, there will always be exceptions to these rules and it is therefore important to determine the resistance properties of strains that are associated with raw materials or processing equipment. The heat resistance of various groups of spore-forming bacteria is covered by Brown (2000).

Studying the heat resistance of bacterial spores is notoriously difficult because of the influence of different factors such as sporulation temperature, sporulation medium, heating medium, heating method and recovery conditions (see Russell, 1982, for more information). There can be significant advantages to be gained in destroying spore-formers by making use of the interaction between pH and temperature. Low pH values have been shown to increase the cidal effects of heat for some spore-formers (Cook and Gilbert, 1968; Gibriel and Abd-El Al, 1973). In terms of reproducibility and comparisons of heat resistance between strains used, there are a number of uncertainties. Even repeating the same heating experiment with different spore crops of the same strain, using exactly the same conditions of preparation and heating, can give different heat resistance results. It can, however, be concluded that
<table>
<thead>
<tr>
<th>Organism</th>
<th>$D$ (min)</th>
<th>$z$ (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermoanaerobacterium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thermosaccharolyticum</td>
<td>3000$^3$</td>
<td>90$^3$</td>
<td>1$^{Ingram}$ (1969)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2$^{Xezones et al.}$ (1965)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>3$^{Ashton}$ (1981)</td>
</tr>
<tr>
<td><strong>Desulfitomaculum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nigrificans</td>
<td>2–3$^1$</td>
<td>195$^2$</td>
<td>1$^{Ingram}$ (1969)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55$^3$</td>
<td>2$^{Donnelly and Busta}$ (1980)</td>
</tr>
<tr>
<td><strong>Bacillus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sporothermodurans</td>
<td>0.32–0.57</td>
<td></td>
<td>Meier et al. (1996)</td>
</tr>
<tr>
<td><strong>Geobacillus</strong></td>
<td>3000$^1$</td>
<td>4–5$^1$</td>
<td>1$^{Ingram}$ (1969)</td>
</tr>
<tr>
<td>stearothermophilus</td>
<td></td>
<td>1–2$^2$</td>
<td>2$^{Cook and Brown}$ (1960)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.7$^3$</td>
<td>3$^{Davies et al.}$ (1977)</td>
</tr>
<tr>
<td><strong>B. coagulans</strong></td>
<td>13$^4$</td>
<td>3.1$^3$</td>
<td>1$^{Ingram}$ (1969)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2$^{Brown &amp; Ayres}$ (1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3$^{Hersom and Hulland}$ (1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4$^{York et al.}$ (1975)</td>
</tr>
<tr>
<td><strong>C. sporogenes</strong></td>
<td></td>
<td>0.1–1.5$^1$</td>
<td>1$^{Ingram}$ (1969)</td>
</tr>
<tr>
<td><strong>C. butyricum</strong></td>
<td>23$^2$</td>
<td>0.1–0.5$^1$</td>
<td>1$^{Ingram}$ (1969)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10–15$^3$</td>
<td>2$^{Russell}$ (1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3$^{Hersom and Hulland}$ (1980)</td>
</tr>
</tbody>
</table>
Table 21.3 Continued

<table>
<thead>
<tr>
<th>Organism</th>
<th>( D ) (min)</th>
<th>( z ) (C(^{°}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80(^{°})C</td>
<td>85(^{°})C</td>
<td>90(^{°})C</td>
</tr>
<tr>
<td><em>C. tyrobutyricum</em></td>
<td>6.5–21</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. pasteurianum</em></td>
<td>0.1–0.5(^{1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. beijerinkii</em></td>
<td>2–4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alicyclobacillus acidoterestris</em></td>
<td>41.2–54.3(^{3})</td>
<td>56–57(^{1})</td>
<td>16–23(^{1})</td>
</tr>
<tr>
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</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>13(^{1})</td>
<td>4–8(^{3})</td>
<td></td>
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<tr>
<td><em>B. subtilis</em></td>
<td>11(^{1})</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Paenibacillus macerans</em></td>
<td>0.1–0.5(^{1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>18.5(^{2})</td>
<td>5.8(^{2})</td>
<td>1.8(^{2})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4–15.7(^{3})</td>
<td></td>
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<tr>
<td></td>
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</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>16.5</td>
<td>5.1</td>
<td>1.4</td>
</tr>
<tr>
<td><em>B. laterosporus</em></td>
<td>20.5</td>
<td>6.4</td>
<td>2.1</td>
</tr>
<tr>
<td><em>B. psychrosaccharolyticas</em></td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. globisporus</em></td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. macquariensis</em></td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. putrefaciens</em></td>
<td>8–14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature range for vigorous growth (°C)</td>
<td>Acidity status of food</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------</td>
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</tr>
<tr>
<td></td>
<td>High-acid (pH &lt;3.7)</td>
<td>Acid (pH 3.7–4.6)</td>
<td>Low-acid (pH &gt; 4.6)</td>
</tr>
<tr>
<td>Thermophilic (55–35)</td>
<td><em>Alicyclobacillus acidoterrestris</em></td>
<td><em>Bacillus coagulans</em></td>
<td><em>T. thermosaccharolyticum</em></td>
</tr>
<tr>
<td></td>
<td><em>A. acidocaldarius</em></td>
<td><em>B. smithii</em></td>
<td><em>Desulfitomaculum nigrificans</em></td>
</tr>
<tr>
<td></td>
<td><em>A. acidiphilus</em></td>
<td><em>Thermoanaerobacterium thermosaccharolyticum</em></td>
<td><em>Geobacillus stearothermophilus</em></td>
</tr>
<tr>
<td>Mesophilic (40–15)</td>
<td><em>Clostridium butyricum</em></td>
<td><em>C. sporogenes</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>C. pasteurianum</em></td>
<td><em>C. putrificum</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Paenibacillus macerans</em></td>
<td><em>B. licheniformis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. polymyxa</em></td>
<td><em>B. subtilis</em></td>
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<tr>
<td></td>
<td></td>
<td><em>B. amyloliquefaciens</em></td>
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<tr>
<td>Cold-tolerant (30–&lt;5)</td>
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</table>
sterilisation processes designed to destroy spores of mesophiles will easily
destroy spores of psychrophilic strains but pasteurisation processes used to
treat milk and other dairy products are insufficient for complete inactivation.
The fundamental considerations for calculating and setting heat processes
are described by Ingram (1969).

The mechanism of thermal inactivation of bacterial spores has been the
subject of much debate over the past 40 years. Heat resistance is thought to
be related to relative mineralisation and dehydration of the spore, protection
of the spore DNA against depurination by the binding of small acid soluble
proteins (SASP) and sporulation temperature but the actual mechanism of
heat killing remains unknown (Setlow and Setlow, 1998). There is some
evidence to support that protein denaturation takes place in heat-killed spores
and it has been suggested that some crucial spore protein is the initial
target for heat-killing (Setlow and Setlow, 1998). Exactly why there are
differences in the heat resistance of spores of different organisms remains
unclear. It is noteworthy that some organisms are particularly heat resistant,
for example $D$ values of 195 min at 121°C have been recorded for $T.$
thermosaccharolyticum (see Table 21.3). Heat resistances of this magnitude
make it incredibly difficult to set thermal processes for control that will not
also destroy the food being processed. In some cases, other methods for
control of thermophilic spore-formers, such as inhibition of germination and
outgrowth (e.g. through use of sucrose esters) are required in foods at risk of
spoilage by these organisms.

The other methods used to inactivate bacterial spores are high-pressure
treatment and irradiation. In general, bacterial spores are more resistant than
vegetative cells to ionizing radiation. Generally speaking, radiation doses
between 10–50 kGy are required to inactivate bacterial spores, with typical
$D$ values of 1–4 kGy, depending on the organism. The $D$ values for vegetative
organisms are generally below 0.8 kGy. The food matrix, as with other
processes, can influence the efficacy of treatment. For example, $D$ values for
spores $C.$ sporogenes ranged from 2.8 kGy in phosphate buffer to 10.1 kGy
in chicken fat (Shamsuzzaman and Lucht, 1993). There do not appear to be
the same differences between different types of spores (e.g. between non-
proteolytic and proteolytic $C.$ botulinum) as demonstrated in heat resistance.
Even though relatively high doses, applied with high-energy electromagnetic
radiation (gamma-rays of $^{60}$Co or X-rays, or electrons from electron
accelerators) are required to inactivates spores, irradiation offers a very effective
solution for the problem of microbiological contamination and is accepted
by health organisations and regulatory bodies. An excellent review of
irradiation inactivation of foodborne microorganisms is provided by Monk
et al. (1995).

High hydrostatic pressure is another technique that can be applied to
foods to effect inactivation of microbiological contaminants. To inactivate
bacterial endospores, a combination of pressure and moderate heat is required
(Mallidis and Drizou, 1991). Inactivation can also be enhanced through use
of nisin or by acid pH. Pressure resistance varies dramatically within the same species and between species and it is important to use a large number of different strains when evaluating processes that are to be used for the preservation of foods. Spores of *Bacillus* and *Clostridium* species are generally inactivated with pressures ranging from 500 to 800 MPa at temperatures ranging from about 60 to 80 °C with higher temperatures affording more lethal effects. For vegetative cells, pressures of around 500 MPa at temperatures of 50 °C will provide a significant (e.g. 6-log reduction) kill. The pressure resistance of bacterial spores does not correlate with heat resistance properties. The pressure resistance of a large number of food isolates of the genus *Bacillus* was recently reported by Margosch et al. (2004). An update on non-thermal technologies, including high hydrostatic pressure, irradiation, pulsed electric fields and ultrasound is provided by Barbosa-Cánovas and Rodriguez (2002) and Raso and Barbosa-Cánovas (2003).

Chemical disinfectants commonly used to destroy vegetative cells are sometimes effective against spores of spore-forming bacteria but generally speaking, much higher concentrations and longer contact times are required for sporicidal activity compared with bactericidal activity (Bloomfield and Arthur, 1994). This is due to the protective spore coat. The chemical agents that are shown to have comparatively rapid sporicidal activity include glutaraldehyde, alkalis, β-propiolactone, ethylene and propylene oxides in solution, iodine, hydrogen peroxide, chloramines and peroxy acids. Chemical agents that are less effective, requiring longer exposure times, include formaldehyde, methyl bromide, silver compounds, chlorine and bromine in solution. Sporicidal concentrations of chlorine vary between 2 and 50 ppm and like other sporicidal agents, efficacy depends on pH and temperature, with higher temperatures leading to higher inactivation rates. Agents effective in destroying vegetative cells but having no effect on the killing of spores include mercury compounds, acids, phenolics, alcohols, quaternary ammonium compounds, chlorhexidine and bisguanides. The efficacy of various chemical agents against spores is reviewed by Roberts (1969), Waites (1985) and Russell (1990).

21.4 Implications for control in foods affected

The key considerations for growth of spore-forming bacteria in food products are related to their physiological characteristics, such as range of growth temperature, tolerance of low pH environments and resistance to heating. Spore-forming bacteria are of more limited relevance to chilled foods since there are relatively few organisms (e.g. psychrotolerant *Bacillus* spp. and psychrotolerant clostridia) capable of growth at chill temperatures and then their growth is usually slow, so they are mostly relevant to medium/long shelf-life products. The shelf-life of these products is often determined by growth of these spore-formers so they are often controlled by setting ‘use-
by’ dates. This type of spoilage is not so common nowadays, although modern processing trends to reduce salt and nitrite would tend to favour growth of this type of organism.

Many spore-forming bacteria are mesophilic but they tend to constitute a sub-set of spoilage flora in unheated foods that are less capable, compared with non spore-formers, of causing spoilage through rapid growth. In foods that are heated to eliminate vegetative cells, spore-formers are selected and become the predominant concerns in foods held above chill temperatures. At temperatures above about 40°C, the bacterial spore-formers tend to predominate whether foods have been heated or not.

Considering pH, foods tend to be segregated into high-acid (pH < 3.7), acid (pH 3.7–4.5) and low-acid (pH > 4.5) foods. The only spore-forming bacteria of relevance to high-acid products are *Alicyclobacillus* spp., capable of growth at pH values down to 2.0. These organisms pose a considerable challenge to manufacturers of high-acid products that are normally pasteurised, such as fruit drinks, even though they are not capable of growth below about 20°C. Storage of products at temperatures below 20°C provides an option for controlling these microorganisms but this may be difficult because of the costs involved in keeping these products at lower-than-ambient temperatures. Alternatively, the products could be processed at higher temperatures (e.g. above 100°C) but this is likely to impart unacceptable organoleptic changes to the product. The design of pasteurisation processes for fruit products has been covered by Silva *et al.* (2000) and Silva and Gibbs (2001). The different spore-forming bacteria important in the spoilage of heated foods are summarised in Table 21.4.

In acid foods, there are a number of spore-forming bacteria that need to be controlled. The major causes of concern are *B. coagulans*, capable of growth down to about pH 4.1 and the mesophilic anaerobes *C. butyricum* and *C. pasteurianum*, capable of growth down to about pH 4.0. *Bacillus coagulans* is more heat resistant than the clostridia and therefore tends to be the main target of the product and process design for acid foods. Interactions between different factors, such as heating temperature/time, pH, presence of acids and to a lesser extent *a*<sub>w</sub>, are important for the stability of acid and acidified foods. The pH and acid content of low pH produce, such as tomatoes, varies between species and strains of fruit and also changes with ripeness of fruit, with riper fruit having a higher pH than less-ripe fruit. For tomatoes, the pH can range from about 4.1 up to about 4.8 or even higher. For safety, to remove the potential for growth of *C. botulinum*, the pH of acid and acidified foods is set to below 4.6. Consequently, it is important to acidify tomato products (usually with citric acid) so that their pH is below 4.5. In addition, the risk of spoilage is reduced if the pH is further reduced to 4.3 or below. This is because of the interactive effects between pH, acid and temperature, where heat-injured spores are unable to germinate and grow in these products. Acid foods with a pH < 4.3 usually require only 5 min at 93°C whereas products with pH 4.3–4.5 require temperatures around 93°C for 10 min (York
et al., 1975). For products containing sugar, such as canned pears, $a_w$ can also be included in the preservation system to prevent growth of butyric anaerobes (Jakobsen and Jebsen, 1975).

Other concerns in these products include *P. polymyxa* and *P. macerans* but since these are also more heat sensitive than *B. coagulans* (like the butyric anaerobes above), they will be controlled by processes used to control *B. coagulans*. Some strains of more aerobic members of the genus *Bacillus* such as *B. subtilis* and *B. licheniformis* may survive processes used for acid and acidified products but growth of these organisms is dependent on the availability of oxygen (Rodriguez et al., 1992, 1993). In canned products, these organisms are not known to be a significant cause of spoilage but it is possible that flexible packs/pouches that are becoming more popular will not be as stable as canned products receiving a similar heat process because of ingress of oxygen into the packs. Growth of these aerobic spore-formers in acid-products results in an increase in pH (Rodriguez et al., 1993) and can therefore also impact on the safety of these products. Although *T. thermosaccharolyticum* can grow down to pH levels relevant to acid foods, growth of these organisms is controlled through cooling of products and holding temperatures below 30°C.

For non-refrigerated low-acid foods, the main spore-former spoilage concerns tend to be mesophilic *C. sporogenes* and some *Bacillus* spp., and the three thermophilic species *G. stearothermophilus*, *T. thermosaccharolyticum* and *D. nigrificans*. None of the thermophiles is able to grow at $\leq 30^\circ$C, so proper cooling and storage will prevent these organisms from causing spoilage. However, some strains of *G. stearothermophilus* are capable of growth down to about $30^\circ$C and these organisms pose a greater risk for spoilage. Generally speaking, in temperate climates where ambient temperatures remain below about 35°C, it is rarely necessary to give severe heat treatments sufficient to destroy spores of thermophilic organisms. The thermal processes set will normally target the more resistant *Bacillus* spp. For products susceptible to spoilage with thermophiles, additional measures for control include selection of good-quality ingredients and pre-sterilisation of ingredients. Hot-vended products are particularly vulnerable to these organisms. Susceptibility to spoilage can be assessed by incubation of containers at temperatures able to support growth of thermophiles. However, even though incubation may show growth of thermophiles, this does not necessarily mean the product will spoil, because of the normal storage of these containers at ambient temperature.

For dairy foods, such as milk, cream and other dairy products, various factors determine the shelf-life and spoilage flora that develop. These products usually receive a pasteurisation at some stage during processing designed originally to remove infectious vegetative microorganisms such as *Coxiella burnetti* and *Mycobacterium tuberculosis* and the heat treatment also inactivates enzymes that occur naturally in milk. In recent years, the development of pasteurisation and other heat processes has transformed the milk industry
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allowing much larger distribution capabilities. These heat treatments have been categorised (Boor and Murphy, 2002) into:

- pasteurisation (e.g. heating to 71.1°C for 15 s) – for limited shelf-life (10–20 days) under refrigerated storage;
- ultra-pasteurisation or UHT pasteurisation (e.g. 138°C for 2 s) for products with extended shelf-life (e.g. 30–90 days) under refrigerated storage;
- ultra-high temperature sterilisation (e.g. 135–150°C for 2–8 s) for ambient-stable products;
- in-pack sterilisation (e.g. 105–120°C for 20–40 min) for ambient-stable products.

The last two of these processes are designed to effect a 9-log inactivation of spores of thermo philic bacteria normally associated with raw milk (Burton, 1984; Hinrichs and Kessler, 1995). The importance of good refrigeration conditions for storage is ably demonstrated by Hanson et al. (2005) who showed that after various heat treatments, storing at 10°C allowed numbers of surviving spore-formers to increase to high numbers (>10⁶/ml) after 10 days whereas storage at 6°C resulted in little or no increase.

There are other methods used to control growth of bacterial spore-formers in foods. Many of these make use of a combination of conditions, such as reduced water activity and milder heat treatment, or reduced pH and milder heat. Braithwaite and Perigo (1971) and Bean (1983) carried out extensive studies on the combined effects of heat pH and water activity on spores from a large number of Bacillus spp. and Clostridium spp., identifying conditions that required less heat treatment than would normally be required. Combination effects are also used in cured meats, where NaCl, NaNO₂, pH 6 and only one-tenth of the heat treatment used for uncured meats can provide a safe and stable product (Ingram, 1969). In this scenario, the important spoilage organisms are Bacillus spp. capable of growth at high NaCl concentrations and can reduce nitrate. Since spores of these organisms are relatively heat sensitive, they are controlled with moderate heat treatments (Ingram, 1969).

A number of other antimicrobial agents may be used to control the growth of spore-forming bacteria. As with other methods of preservation already mentioned above, many of the published studies refer to pathogenic spore-forming bacteria, such as C. botulinum but this notwithstanding, there is some information available on controlling growth of spoilage spore-formers. Cook and Pierson (1983) reviewed the inhibition of bacterial spores by antimicrobials and Gould (1964) studied the effects of various food preservatives on growth from spores of six Bacillus species. The preservatives considered in these studies include substances such as nisin, nitrite, NaCl, sorbate and benzoate, among others. Control of late-blowing of hard cheese is achieved through hydrogen peroxide/catalase, addition of lysozyme to milk (IDF, 1987), bactofugation of the cheese milk (Lembke et al., 1984) or addition of nitrate (Waltstra et al., 1993). Depending on national legislation, nisin may also be used to control growth of spore-forming bacteria in foods,
such as cheeses, by direct addition or by use of nisin-producing starter cultures (Rilla et al., 2003). Application of nisin for preservation of fruit drinks has also been investigated by Yamazaki et al. (2000).

One aspect of bacterial spores that sometimes gets overlooked is the deliberate inclusion of these in cleaning products, because of the ability of some spore-forming organisms to produce significant amounts of fat-degrading enzymes. It is important that these products are used in an appropriate manner and are not used in close proximity to foods that may be susceptible to spoilage caused by these organisms.

21.5 Specific detection, identification and enumeration methods

21.5.1 Aerobic and facultatively anaerobic spore-formers

Members of the genus *Bacillus* and closely related genera are isolated according to their growth characteristics or the type of spoilage. The methods used to isolate and enumerate organisms associated with spoilage of high-acid, acid and acidified and low acid products make use of the pH range, growth temperature range and type of spoilage (e.g. flat-sour) of the particular groups involved. The most general test for members of the genus *Bacillus* is for ‘aerobic mesophilic spore-formers’ and the only species for which a specific selective medium exists is *B. cereus*, although a selective medium has been reported for heat resistant spores of *B. coagulans* (Nakajo and Moriyama, 1994). Aerobic spore counts can be carried out using a variety of methods and usually use dextrose tryptone agar or tryptone–glucose extract agar. The various methods and media used for different types of *Bacillus* spp. are described by Jensen (2000a). For psychrotrophs, a modified Mikolajcik method developed by Feijoo and Bodyfelt (1990) may be used, involving a heat shock of 75°C for 20 min, storing samples at 7.2°C for 8–10 days and then plating onto Standard Methods agar. A method typically used for the isolation of mesophilic aerobic spore-forming organisms involves plating onto tryptone glucose extract agar plates after heating to 80°C for 30 min, and then incubating aerobically at 35°C. For isolation of thermophiles, incubation at 50–55°C for 2–3 days on dextrose-tryptone agar is used after a more severe thermal treatment, such as 100°C for 5 min. For *G. stearothermophilus*, a standard procedure used is 30 min at 100°C or 10 min at 110°C, followed by rapid cooling (Kotzekidou, 2000). ‘Rope’-causing spore-formers can be isolated using tetrazolium salts in the non-selective agars referred to above (see Jensen, 2000a).

Following this, a number of different approaches may be used to identify the isolates and these include use of molecular techniques and more conventional biochemical/phenotypic properties. Unfortunately, the conventional methods are rather limited in their ability to distinguish between different species (van der Vossen and Hofstra, 1996) and are sometimes at
odds with the identification or characterisation provided by more recently developed techniques, such as polymerase chain reaction (PCR) and DNA–DNA hybridisation. It is also the case that there are no selective media able to detect species within this group of organisms. Nevertheless, classical methods are still useful, particularly where methods such as 16S rRNA/DNA sequencing is insufficient or unsatisfactory. An alternative method that has been applied to differentiate the different *Bacillus* spp., for identification and taxonomy is matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) protein profiling and this method has been shown to correlate well with *gyrB* sequence analysis and DNA–DNA hybridisation for *B. pumilus* (Dickinson *et al.*, 2004). Identification of aerobic spore-forming bacteria has always been a difficult task and there are a number of sophisticated techniques now available but these are not always suitable for routine application, so phenotypic characterisation remains an important tool for identification (Reva *et al.*, 2001).

The methods used to isolate *Alicyclobacillus* spp., the only spore-formers capable of spoiling high-acid products, are either media-based or filtration-based. These organisms are unable to grow on normal agar media (e.g. nutrient agar, brain heart infusion, trypticase soy agar) even when these are acidified to low pHs. As with other methods for isolation of spore-former, there is an initial heating step, where the sample is heated at 80°C for 1–10 min. The media that are commonly used include: *Alicyclobacillus acidocaldarius* medium (AAM or *Bacillus acidocaldarius* medium, BAM), first proposed by Darland and Brock (1971) and modified since by inclusion of trace elements by Farrand *et al.* (1983) and Silva *et al.* (1999); orange serum agar (OSA) supplemented with sucrose (Jensen, 2000b); potato dextrose agar (PDA), commonly used to culture yeasts and moulds, acidified to 3.5 (Splittstoesser *et al.*, 1998); yeast–starch–glucose (YSG) agar, adjusted to pH 3.7 (Motohiro and Hiroko, 1994); HGYE medium proposed by Hiraishi *et al.* (1997); and K agar, used by Walls and Chuyate (2000). The filtration methods usually involve filtration through a 0.45 µm pore-size filter (Vieira *et al.*, 2002) and this has been found to improve recovery compared with plating methods (Pettipher and Osmundson, 2000) and is now commonly used in the fruit industry.

Identification methods for *Alicyclobacillus* spp. vary in their power of discrimination and complexity with some involving use of PCR methods (Cerny *et al.*, 2000; Murakami *et al.*, 1998). DNA–DNA homology and comparative 16S ribosomal sequence analysis (Yamazaki *et al.*, 1996) or randomly amplified polymorphic DNA (RAPD) analysis (Yamazaki *et al.*, 1997), and others using more pragmatic approaches, such as direct epifluorescent filter technique or off-odour production on plates (Pettipher and Osmundson, 2000), or standard biochemical and morphological tests.

The main aerobic/facultatively anaerobic organism responsible for causing spoilage of acid and acidified products is *B. coagulans*. The method usually used for isolation of this organism is acidified proteose-peptone agar described by Stern *et al.* (1942). For heat-treated spores, dextrose tryptone agar is used
for recovery (Thompson, 1981). For thermophilic species, primary isolation at 55°C will encourage growth of these organisms, which include *B. licheniformis*, *B. subtilis*, *G. stearothermophilus*, *B. coagulans*, *Br. brevis*, *B. pumilus* and *P. macerans*. A method for screening shelf-stable hermetically sealed products for flat-sour organisms was developed by Demaré et al. (1985), involving in-product catalase measurements. Phenotypic tests that can be used to identify *G. stearothermophilus* are described by Kotzekidou (2000) and include acid and gas production in phenol red carbohydrate broth, hydrolysis of casein and starch, utilisation of citrate and formation of dihydroxyacetone.

Identification of *B. coagulans*, other species of the genus and closely related genera is usually carried out using biochemical tests (e.g. API 20E + 50CHB systems or the Vitek system using the BAC card or the Biolog system) together with microscopy, casein hydrolysis and growth characteristics (temperature and anaerobic growth). Some key differences between *B. coagulans* and other closely related organisms are provided by De Clerck et al. (2004a). Other methods used to differentiate between these organisms include fatty acid analysis (Kampfer, 1994), DNA-base composition (De Bartolomeo et al., 1991), 16S rRNA gene sequencing (Shida et al., 1997), RAPD-based approaches (e.g. Ronimus et al., 2003) or combinations of these.

A recent application using PCR of 16S rRNA amplicons (Rückert et al., 2005) demonstrated detection and enumeration of thermophilic bacilli in milk powders. PCR detection methods using primers that target species-specific 16S rRNA gene sequences have been developed for a number of spoilage aerobic spore-formers, including *P. macerans* (Vollú et al., 2003). A simplified technique using morphological and physiological tests has also been proposed by Reva et al. (2001) and this allows relatively rapid identification based on phenotypic properties. MALDI-TOF MS (Dickinson et al., 2004) is reported to be more accurate than Biolog metabolic profiling and more discriminating than 16S rRNA sequence analysis, but this method requires development of a standardised MALDI profile database to be of greater value.

### 21.5.2 Anaerobic spore-formers

Isolation of anaerobic spore-forming bacteria is often hampered by the exacting requirements for sporulation of some of these species and many will go undetected if good recovery/isolation conditions are not used. It is often necessary to use selective-diagnostic media for this reason. An early step in isolation is use of reinforced *Clostridium* medium (RCM) broth, differential reinforced clostridial medium (DRCM), H2S producing medium or cooked-meat medium. Enumeration and isolation of anaerobes can be carried out using decimal dilutions and RCM agar or with a most probable number (MPN) technique using DRCM tubes (Gibbs and Freame, 1965). Many clostridia reduce sulphite to sulphide and produce black colonies (large zones of FeS) in iron-containing media under anaerobic conditions. Growth of
Enterobacteriaceae can be suppressed by inclusion of polymyxin and sulfite-reducing *Bacillus* spp. can be distinguished by sensitivity to metronidazole. The availability of gas-packs and oxygen-impermeable bags has made the culturing of anaerobes much easier in recent years.

Butyric anaerobes may be isolated by heating product to 76.6°C for 10 min and using thermooxidurans agar overlaid with thioglycollate agar (NCA, 1961). Growth with gas production indicates presence of butyric anaerobes. Another isolation medium that can be used to isolate butyric anaerobes and developed by the Continental Can Co. is described by Morton (1998). Media used for anaerobes can be made more specific for *C. tyrobutyricum* by replacing glucose with lactate and adjusting the pH to 5.3–5.5. A review of the methods available for isolation and enumeration of *C. tyrobutyricum* is provided by Bergère and Sivelä (1990).

Various media have been developed for the isolation of *D. nigrificans*. Donnelly and Busta (1981) compared different procedures for the isolation of *D. nigrificans* from soy products and reported that there was little difference in the performance of these methods, which included beef extract tryptone iron (BETI) agar and Baars broth, and soytone–sodium metabisulphite–ferric ammonium citrate (SoS, soytone sulphite agar), although SoS recovered more spores than sulphite agar.

For isolation of non-hydrogen sulphide-producing thermophilic anaerobes such as *T. thermosaccharolyticum*, the recommended medium is PE-2 medium, described by Folinazzo and Troy (1954). Liver broth is also used for this purpose, although this is more difficult to prepare and can inhibit recovery of antibiotic-sensitive organisms (Ashton, 1981).

Identification of clostridia is normally carried out by assessing the phenotypic properties of isolates. These include gelatin liquefaction, indole production, motility testing, location of spores, nitrate reduction and fermentation of carbohydrates. End-products of fermentation may also be analysed using gas-liquid chromatography (Usha and Murugesan, 1983). These methods can be found in the Virginia Polytechnic Institute (VPI) manual (Holdeman et al., 1977). Alternatively, biochemical reaction profiles provided by systems such as API 20 A can also be used. As with *Bacillus* and other closely related aerobic spore-formers, new methods are available for identifying and characterising anaerobes. These include antibody (e.g. enzyme-linked immunosorbent assay, ELISA) methods and DNA-based methods. DNA probes and PCR techniques based on specific 16S rRNA sequences have been developed and have been used successfully for identification of various clostridial species, such as *C. tyrobutyricum* (Herman et al., 1995).

### 21.6 Future trends

The various forms of spoilage caused by spore-forming bacteria are largely preventable by a wide-range of preservation techniques, including chilling,
curing, conserving, vacuum packing and acidifying, and a smaller range of inactivation procedures. Some of these more recently introduced procedures, such as vacuum packing and chilling, have led to the appearance of new types of spoilage such as ‘blown pack’ spoilage caused by psychrotolerant clostridia. These newer processes are part of the general trend towards the use of fewer preservatives and lower heat treatments in foods, to provide ‘healthier’ and ‘fresher’ foods. This trend is likely to continue and may provide other new niches for other new forms of spoilage to occur. For example, the recent emphasis on reduction of NaCl in processed foods by the Foods Standards Agency in the UK is commendable but care should be taken that removal of preservatives does not impact on the susceptibility of these foods to growth of spoilage and pathogenic microorganisms.

One of the major growth areas in food in recent years has been chilled foods, such as ‘sous vide’ products or refrigerator-processed foods of extended durability (REPFEDs). These products are susceptible to spoilage by spore-forming bacteria and the shelf-life of these products is currently determined by these organisms, because of the way they are processed. Product developers and marketing people are continuously looking to extend the shelf-life of these products to >60 days at chill temperatures. There is greater emphasis on looking for new methods to control spore-forming bacteria in these products to provide assurance for these long shelf-life products. Invariably, there will be a combination of a heat treatment (e.g. pasteurisation) and use of inhibitory combinations to prevent outgrowth of spore-forming bacteria that are able to survive the heat applied to a food. Development of milder preservation techniques must be carefully considered in case these allow microorganisms to more easily overcome the applied stresses (Knochel and Gould, 1995).

It is also the case that advances in preservation techniques, through development and application of predictive models (e.g. as described by McClure et al., 1994) have facilitated application of novel combination treatments, offering a wider choice of options that are more attractive for process developers. Recent developments in risk assessment have also impacted on product and process development by allowing food scientists to take account of microbial loads and to refine the methods of control so that they do not over-process the foods in question. While these developments should result in foods that are still safe and stable, it is likely that they will be less robust compared with conventionally-processed foods and there is potential for the processes to go wrong if incorrect assumptions are made or if there is poor control in one or more parts of the process. There is increasing pressure on raw material suppliers to provide less heavily contaminated materials and advances in hygiene and handling practices can lead to cleaner raw materials but it is important to have systems in place to ensure that standards are maintained since these steps in the process effectively become more important for the targets of critical control points.

One other relatively recent development in food processing is the advent of flexible pouch technology. The importance of low redox potential in
canned foods has probably contributed to their safety and stability and many companies are now switching over to the use of flexibles for primary packaging. If materials are used that allow ingress of gases, the contents of these packs will increase their redox status over time and this may make these products more susceptible to growth of more aerobic spore-forming bacteria (such as *B. subtilis* and *B. licheniformis*) that have survived the heat process. To assess the stability of these new products, it is important to carry out studies that extend for the expected shelf-life of the products and to avoid use of ‘accelerated’ shelf-life studies (e.g. using higher than expected storage temperatures) that may misrepresent the actual redox status of the food when it is in the marketplace.

New technologies and ‘natural’ preservatives have promised much in the past two decades and these have been driven by the demand for fresher, higher-quality foods. There is little or no correlation between responses to these non-thermal technologies and much better characterised heat resistance, and this means that application of new technologies poses difficult challenges because of the variable and unpredictable responses seen so far. It is also true that ‘natural’ preservatives have probably not been as successful in application compared with the promise they sometimes show in laboratory studies. For this reason, it is important that results from laboratory studies are validated quickly in foods before effort is wasted. These difficulties would be more easily overcome if more were known about the mechanisms of action of factors acting in concert with other factors and interdependencies between different factors.

With the continued emphasis on reducing preservatives and heat treatments, and use of combination effects, it is increasingly important to understand the interactions and mechanisms involved, particularly where synergy is identified. At present, there are no accepted methods to calculate total effects of combination treatments, as there are for more conventional process approaches. However, there is likely to be more effort devoted to methods that can take account of injury effects on spore-formers, in combination with other factors such as storage at chill temperatures, as demonstrated recently by Membré *et al* (2006). Bacterial spore-forming organisms are likely to remain the main targets for the preservation of many foods because of their remarkable resistance properties and their widespread occurrence in the environment and future strategies aimed at assuring food safety and stability will have to remain focused on these important organisms.

### 21.7 Sources of further information and advice

In the past 40 years, a great deal of information and understanding of bacterial spores has been generated and many aspects related to spore development, germination, outgrowth are available in excellent text books and articles including Gould and Hurst (1983) and Gould (2000). Information on the
recovery, isolation, culturing and identification/characterisation of spore-forming bacteria can be found in the *Encyclopaedia of Food Microbiology* (Robinson *et al.*, 2000). The destruction of bacterial spores by various technologies and factors is covered in depth by Russell (1982). Various fundamental and applied aspects of bacterial spores, such as germination triggers, resistance mechanisms and response to different ‘stresses’ are covered by Gould *et al.*, (1994).

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22

Enterobacteriaceae
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22.1 Introduction

The family Enterobacteriaceae comprises a large group of Gram-negative, non-spore-forming, facultatively anaerobic bacteria. Owing to their medical and economic importance members of the Enterobacteriaceae include some of the most thoroughly studied microorganisms, and several of the important human pathogens including Salmonella enterica serovar Typhi, Shigella dysenteriae, Yersinia pestis and a range of pathogenic Escherichia coli, including E. coli O157:H7. Besides their clinical importance, some members of this family are important food spoilage organisms, responsible for substantial economical losses in some sectors of the food industry. This chapter will describe some of the characteristics of the Enterobacteriaceae including recent changes in taxonomy, information on some newly described genera and species, the habitats of these bacteria and their natural associations with foods as well as their importance as indicator organisms. Principles behind methods for their enumeration will be explained together with types of spoilage commonly associated with these bacteria and ways of preventing spoilage occurring. Lastly, this chapter provides additional information on factors affecting growth and survival of various members of the Enterobacteriaceae, future trends and useful sources of information on this group of important bacteria.

22.2 Taxonomy of the Enterobacteriaceae

In 1937 the name Enterobacteriaceae was first proposed by O. Rahn (Rahn,
Enterobacteriaceae. At the time this family contained the single genus *Enterobacter* and numerous bacteria that shared the ability to ferment glucose with the production of gas. Besides including several genera which still remain within the Enterobacteriaceae, this family previously encompassed flavobacteria and pseudomonads. The type genus is *Escherichia*; however, there have been several changes and the use of the name Enterobacteriaceae has been challenged twice (Lapage, 1979; Goodfellow and Trüper, 1982). Central to the argument is that the family name Enterobacteriaceae has not been formed in accordance with the rules of bacteriological nomenclature. Under these rules the name of the family should be formed from the name of the type genus by adding the suffix *aceae*. Therefore, either the type genus should have changed to *Enterobacter* or the family should be named Enterobacteraceae, or the family should have been renamed Escherichiae to reflect the chosen type genus. Both proposals have been rejected by the Subcommittee on the Taxonomy of Enterobacteriaceae (Brenner, 1983) and the family name has remained Enterobacteriaceae, not least because of its universal use and wide acceptance.

Bacterial classification until the early 1960s was largely based on cultural observations and phenotypic characteristics. Improved identification and assignment of correct genus and species via genetic relatedness became possible with the advent of techniques based on nucleic acids such as DNA–DNA hybridisation and guanine plus cytosine (G+C) determination. DNA–DNA hybridisation has revealed the relatedness between various members of the Enterobacteriaceae (Brenner, 1992a,b) and comparison of portions of the *E. coli* genome, including the *trp* operon, with corresponding DNA sequences in other enteric bacteria has revealed how some regions of their genomes are homologous whereas others are more divergent, reflecting differences in the evolutionary origins of these segments of DNA (Riley and Anilionis, 1980).

During the 1980s high-resolution polyacrylamide gel electrophoresis of proteins became an additional taxonomic tool used to identify and type bacteria. Identification and characterisation of bacteria have been greatly improved by the introduction of methods based on fragment analysis and techniques using polymerase chain reaction (PCR) and sequencing. There are now numerous genetic subtyping methods, some of which have been used to characterise and compare the relationship between members of the Enterobacteriaceae. The principles behind some of these methods and further information about their application are described in a recent review (Fitzgerald et al., 2003).

Compared with traditional phenotypic methods, nucleic acid sequences contain more evolutionary information. This is particularly true of highly conserved regions of the genome, especially sequences relating to protein-encoding genes and housekeeping genes so long as they are universally distributed and contain sufficient sequence variation to enable separation of genera and species. Fundamental to analysis of phylogenetic relationship
between bacterial taxa is comparison of rRNA sequences. A commonly used molecule for this purpose is 16S rRNA. Sequencing of 16S rRNA genes (16S rDNA) has now become a valuable tool in bacterial taxonomy for determining relationships between bacterial groups. Unlike families such as Pseudomonioniaceae, Bacilliaceae and Clostrediaceae, members of the Enterobacteriaceae had not been subjected to extensive analysis of 16S rDNA. This technique has been used successfully to compare the relationship between members of the Enterobacteriaceae (Spröer et al., 1999) as well as specific genera described later. However, there can be limitations using 16S rRNA sequences, which may give poor separation of some taxa at or below the species level. One problem relates to the presence of multiple copies of rRNA operon in the genomes of some bacteria, for example E. coli, which has seven copies. Consequently, variation in the 16S rRNA gene sequences in different operons on the same genome may be overlooked if a single or consensus sequence is chosen for comparisons. Therefore, any representative sequence should ideally be derived from multiple isolates and from each of the different operons. An alternative is to use comparison of 23S rRNA sequences which are twice as long and contain more variable regions. One study (Christensen et al., 1998) examined both 16S and 23S rRNA sequences to successfully separate two Salmonella species and revealed how Salmonella is closely related to E. coli and Shigella, which supports a previous evolutionary hypothesis that E. coli evolved from Salmonella (Lawrence and Ochman, 1998). Furthermore, the 23S rRNA sequence comparison in this example also enabled further separation of different Salmonella serotypes which did not occur with the 16S rRNA analyses.

While phylogenetic studies on Enterobacteriaceae using rRNA sequences have become popular, the phylogenetic trees obtained may not always agree with the classical taxonomy. A different approach being used to examine phylogenetic relationships between different bacteria, including members of the Enterobacteriaceae, is to compare sequences of specific genes, often in association with analysis of 16S rRNA sequences. For example phylogenetic trees based on gyrB sequences have been reported to be more reliable than those based on 16S rRNA for determining relationships between Serratia species and other members of the Enterobacteriaceae (Dauga, 2002). Sequencing the partial sequence of the gene encoding translation of initiation factor 2, an essential protein involved in the initiation of protein synthesis, has also provided useful information about the relationships between members of the Enterobacteriaceae, which has further supported evidence acquired by sequencing of 16S rRNA (Hedegaard et al., 1999).

In addition to sequencing specific genes, some species belonging to the family Enterobacteriaceae have had their complete genomes sequenced. This has revealed much about the relatedness of different genera and the mobility of certain genes, especially those associated with virulence and pathogenicity. One of the first complete genomes to be fully sequenced and published belonged to a strain of E. coli K12 (Blattner et al., 1997) which has been
Enterobacteriaceae used as a model organism for the study of bacterial genetics and metabolism. The complete genome sequence from this strain has been used as a benchmark against which many other bacterial genomes have been compared. Several complete genome sequences belonging to members of the Enterobacteriaceae, especially the important human pathogens in this family, have now been published. Notable examples include strains of *Salmonella enterica* serovar Typhimurium (McClelland et al., 2001) and *Salmonella enterica* serovar Typhi (Parkhill et al., 2001a), two strains each of *Yersinia pestis* (Parkhill et al., 2001b; Deng et al., 2002), *E. coli* O157:H7 (Hayashi et al., 2001; Perna et al., 2001) and *Shigella flexneri* (Jin et al., 2002; Wei et al., 2003) and a strain of uropathogenic *E. coli* (Welch et al., 2002). With time, more complete genome sequences belonging to other strains of these and other genera and species will become available, further increasing our knowledge of these organisms and how they cause human disease.

As a consequence of the introduction of new molecular methods, particularly the development of more discriminatory techniques, together with the publication and comparison of whole bacterial genome sequences, it is inevitable that there will be future changes to the family Enterobacteriaceae, including the introduction of new genera and species and reclassification of some existing ones. An important change in nomenclature to occur recently as a result of using genetic-based classification methods occurred with *Salmonella* (Brenner et al., 2000). Previously the genus *Salmonella* comprised over 2400 serotypes (serovars), which were once considered separate species based on the combination of somatic O and flagella H antigens expressed by these bacteria. However, DNA–DNA hybridisation studies have shown that all *Salmonella* form a single DNA homology group comprising two species. The first species, *S. enterica*, comprises six groups, whereas the seventh group has been assigned to the second species, *S. bongori* (Table 22.1). Other changes to the family Enterobacteriaceae include the generation of the new genus *Photorhabdus* to accommodate *Xenorhabdus luminescens* strains, based on DNA relatedness data (Akhurst et al., 1996).

More recently comparative analysis of the 16S rRNA and *rpoB* (encoding the bacterial RNA polymerase β-subunit) genes has supported the division of the genus *Klebsiella* and the creation of a new genus *Raoultella* (Drancourt et al., 2001). Members of the genus *Erwinia* have always been important because of their ability to cause disease in plants. Since the introduction of genetic-based methods, particularly analysis of 16S rRNA sequences and other gene sequences, some species formerly belonging to the genus *Erwinia* have now been assigned a new genus *Brenneria*, which together with *Erwinia* encompass all of the necrotic phytopathogenic species, and a second new genus, *Pectobacterium*, which now includes the soft rotting phytopathogenic species (Brown et al., 2000).
### Table 22.1 Members of the family Enterobacteriaceae

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species/Strains</th>
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<tbody>
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Table 22.1  Continued

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<td>pseudotuberculosis</td>
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<td>ruckeri</td>
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22.3  Properties of the Enterobacteriaceae

Currently the family Enterobacteriaceae comprises at least 34 genera, 149 species and 21 subspecies (Table 22.1). Cells are typically 0.3–1.8 µm, straight, rod-shaped although longer filamentous forms can occur in many genera or if cells are exposed to certain environmental conditions. Cells can be non-motile or motile, often by peritrichous flagella. One exception is Tatumella which exhibit motility by means of polar, subpolar or lateral flagella among 50% of strains when grown at 25°C.

Given the number and diversity of species within the Enterobacteriaceae it would be misleading to generalise about optimum growth conditions and limits of certain factors which affect growth. Within the FORECAST predictive modelling system (see p. 110), the growth of Enterobacteriaceae, comprising a cocktail of five representative species (P. mirabilis, K. pneumoniae, Cit. freundii, Ent. cloaceae and H. alvei) is predicted for a variety of factors including temperature (0–30°C), water activity (a_w) (0.94–1.0), % aqueous NaCl (0.5–10) and pH (4.0–7.0). With regard to temperature, some species grow better, or are metabolically more active, at 25–30°C although the majority of species grow well at 37°C. However, within the Enterobacteriaceae psychrotrophic strains are reported to exist (Michener and Elliott, 1964;
Kraft, 1992) and growth of some coliform bacteria at temperatures as low as –1.5 to 1.5°C in milk has been reported (Eddy and Kitchell, 1959). At these lower temperatures growth rates are slower, although in raw milk during refrigeration at 3–5°C coliforms have been reported to increase 100 or even 1000-fold in 3 days (Panes and Thomas, 1968). Reducing the temperature typically increases the generation time which results in slower growth. Reported generation times for some psychrotrophs between 0°C and 32°C have been reported by Tompkin (1973). In the review by Tompkin a strain of Enterobacter (Aerobacter) aerogenes is reported to have the following generation times: 37.7h (0°C), 12.2h (4–5°C), 4.1h (10°C), 2.2h (14–15°C), 1.3h (20°C) and 0.8h (30–32°C). As with most Gram-negative bacteria, freezing will reduce the initial population but those bacteria that survive can remain viable for prolonged periods of frozen storage. In ground beef patties, frozen storage (–20°C) has been shown to result in a 1–2 log 10 cfu/g reduction of E.coli O157:H7 after 1 year (Ansay et al., 1999).

While some strains may tolerate and even grow at temperatures as high as 50°C members of the Enterobacteriaceae are not particularly heat resistant and these bacteria would be killed by pasteurisation temperatures (typically >72°C) used in many food processes. Approximate D values at a_w 0.95 and pH ca 7 published in Mossel et al., (1995) for E. coli is 4–6 min at 56°C and ca 2 min at 60°C, whereas for Salmonella they are 0.1–2.5 min at 60°C, 0.07 min at 65°C and 0.3min at 70°C. In contrast Salmonella Senftenberg 775W, which is reported to be more heat resistant than other salmonellas is reported to have a D value of 6.0 to 10 min at 60°C. Although there are occasional strains that appear to show greater tolerance of heat compared with others these are very much the exception. The majority of the Enterobacteriaceae are not exceptionally heat resistant, which therefore makes them suitable indicator organisms for cooked and other heat processed foods. However, heat resistance can be influenced by other factors, notably a_w and prior exposure to low temperatures. Cells in stationary phase of growth also tend to be more resistant to heat as well as other factors. A study of thermal resistance of E. coli O157:H7 in beef burgers revealed greater heat resistance of this bacterium when cells had been previously exposed to frozen storage compared with those that had been stored at 15°C prior to cooking (Jackson et al., 1996). Under normal cooking conditions, a 4 log cycle reduction in the E. coli O157:H7 population in beef burgers was achieved when the burgers were heated to an internal temperature of 68.3°C (Juneja et al., 1997).

The optimum a_w for Enterobacteriaceae is normally between 0.94 and 0.99, but as with freezing these bacteria can survive in low a_w foods for long periods. In chocolate (typical a_w 0.4) Salmonella has been reported to survive for over 12 months (Tamminga et al., 1977) and strains of pathogenic E. coli, including E. coli O157:H7 can survive for similar periods in ambient stored chocolate (Baylis et al., 2004). Furthermore, in this and other studies the decline and survival of many bacteria, including E. coli O157:H7 is noticeably longer if the temperature is lowered (Deng et al., 1998; Baylis
Enterobacteriaceae (et al., 2004). The pH range for growth of Enterobacteriaceae is usually between 4.4 and 9.0, although some strains of *E. coli* O157:H7 are reported to tolerate acidic conditions below pH 4 and have been responsible for outbreaks associated with fermented meats and other low-pH foods (Duffy and Garvey 2001).

A physiological response of many bacteria, which is of particular concern when this occurs in foodborne pathogens, is their ability to survive adverse environmental conditions after prior exposure to non-lethal stress such as cold, heat, acid, etc. This can induce expression of stress proteins by the cell, which can in turn enable them to survive adverse conditions or treatments that would otherwise be lethal. This cross-protection, whereby exposure to one stress factor can bring about enhanced resistance against another one, can result in greater heat resistance and acid tolerance of these bacteria. This phenomenon has been studied in many pathogens, including *E. coli* O157: H7 which exhibits greater heat and acid resistance in foods after exposure to sublethal treatments and stresses (Ryu and Beuchat, 1998; Duffy and Garvey, 2001).

Members of the Enterobacteriaceae are chemoorganotrophic, having both a fermentative and a respiratory type of metabolism. A number of carbohydrates are catabolised, with the production of acid and some species also produce gas, although some are anaerogenic, and fail to produce gas under certain conditions. The fermentation of D-glucose is one characteristic property of the Enterobacteriaceae that is used in many media for their isolation and enumeration. Besides their ability to ferment D-glucose, another characteristic feature of bacteria in this family is the absence of cytochrome oxidase activity. The oxidase test is therefore an important test to differentiate Enterobacteriaceae from bacteria belonging to other families, namely the Vibrionaceae and Pasteurellaceae, as well other facultatively anaerobic Gram-negative rods, present in other families (Brenner, 1992b). The majority of the Enterobacteriaceae are catalase positive, with the exception of *Shigella dysenteriae* O group 1 and *Xenorhabdus* species (Holt et al., 1994). Nitrate reduction is also another common feature of members of the Enterobacteriaceae, with the exception of *Arsenophonus*, some *Erwinia* species, the majority of *Xenorhabdus* species and some strains of *Klebsiella pneumoniae* subsp. *ozaenae*, *Pantoea* and *Yersinia* (Holt et al., 1994).

Within this family there are several genera that also have the ability to rapidly ferment lactose, normally within 24 h. These include *Enterobacter*, *Escherichia*, *Citrobacter* and *Klebsiella*. Bacteria belonging to this group are collectively termed coliform bacteria, although unlike the Enterobacteriaceae family to which they all belong, which is a well-defined taxonomic group, coliforms are an ill-defined group of bacteria, although they remain a popular group of indicator organisms used by the food and water industry (see below). More recently, coliform bacteria have been defined by the presence of β-galactosidase activity, although this can cause confusion because some slow lactose fermenting species such as *Hafnia alvei* and
Serratia spp. which display β-galactosidase activity would be regarded as coliforms on this basis, yet they may fail to ferment lactose rapidly. Consequently, these features that are commonly employed as diagnostic features for coliform bacteria can influence the results of some coliform methods.

22.4 Distribution and habitats of the Enterobacteriaceae

Members of the Enterobacteriaceae are widely distributed in nature and the environment. Some species are human and animal pathogens, whereas others are pathogenic to plants and insects. Plant pathogens, which are of economic importance owing to the threat to agricultural crops, include members of the extensively studied genus Erwinia, of which some species have now been assigned to the genera Brenneria and Pectobacterium (Brown et al., 2000).

The natural habitats of members of the Enterobacteriaceae and reported isolation sources are given in Table 22.2. Owing to their widespread distribution it is therefore inevitable that some members of the Enterobacteriaceae will enter the food chain where they can be responsible for causing foodborne disease and food spoilage.

22.5 Pathogenic Enterobacteriaceae

Although some members of Enterobacteriaceae are isolated from human clinical specimens from specific parts of the body, for example the respiratory tract of humans, others are opportunistic pathogens and some represent important human pathogens capable of causing serious infections in humans. For example Yersinia pestis is the causative agent of bubonic plague and Salmonella Typhi and Paratyphi are responsible for typhoid and paratyphoid fever, respectively. There are also over 2000 serotypes of non-typhoid Salmonella which are widely distributed in animals and the environment. These bacteria are second only to Campylobacter as leading causes of gastrointestinal infections in people in the UK and USA. Some serotypes also appear to show close host specificity with certain animals. Consequently contact with these animals, their environment or foods derived from them provide a source of transmission of infection to people. More recently the World Health Organization recognised Enterobacter sakazakii as an emerging pathogen. This opportunistic pathogen represents a risk to new-borns, especially low birth weight and immuno-compromised infants. It can cause severe neonatal sepsis and meningitis and has been isolated from infant formula that has been shown to be a vehicle for Ent. sakazakii infection, although poor treatment of the product and environmental contamination have been shown to contribute to contamination and subsequent infections.
### Table 22.2  Natural habitats and isolation sources

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<thead>
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<th>Genus</th>
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<td>Arsenophonus</td>
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<td>Budvicia</td>
<td>Fresh water (isolates rare in human faeces and animals)</td>
</tr>
<tr>
<td>Buttiauxella</td>
<td>Fresh water</td>
</tr>
<tr>
<td>Cedecea</td>
<td>Human clinical (respiratory tract)</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>Human faeces and clinical specimens, sewage, soil, water, food</td>
</tr>
<tr>
<td>Edwardsiella</td>
<td>Cold-blooded animals (gastrointestinal (GI) tract and faeces) and their environment especially fresh water. Warm-blooded animals and humans</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>Widely distributed in nature; soil, fresh water, plants vegetables, sewage, animal and human faeces. Human clinical specimens and respiratory tract</td>
</tr>
<tr>
<td>Erwinia, Brenneria, Pectobacterium</td>
<td>Plants as pathogens, saprophytes or normal flora (rarely isolated from humans)</td>
</tr>
<tr>
<td>Escherichia</td>
<td>GI tract of humans and other warm-blooded animals (water, food and soil via faecal contamination), <em>E. blattae</em> (hind gut of cockroaches)</td>
</tr>
<tr>
<td>Ewingella</td>
<td>Human clinical specimens and respiratory tract. Occasionally found in molluscs</td>
</tr>
<tr>
<td>Hafnia</td>
<td>Human and other animal faeces (including birds), sewage, soil and dairy products</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>GI tract and respiratory tract of humans and other animals, faeces, soil, water, fruits and vegetables, grain and human clinical specimens.</td>
</tr>
<tr>
<td>Kluyvera</td>
<td>Human clinical specimens and respiratory tract, food, milk, water, soil and sewage</td>
</tr>
<tr>
<td>Lelercia</td>
<td>Human clinical specimens, food, water and environmental sources</td>
</tr>
<tr>
<td>Leminorella</td>
<td>Isolated from human faecal and urine samples</td>
</tr>
<tr>
<td>Moellerella</td>
<td>Human faeces and water</td>
</tr>
<tr>
<td>Morganella</td>
<td>Faeces of humans, dogs and other mammals and reptiles</td>
</tr>
<tr>
<td>Obesumbacterium</td>
<td>Associated with brewery contamination</td>
</tr>
<tr>
<td>Pantoea</td>
<td>Plant surfaces, seeds, soil and water. Wounds, blood and urine of humans and other animals</td>
</tr>
<tr>
<td>Photorhabdus, Xenorhabdus, Pragia</td>
<td>Symbiotically associated with specific nematodes belonging to family Heterorhabditidae (<em>Photorhabdus</em>) and Steinernematidae (<em>Xenorhabdus</em>) Isolated from drinking water</td>
</tr>
<tr>
<td>Proteus</td>
<td>Intestines of humans and a wide range of animals, soil, manure and polluted waters. Gypsy moth larvae (<em>P. myxofaciens</em> only)</td>
</tr>
<tr>
<td>Providencia</td>
<td>Human clinical specimens and penguins</td>
</tr>
<tr>
<td>Rahnella</td>
<td>Fresh water and occasionally human clinical specimens</td>
</tr>
<tr>
<td>Raoutella</td>
<td>Botanical and aquatic environments (<em>planticola &amp; terrigena</em>) human clinical specimens (<em>ornithinolytica</em>)</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Humans and other warm-blooded animals, cold blooded animals, foods, water and environmental sources. Common cause of gastroenteritis some serovars cause typhoid/paratyphoid fever</td>
</tr>
<tr>
<td>Serratia</td>
<td>Human clinical specimens, plant surfaces, soil, water and environmental sources, digestive tracts of rodents and insects. Some strains cause mastitis in cows</td>
</tr>
<tr>
<td>Shigella</td>
<td>Intestinal pathogen of humans and other primates. Cause of bacillary dysentery</td>
</tr>
<tr>
<td>Tatumella</td>
<td>Human clinical specimens, especially respiratory tract, occasionally blood and found in animals</td>
</tr>
<tr>
<td>Yersinia</td>
<td>Widely distributed and wide range of habitats including humans, animals (especially birds and rodents), dairy products and other foods, soil and water</td>
</tr>
<tr>
<td>Yokenella</td>
<td>Human wounds and insect intestines</td>
</tr>
</tbody>
</table>
Perhaps the most extensively studied bacteria is *E. coli*, which is genetically diverse and includes several pathotypes that are responsible for distinctive clinical diseases or syndromes (Nataro and Kaper, 1998; Baylis *et al.*, 2006). The extraintestinal *E. coli* include uropathogenic *E. coli* (UPEC) which are responsible for a large proportion of urinary tract infections and the meningitis-associated *E. coli* (MAEC) which cause meningitis in neonates. By comparison, the intestinal pathogenic *E. coli* (IPEC) are responsible for a range of diarrheagenic diseases and syndromes in people which can be transmitted by contaminated food and water. The six most important pathotypes associated with food are enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enterohaemorrhagic *E. coli* (EHEC). The latter represents a subgroup of a much larger group of *E. coli* termed Shiga toxin-producing *E. coli* (STEC), which are characterised by their ability to produce potent toxins termed Verocytotoxins or Shiga toxins, so termed because of their structural similarity with Shiga toxin from *Shigella dysenteriae* I. Perhaps the most important pathogens of recent times are the EHEC strains belonging to serotype O157:H7/(H–) which have been responsible for many large food- and waterborne outbreaks worldwide. This pathogen shows a high level of infectivity and is responsible for serious disease in humans, including haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS), which can result in renal failure. There are now over 200 serotypes of STEC but the clinical significance of many remains unknown.

Other important human pathogens within the Enterobacteriaceae include *Shigella* spp. which are responsible for bacillary dysentery. Genetically this genus appears to be very closely related to *E. coli* and it has been proposed that *Shigella* spp. have evolved from *E. coli* and that they constitute a single species. Another important foodborne pathogen is *Yersinia enterocolitica* which is commonly associated with pork and other meats. However, unlike other members of the Enterobacteriaceae these specific genera or species are regarded as foodborne pathogens and they require specialist approaches and specific methods for their detection, isolation and confirmation. Consequently, laboratories would test for these organisms separately whereas the Enterobacteriaceae are now used as indicator organisms and tests are often performed routinely for these bacteria.

### 22.6 Enterobacteriaceae in foods

Members of the Enterobacteriaceae can contaminate foods directly or indirectly from various natural sources or they may themselves represent the natural flora of the food. This is particularly true of unprocessed or raw foods that may be naturally contaminated by members of the Enterobacteriaceae as well as by other bacteria, yeasts and moulds. Those Enterobacteriaceae responsible for some characteristic types of food spoilage will be described
later, whereas this section will mention some of the common associations between members of the Enterobacteriaceae with particular foods.

Genera that appear to be widely distributed and therefore commonly encountered in foods include *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Serratia* and *Yersinia* although others, including *Escherichia*, *Proteus* and *Salmonella*, can enter the food chain or may be associated with particular foods via faecal contamination. These as well as a number of other genera, including *Pantoea*, may originate from soil, contaminated water or plants. Foods of vegetable or plant origin may also harbour *Erwinia*, *Brenneria* or *Pectobacterium* spp., because of the close association that exists between these organisms and plants.

### 22.6.1 Milk and dairy products

Milk and dairy products, particularly raw milk, contain Enterobacteriaceae at variable levels. At the time of collection of farm bulk tank milk psychrotrophic coliform bacteria may constitute 5–20% of the psychrotrophic microflora (Panes and Thomas, 1968). In some cheese-making processes these bacteria can contribute to the flavour and texture, but their presence, particularly at high levels, may be less desirable owing to their ability to cause spoilage. In raw milk pathogenic Enterobacteriaceae may also be present, including strains of pathogenic *E. coli*, *Salmonella* and *Yersinia*. Psychrotrophic Enterobacteriaceae strains may also be present in milk and dairy products and they too can give rise to spoilage. In cheese-making the formation of volatile compounds by Enterobacteriaceae is an important contributor to flavour and odour of cheese. These compounds, which include aldehydes, ketones, sulphur compounds, alcohols and aromatic compounds are produced by the metabolism of Enterobacteriaceae. In one study comparing 10 Enterobacteriaceae strains, a strain of *Ent. sakazakii* was shown to produce the highest amount of volatile compounds in fresh cheese (Morales *et al.*, 2004). Genera belonging to the family Enterobacteriaceae isolated from milk and dairy products include *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella* and *Serratia*, with *Enterobacter* and *Klebsiella* the most frequently isolated members in refrigerated raw milk (Cousin, 1982).

### 22.6.2 Meat, poultry and associated products

Some members of the Enterobacteriaceae, particularly *E. coli*, make up part of the natural gut flora of many animals including ruminants, such as cattle and sheep, as well as pigs, chickens and other farm animals. It is therefore inevitable that these bacteria will enter the food chain, although good slaughter practices and hygiene should restrict their spread and contamination to meat and meat products. Raw meats can become contaminated by Enterobacteriaceae from various sources in the slaughter environment. In one study a major source of carcass contaminations by bacteria, including Enterobacteriaceae
was reported to be the area behind the hide puller and also via split saw sprinkles (Gustavsson and Borch, 1993). Airborne contamination may also occur in slaughter environments and while puncture of the intestine or spillage of its contents during evisceration may give rise to massive contamination of the carcass and associated offals by enteric bacteria; this is fortunately a rare event. Poultry carcasses can become contaminated during processing, handling and evisceration, which may provides opportunities for contamination by gastrointestinal bacteria, including enteric pathogens, particularly Salmonella and Campylobacter. Scald tanks also provide a source of contamination by Enterobacteriaceae.

Freshly slaughtered meat produced under hygienic conditions is normally contaminated by low levels of Enterobacteriaceae. On cattle and sheep carcasses the incidence of Enterobacteriaceae is normally low and acceptable limits for levels of Enterobacteriaceae on beef carcasses have been defined in legislation (2001/471/EC). These limits are graded as acceptable ($<1.5\log_{10}\text{cfu/cm}^2$) marginal ($1.5\text{ to } 2.5\log_{10}\text{cfu/cm}^2$) and unacceptable ($>2.5\log_{10}\text{cfu/cm}^2$) (Anon., 2001c). However, there are currently no European regulations specifying acceptable limits for Enterobacteriaceae in minced beef products and in these types of meat contamination by Enterobacteriaceae can be high, although the levels may vary according to the hygiene and quality of the meat. Ground meats generally consist of trimmings from various cuts that may have been handled excessively and high levels of bacteria can be introduced via contaminated equipment such as meat grinders, knives and storage utensils. The greater surface area of ground meat also contributes to the higher microbial loading.

In a study of 1030 samples of raw minced beef and beef burgers from retail outlets in the Republic of Ireland Enterobacteriaceae counts were reported to range from $0.52\log_{10}$ to $6.98\log_{10}\text{cfu/g}$ (mean average $2.20$ to $4.64\log_{10}\text{cfu/g}$) with unpacked minced beef containing the highest count ($4.05$–$5.00\log_{10}\text{cfu/g}$) and frozen burgers the lowest with $0.52$ to $5.02\log_{10}\text{cfu/g}$ (average $2.38\log_{10}\text{cfu/g}$) (Crowley et al., 2005). In a study of 40 minced beef samples in Sweden the Enterobacteriaceae counts were reported to range from $3.50$ to $6.80\log_{10}\text{cfu/g}$ (Lindberg et al., 1998).

Various members of the Enterobacteriaceae contaminate raw meat and poultry, in particular species commonly associated with the gastrointestinal tract, including E. coli, which is often used as an indicator of faecal contamination in these products, and some of the important enteric pathogens such as Salmonella and Yersinia enterocolitica. In ruminants such as sheep and cattle E. coli O157:H7 can be carried in the gastrointestinal tract of these animals and meats derived from them can therefore become contaminated by this pathogen. Species frequently encountered in minced beef in one study were Serratia liquefaciens, Hafnia alvei and Rahnella aquatilis and some of these isolates were found to harbour toxin-encoding genes and other putative virulence factors (Lindberg et al., 1998). However, in meats psychrotrophic Enterobacteriaceae can multiply during refrigerated storage.
and levels can therefore increase so their hygienic significance must be interpreted accordingly.

22.6.3 Fish and seafoods
Fish and other seafoods can become contaminated by Enterobacteriaceae through various routes. Some Enterobacteriaceae may be present in the gastrointestinal tract of cold-blooded animals including fish. These bacteria may also be present in the aquatic environment and in polluted waters fish and other sea foods, particularly filter feeders, may become contaminated by Enterobacteriaceae of faecal origin, including *E. coli* and *Salmonella*. Fish can also become contaminated in the processing environment and subsequent growth and metabolic activity of these bacteria can lead to deterioration in the quality of fresh fish. The Enterobacteriaceae isolated and identified from whole fresh fish include *Rahnella aquatilis*, *Moellerella wisconsensis*, *Hafnia alvei* and *Citrobacter freundii* (Lindberg et al., 1998). In another study genera isolated from fresh-water fish included *Pantoea*, *Proteus*, *Serratia*, *Enterobacter* and *Escherichia* with *Hafnia alvei*, *Enterobacter cloacae* and *Citrobacter freundii* the most common species isolated (González-Rodriguez et al., 2001). Enterobacteriaceae have also been reported to be a dominant microflora in whole, gutted and filleted sea bass but numbers are generally less than those of pseudomonads and other bacteria associated with spoilage (Papadopoulous et al., 2003; Paleologos et al., 2004). In fish perhaps one of the most important features, besides deterioration in the organoleptic properties of the product, is the production of histamine by histidine decarboxylase activity of some members of the Enterobacteriaceae, which is described in Section 22.10.

22.6.4 Fruit and vegetables
Fruit and vegetables can become contaminated by a range of bacteria including *Salmonella*, *E. coli* O157 and other enteric pathogens. Such contamination has resulted in food poisoning outbreaks associated with contaminated fresh produce (Everis, 2004). Some bacteria, including members of the Enterobacteriaceae, show a natural association with plants or the environment in which they are grown and harvested, and some of these species can be responsible for spoilage and deterioration of these foods. Fruit and vegetables can become contaminated directly or indirectly by a variety of sources. These bacteria may be present in the soil where they may contaminate root vegetables or come into contact with leafy vegetables and the exterior of plants. They may enter plant materials during growth or they can contaminate plants and fruits directly or indirectly via insects, birds and animals, which can act as vectors for a range of human pathogenic bacteria. Faecal contamination via contaminated soil and irrigation water may also contribute to the flora on fruit and vegetables. The relationship that exists between certain bacteria, including members of the Enterobacteriaceae with vegetable
foods, is the subject of a review which gives further details on these natural associations (Lund, 1992). As a result of the many natural associations between these bacteria and some plants the hygienic significance of these bacteria in vegetables and foods of plant origin must be made with care (see Section 22.7 on indicator organisms).

Enterobacteriaceae and pseudomonads represent the two major groups of saprophytic bacteria found on the surfaces of freshly harvested plants. Furthermore, Erwina spp., which are also important plant pathogens, are commonly found in association with vegetable foods. One species in particular, which is a common cause of bacterial spoilage of vegetables, is Erwina (Pectobacterium) carotovora (ICMSF, 1998). In one study (Riser et al., 1984) the bacteria, which were all members of the Enterobacteriaceae, most commonly isolated from hydroponically grown lettuce, were Citrobacter freundii, Enterobacter cloacae and Enterobacter (Pantoea) agglomerans. A study of 146 coliform isolates from fresh produce showed that the predominant genus was Klebsiella (64%), whereas 14% were Escherichia, 14% were Enterobacter and 8% were Citrobacter (Duncan and Razzell, 1972). These same genera were also identified as common coliforms during a survey of 120 lettuce and 89 fennel samples in Italy (1973–1975) which also isolated E. coli and Salmonella in half of the samples and showed that Citrobacter occurred at higher frequency among faecal coliforms on lettuce during the summer months (Ercolani, 1976). A later study of 144 samples of lettuce from 16 university restaurants reported total coliform counts ranging from \( < 0.47 \) to \( > 3.38 \log_{10} \) most probable number (MPN)/g and 25.7% of these samples were contaminated with E. coli. Other members of the Enterobacteriaceae present in these samples included Citrobacter freundii (14.6%), Klebsiella pneumoniae (8.3%) Enterobacter cloacae (4.2%) and Providencia spp. (1.4%) (Soriano et al., 2000).

22.6.5 Other foods
The Enterobacteriaceae are widely distributed throughout nature and consequently many foods can become contaminated with them. Some of the foods commonly associated with contamination by these bacteria are given above. However, they can also survive in many other foods, depending on the intrinsic and extrinsic factors, and may even multiply and cause spoilage under the right conditions. Some of these other foods are described in Section 22.9 on spoilage by Enterobacteriaceae.

22.7 Indicator function of the Enterobacteriaceae and their relevance and survival in foods

Various groups of bacteria or individual species are used to provide evidence of poor hygiene, inadequate processing or post-process contamination of
Enterobacteriaceae

foods. Furthermore, some bacteria, notably *E. coli*, are present in the gastrointestinal tract of many animals including humans so their presence can be used to indicate potential faecal contamination. These organisms are commonly termed indicator organisms, although the significance of these organisms in certain foods, particularly unprocessed foods or foods of vegetable or plant origin, must be interpreted with care (Baylis and Petitt, 1997). Besides their use as indicator organisms, some groups of bacteria or individual species are used to assess the potential risk of closely related pathogens being present in food and water. Under these circumstances the organisms are often referred to as index organisms (Mossel, 1978, 1982). *E. coli* is a good example of an organism that can be used as an indicator of possible faecal contamination as well as an index organism for other enteric pathogens such as *Salmonella* in the same food. However, enteric pathogens in foods will often be present at low levels and therefore require specialist methods which can take days to isolate them. By comparison, tests for *E. coli* are simple and can take as little as 24h to obtain a presumptive count or detection result. For this reason methods for indicator or index organisms provide a simple approach for routine screening of foods. Besides food safety, indicator groups including Enterobacteriaceae provide a measure of food quality and spoilage potential.

Perhaps the most common group of bacteria employed as indicator organisms by the food industry are the coliforms, which can be regarded as a subgroup within the Enterobacteriaceae. However, unlike Enterobacteriaceae, which are well defined taxonomically, coliforms are ill defined and do not represent a true taxonomic group. Instead they are commonly defined by their ability to ferment lactose rapidly producing acid and gas, typically within 24h, which are diagnostic features exploited by traditional culture media used for their detection and enumeration. In the past, coliforms have been defined as bacteria that give a positive result to a coliform test (Anon., 1978), although under this definition lactose fermenting bacteria outside the Enterobacteriaceae, notably *Aeromonas* spp., would be included.

Using rapid gas and acid production from lactose fermentation as a diagnostic feature of the coliform group, the genera that would commonly be expected to belong to this group would include *Enterobacter*, *Klebsiella*, *Citrobacter* and *Escherichia*, particularly *E. coli*. However, species belonging to other genera, for example *Erwina* and *Serratia*, can also ferment lactose, albeit slowly, whereas some strains of *Citrobacter* and *Klebsiella*, as well as *Salmonella Arizonae* and *Hafnia alvei*, show delayed or variable lactose fermentation ability. Consequently, it has not been easy to properly define the coliform group or the genera or species belonging to it. Water microbiologists have attempted to improve the definition of coliforms by making possession of β-galactosidase activity a requirement (Anon., 1994). This definition was intended as a practical working definition of coliform bacteria and not a taxonomic one. Furthermore, while this criterion helps to provide a definition that is not method related, slow lactose fermenting
strains, which may not produce acid or gas by the traditional methods, would therefore be included. This therefore broadens the group to include more genera and this must be appreciated when coliform results from methods based on lactose fermentation are compared with those obtained using chromogenic media.

In addition to the poorly defined coliform group, the use of the term faecal coliform provides even greater confusion and misunderstanding. The purpose of looking for these bacteria is to assess possible faecal contamination of foods by restricting the search to genera that are commonly associated with the gastrointestinal tract and faeces. This is achieved using traditional culture-based methods and by using lactose fermentation and gas production at 44.5°C within 24 ± 2 h as diagnostic for these bacteria. Not surprisingly the most common faecal coliform isolated by these methods is *E. coli*. However, although these methods will provide better specificity for assessing faecal contamination, besides coliforms of faecal origin such as *E. coli*, some non-faecal coliforms are also capable of growth and lactose fermentation at these elevated temperatures, for example some *Klebsiella* spp. The term thermotolerant coliform, which is now being used, is therefore a more appropriate description for these bacteria.

In the water and dairy industry tests for coliforms have remained popular, not least because there are specific guidelines and regulations that specify tests for coliforms, but also because they have been used historically, and therefore remain a popular indicator group. In the food industry to improve assessment of food safety and quality there has been a gradual move towards testing for total Enterobacteriaceae rather than limiting testing for coliforms. Irrespective of whether foods are tested for coliforms or Enterobacteriaceae the significance of the results should always be put into context with the food and any natural association that may exist between the bacteria present and the food.

In processed foods, particularly foods subjected to heat treatment, these bacteria can provide a reliable indication of process failure, under-processing or post-process contamination, depending on the initial contamination level and treatment. However, with unprocessed foods, particularly foods of plant origin such as vegetables the presence of coliforms may be unavoidable and would not necessarily indicate poor hygiene or unacceptable quality (Splittstoesser and Wettergreen, 1964). While certain Enterobacteriaceae are normal inhabitants of the intestinal tract of warm and cold-blooded animals and birds, others, such as *Erwinia* spp., may be associated with plants and the soil. It is therefore inevitable that certain foods will become contaminated by these bacteria, although the number present and their potential to spoil the product will become important considerations.

Unlike water, where indicator bacteria have little or no chance of multiplication, in certain foods they may continue to increase in number. Coliform bacteria are reported to grow at temperatures as low as –2°C and as high as 50°C. Even at refrigeration temperature some Enterobacteriaceae
Enterobacteriaceae can continue to grow (Mossel and Zwart, 1960). These so-called psychrotrophic Enterobacteriaceae are widely distributed and can therefore be found in a variety of foods including milk, meat and poultry. Depending on the substrate growth of coliforms, including *E. coli* and *Ent. aerogenes* can occur below 5°C (Michener and Elliott, 1964). Multiplication of coliforms in milk stored at 3–5°C has been reported (Panes and Thomas, 1959) and in meats, strains of coliforms have been reported to grow between –1.5°C and +1.5°C, although, unlike true psychrophiles the optimum growth temperature of the majority of these strains was nearer 37°C than 30°C (Eddy and Kitchell, 1959).

The ability of these bacteria to multiply in certain foods, even under chilled storage, makes interpretation of results more difficult because the numbers present in these foods may not always reflect the initial level of contamination. Moreover, in certain foods, particularly raw meats, both the Enterobacteriaceae and coliforms may be of limited value as indicators of faecal contamination. This is because of the ability of psychrotrophic strains to multiply and because these groups include bacteria that are not always of faecal origin and, unlike faecal coliforms, many do not grow at 44°C (Eddy and Kitchell, 1959; Newton, 1979; Shaw and Roberts, 1982). By comparison, *E. coli* is generally of faecal origin and growth of this organism under refrigeration conditions remains minimal, therefore making this a suitable indicator of faecal contamination in refrigerated raw meat (Shaw and Roberts, 1982; Harris and Stiles, 1992) as well as in fresh poultry stored at 6°C (Zeitoun *et al.*, 1994).

In perishable foods, such as those with a pH of over 4.5, faecal indicator organisms will continue to multiply unless unfavourable conditions prevail (Buttiaux and Mossel 1961). Enterobacteriaceae including coliforms typically grow over a pH range of 4.4 to 9.0. However, some strains of *E. coli* O157:H7 are reported to grow at pH levels below pH 3.5 and can therefore pose a potential safety risk in some acidified foods such as fermented meats (Duffy and Garvey, 2001). Significant levels of Enterobacteriaceae in some chilled foods may indicate temperature abuse or improper storage, but care should be taken interpreting such data because of the possibility that psychrophilic Enterobacteriaceae strains have multiplied. In factory environments some strains including species associated with faecal contamination can establish themselves among resident flora and even multiply under suitable conditions (Cox *et al.*, 1988). This is particularly true of vegetable processing plants where these bacteria can be brought into the factory environment on raw materials or attached soil. Consequently, the hygiene significance of these bacteria should be put into context to avoid misleading conclusions, although bacteria should be adequately removed by effective cleaning and disinfection procedures.

The presence of faecal coliforms such as *E. coli* can indicate faecal contamination, especially in foods such as shellfish, fish and certain raw vegetables that have been exposed to contamination from faeces, sewage or
untreated water (Cox et al., 1988; Baylis and Petitt, 1997). Moreover, although *E. coli* can be a valuable index organism, it may not persist for as long as some pathogens in certain environments or foods and therefore the correlation between the presence of this bacterium with other pathogens, including viruses, may be poor, so again interpretation of microbiological data should be made with care (Baylis and Petitt, 1997).

### 22.8 Methods for the detection and quantification of Enterobacteriaceae in foods

The testing of foods for total Enterobacteriaceae, coliforms and *E. coli* has become widely adopted because of their value as indicator organisms and because tests for these bacteria are relatively inexpensive, simple to perform and yield rapid results, often after 24h. Testing for enteric pathogens such as *Salmonella* requires specific methods which are often labour intensive and can take several days to obtain results. Furthermore, pathogenic bacteria in food are often not homogeneously distributed and are present in low numbers, making detection difficult. Many food production sites would also prefer not to isolate enteric pathogens in their on-site laboratory but would instead have testing performed by an external laboratory. For these reasons, testing for index organisms such as *E. coli* is routinely used to provide a convenient assessment of potential faecal contamination. In addition to their indicator function, Enterobacteriaceae and coliforms may be responsible for spoilage of certain foods and tests would be performed to establish their role under these circumstances.

There are numerous published standard methods for Enterobacteriaceae, coliforms and *E. coli* in foods including those published as International Organization for Standardization (ISO) methods (de Boer, 1998). A large proportion of these methods are quantitative because most food manufacturers impose acceptable limits for these bacteria, particularly Enterobacteriaceae and coliforms, although the number allowed will depend on the food. For processed foods or certain high-risk foods one would not expect to find these bacteria and in these situations zero tolerance may be imposed and a detection method used. For quantitative tests the most popular methods are direct plating using pour or spread plate techniques and the MPN technique.

A characteristic feature of the Enterobacteriaceae and coliforms, which is used as a diagnostic feature in many tests for their detection and enumeration, is their ability to produce acid and gas from the fermentation of glucose and lactose, respectively. This characteristic is exploited by most conventional culture methods as well as some proprietary methods, which are described later. Most media use bile salts to inhibit Gram-positive bacteria and other Gram-negative bacteria that are not bile tolerant, unlike members of the Enterobacteriaceae. Two media containing bile and the dye violet red, which have become the most popular media for examining foods for
Enterobacteriaceae and coliforms, are violet red bile glucose agar (VRBGA) and the lactose containing violet red bile agar (VRBA), respectively. Both media are variants of the MacConkey agar, developed by MacConkey for the detection of bile-tolerant Gram-negative bacteria.

A pour plate technique is normally used with both VRBA and VRBGA and plates are incubated aerobically for 24 h at 37°C and inspected for purple-red colonies surrounded by a purplish halo. These typical colonies are the result of glucose or lactose fermentation, which produces acid that is detected in the medium by the pH indicator neutral red. If the purpose of the test is to include psychrotrophic coliforms or Enterobacteriaceae, the incubation temperature may be lowered to 30°C, although if the test is being used as a hygiene indicator, 37°C is probably the preferred temperature. An overlay is recommended to ensure fermentation of the carbohydrates and reduce the likelihood of oxidation as well as improving the specificity of these media and reducing interference from background flora or motile strains. However, bacteria other than Enterobacteriaceae are also capable of growing on VRBA and VRBGA, particularly *Aeromonas* spp., although these bacteria are oxidase positive which enables them to be distinguished from Enterobacteriaceae, and other bacteria generally appear smaller or exhibit colony morphologies that are atypical compared with the target organisms. Consequently, most laboratories would regard the results from both tests as presumptive and, if necessary, further confirmation tests would be performed. These can include production of gas at 37°C in liquid media such as brilliant green bile broth (BGBB) (Anon., 1978) or subjecting a number of typical colonies to biochemical tests to give an identification, which has been incorporated into the ISO colony count method for Enterobacteriaceae which uses VRBGA (Anon., 2004b).

Liquid media can also be used for the detection and enumeration of Enterobacteriaceae, coliforms and *E. coli*. A common approach is to use the MPN technique using multiple tubes filled with an appropriate medium. An alternative to bile salts is to use detergents such as lauryl sulphate, which is used in lauryl sulphate tryptose broth (LSTB) for detecting and enumerating coliforms (Anon., 1991). As with plating media the fermentable carbohydrates used are glucose for Enterobacteriaceae and lactose for *E. coli* and other coliforms. Both carbon sources yield acid that is detected by the indicator system in the medium, resulting in colour change, and gas production can be confirmed by collecting emitted gas in an inverted Durham tube placed in the tube containing the medium. Different sets of tubes, typically three or five, are inoculated with successive dilutions of the food under examination and tubes are inspected for turbidity and acid and/or gas production, depending on the medium and diagnostic system. Tubes are inspected after 24 h and, if necessary, 48 h incubation which is normally at 37°C, although 30°C can be used for psychrotrophic strains. Using the results from the positive tubes, the estimated number of target organisms can be calculated by referring to MPN tables.
Liquid media commonly used to test for coliforms include MacConkey broth, BGBB and LSTB. For detecting and enumerating Enterobacteriaceae in foods a method that employs pre-enrichment in buffered peptone water followed by enrichment in buffered brilliant green–bile–glucose broth (EE broth) and streaking onto selective media was developed (Mossel, 1963). This detection method together with its use as an enumeration method employing the MPN technique has become an ISO method (Anon., 2004a). Owing to its importance as an indicator of faecal contamination and because some strains are themselves pathogenic, many specific media and tests have been developed for \textit{E. coli}. Many of these tests employ the same or similar media developed for coliforms with additional tests performed to confirm the presence of \textit{E. coli}. These tests include acid or gas production at 44°C and production of indole from tryptophan which is typical of biotype I \textit{E. coli} and a feature of up to 98% of \textit{E. coli} strains (Farmer \textit{et al.}, 1985). However, general tests for \textit{E. coli} may not be suitable for pathogenic strains of \textit{E. coli} which require specialist tests (Baylis \textit{et al.}, 2006) and because of its importance as a pathogen methods have been developed specifically for detecting \textit{E. coli} O157:H7 and for other Shiga-toxin producing \textit{E. coli} (STEC) in foods (Baylis \textit{et al.}, 2001).

A period of pre-enrichment in or on a non-selective medium is particularly useful for processed or dry foods where any bacteria present could be sublethally injured and therefore direct enrichment in selective media would be inappropriate. This consideration is particularly important because some methods for coliforms and \textit{E. coli} were developed for water testing so their use with foods directly may be inappropriate. This is particularly true of methods for \textit{E. coli} and faecal coliforms which commonly use elevated incubation temperatures such as 44°C, which gives better selectivity for faecal coliforms including \textit{E. coli}. Under these circumstances incubating food samples in selective media directly at 44°C may result in the failure to recover and detect the target organisms. For this reason tests for \textit{E. coli}, especially those using liquid media, often involve performing a test for coliforms at the lower temperature, for example LSTB at 37°C and then performing a sub culture into medium such as \textit{E. coli} (EC) broth which is then incubated at 44°C and inspected for gas production for up to 48h. Besides gas production, methods for \textit{E. coli} also include a test for indole production at 44°C, although the presence of β-glucuronidase has now become popular, especially with the greater acceptance of chromogenic media. By using 0.45 µm membranes to trap cells and enable physical transfer between media researchers developed a method that was suitable for detecting \textit{E. coli} in frozen and other processed foods (Anderson and Baird-Parker, 1975). This method, which subsequently became a standard (ISO) method (Anon., 1998), incorporates a short 4h growth period at a lower temperature (30°C or 37°C) on a non-selective medium which facilitates repair and recovery of injured cells, followed by transfer of the membrane to the selective bile salt containing medium tryptone bile agar (TBA), and subsequent growth at
44 °C. The presence of typtophan in the medium also enables a test for indole production to be performed.

Although many methods for Enterobacteriaceae, coliforms and *E. coli* are relatively rapid, many lack specificity. Moreover, coliforms remain an ill-defined group and this, together with the limited number of genera within the Enterobacteriaceae able to ferment lactose, has resulted in more tests now being performed for total Enterobacteriaceae. However, it should be realised that these tests will recover a greater number of bacterial species, and in raw foods this may include some *Pseudomonas* spp., which can utilise the glucose in the media, so interpretation of results must be made with care. MPN techniques often provide greater sensitivity compared with plating methods, making them suitable for estimating low-level contamination, but at high contamination levels the results can show greater variation and in some media other bacteria can give rise to false-positive results.

The specificity of many media for coliforms and *E. coli* has been improved by the incorporation of fluorogenic and chromogenic substrates into existing media and the development of chromogenic media specifically for these bacteria (Manafi, 1996). Both types of compound detect enzyme activity that is specific to the target organism or present in a large proportion of the bacteria of interest, and this can be used to improve colony identification and reduce the number of confirmation tests required. Upon cleavage by the enzyme, fluorogenic substrates yield free fluorophores that diffuse into the surrounding medium and fluorescence when illuminated under UV light at a defined wavelength. By comparison, chromogenic substrates yield chromophores that are absorbed onto the bacterial cells, thereby labelling them, and this gives rise to colonies that are a specific colour and easily distinguished from other colonies. In media for coliforms the enzyme of interest is β-D-galactosidase, which is responsible for the breakdown of lactose to galactose and glucose. This enzyme is commonly detected using substrates such as *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL). For *E. coli* the target enzyme is β-D-glucuronidase (GUD) which catalyses the hydrolysis of β-D-glucopyranosiduronic acids to their corresponding aglycons and D-gluconic acid (Manafi, 1996). This enzyme activity is reported to be present in ca 97% of *E. coli* (Kilian and Bülow 1976), but is also found in other members of the Enterobacteriaceae, notably some strains of *Salmonella* and *Shigella* as well as strains of *Hafnia alvei* and other genera (Hartman, 1989; Baylis and Patrick, 1999). Moreover, while the majority of *E. coli* O157:H7 strains are GUD negative, this characteristic is often used to distinguish this pathogen from other *E. coli* strains (Baylis *et al.*, 2001). To detect GUD activity, popular fluorogenic and chromogenic substrates are 4-methylumbelliferyl-β-D-glucuronide (MUG) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG), respectively. The latter is now used in a chromogenic medium based on TBA called tryptone bile x-glucuronide agar (TBX), which is now incorporated into two standard (ISO) methods for enumeration of *E. coli* from foods (Anon., 2001a,b).
By using a cocktail of fluorogenic and chromogenic substrates, a medium was developed for the rapid presumptive identification of different members of the Enterobacteriaceae (Manafi and Rotter, 1991), although the popularity of these compounds remains with methods for coliforms and E. coli, although media are now being developed for a range of bacteria including the important pathogens (Manafi, 2000). Moreover, for routine tests chromogenic media have become more popular than fluorogenic ones as they require no additional equipment, such as a UV lamp to read the results. Furthermore on solid media containing fluorogenic substrates large numbers of fluorescing colonies can be difficult to distinguish from non-fluorescing ones and in liquid media diffusion of fluorescence into the surrounding medium can make interpretation more difficult.

Despite some of these drawbacks fluorogenic and chromogenic compounds have become popular and there are now various proprietary media and tests available, including media incorporating substrates to GUD and β-D-galactosidase which enable simultaneous enumeration and differentiation of E. coli and total coliforms on the same plate. Commercial tests that utilise fluorogenic or chromogenic substrates for the enumeration of Enterobacteriaceae, E. coli and other coliforms are now widely available. A good example is the range of tests available as Petrifilm (3M Health Care) which includes Petrifilm coliform count plates (Blackburn et al., 1996; Park et al., 2001), Petrifilm E. coli count plate (Matner et al., 1990) and more recently Rapid Coliform Count plate (Kinneberg and Lindberg 2002) and Petrifilm Enterobacteriaceae Count plate (Silbernagel and Lindberg, 2002, 2003). These tests comprise ready-made rehydratable selective media under a clear film. The medium is hydrated by the addition of the sample and after incubation visible colonies can be counted. The film traps gas produced from fermentation, which provides an additional diagnostic test. Another commercial system which incorporates fluorogenic or chromogenic substrates but uses an MPN style approach, termed binary detection technology by the manufacturer, is SimPlate (BioControl Inc.). After inoculating the sample into the test medium, which may contain a fluorogenic or chromogenic substrates, depending on the test, this is distributed among 100 individual wells of a SimPlate device. After incubation the numbers of positive wells are counted and the number of target bacteria calculated from a conversion table. SimPlate tests are available for coliforms, E. coli (Townsend et al., 1998) and total Enterobacteriaceae (see Fig. 22.1).

Besides fluorogenic and chromogenic media other approaches to traditional colony count and MPN techniques have been developed. Techniques that rely on impedance or conductance have become popular. These methods are based on metabolic activity of the chosen bacteria in a defined medium, resulting in changes in the electrical conductance of the medium, which is detected by the system. Impedance enumeration relies on detection time being inversely related to sample contamination and with careful calibration of bacterial counts against impedance detection time values, the numbers of
target organisms in a sample can be estimated. Various commercial systems are now available for impedance enumeration of microorganisms, including Enterobacteriaceae, coliforms and *E. coli* (Cousins and Marlatt, 1990; Wawerla *et al.*, 1999). The BioSys Optical System or MicroFoss is a commercial system that is based on a similar principle to impedance but instead, it measures colour change in an optical measurement area caused by the growth and metabolism of the target organisms. This can be the result of pH changes or other biochemical reactions in the medium that bring about a developing colour change, which in turn can be related to number of organisms. As with impedance the numbers are calculated from detection times by comparison against calibration curves produced previously. Tests using this system are available for a variety of organisms including coliforms and *E. coli* (Odumeru and Belvedere, 2002; Firstenberg-Eden *et al.*, 2004).

With the introduction of new technologies, particularly molecular methods, it is no surprise that tests for Enterobacteriaceae, *E. coli* and other coliforms will become more rapid, automated and specific in future. At present techniques such as PCR, which can amplify and detect specific DNA sequences associated with a particular genus, species or group of bacteria, are focused on detection of specific pathogens. PCR tests using universal primers targeted against the Enterobacteriaceae 16S rRNA gene for total Enterobacteriaceae in foods have been developed (Nakano *et al.*, 2003), together with rapid same-day tests to enumerate Enterobacteriaceae in foods using fluorescent *in situ* hybridisation (FISH) technique and Enterobacteriaceae specific probes targeted against 16S rRNA (Ootsubo *et al.*, 2003). Given the specificity of molecular methods, these tests can be easily developed to detect specific genera or species and even genes associated with production of toxins or enzymes. Consequently, some of these tests could be developed to detect bacteria responsible for specific types of spoilage. Diagnostic media have been
developed for the detection and isolation of histamine-producing bacteria (Actis et al., 1999; Mavromatis and Quantick, 2002), including those belonging to the Enterobacteriaceae (see below), although these sometimes yield false positive results.

22.9 Food spoilage by members of the Enterobacteriaceae

The contribution of members of the Enterobacteriaceae to food spoilage has been largely overlooked because of the attention given to the clinical importance of some members of this family. The exception would be Erwina spp. and other genera that are of importance to the agriculture industry, although the economical losses due to food spoilage are now also being realised in the food industry.

Food spoilage involves a combination of microbial, biochemical and chemical activities and the complex interaction of these factors, together with intrinsic and extrinsic factors associated with the particular food. The interactions involved in food spoilage and the foods affected has been the topic of previous reviews (Mossel and Ingram, 1955; Huis in’t Veld, 1996; Jay, 2000). This section will highlight some of the common types of spoilage associated with the Enterobacteriaceae and some of the foods affected. However, for information on specific types of food spoilage, including those involving the Enterobacteriaceae, the reader is advised to consult the relevant chapters in this book. Some of the common food spoilage characteristics directly attributed to Enterobacteriaceae are presented in Table 22.3.

22.9.1 Milk and dairy products

In refrigerated milk, growth of psychrotrophic bacteria, including some strains of Enterobacteriaceae, can result in production of enzymes such as heat-stable lipases and proteinases both of which are able to withstand high temperature–short time (HTST) and ultra-heat (UHT) treatments (Griffiths et al., 1981), which can break down casein and impart off-flavours. The type of off-flavour commonly caused by Enterobacteriaceae in milk is bitterness. This is the result of proteinase activity that results in the formation of peptides, which impart a bitter taste to the milk. These detectable changes generally occur when the population exceeds $10^6$ cfu/ml, and therefore the time this occurs will depend on the initial bacterial load and storage temperature. As well as flavour defects, some Enterobacteriaceae, notably Serratia spp., can give rise to a reddish pigment, while other bacteria may cause other colour defects (ICMSF, 1998).

To prevent flavour defects in milk it is essential that the storage temperature does not enable rapid multiplication of Enterobacteriaceae. The thermostability of lipases and proteinases means that HTST and UHT will not inactivate these enzymes. Prevention of post-process contamination and proper plant hygiene and disinfection are therefore essential to avoid spoilage.
<table>
<thead>
<tr>
<th>Food</th>
<th>Type of spoilage</th>
<th>Cause</th>
<th>Bacterial genera or species involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh vegetables</td>
<td>Soft rot</td>
<td>Formation of pectic enzymes</td>
<td>Erwinia spp., Erwinia carotovora (now Pectobacterium carotovorum)</td>
</tr>
<tr>
<td>Potatoes</td>
<td>‘Black leg’</td>
<td>Enzymatic breakdown</td>
<td>Pectobacterium carotovorum subsp. Pectobacterium carotovorum subsp. atrosepticum, Pectobacterium chrysanthemi (note all previously Erwinia spp.)</td>
</tr>
<tr>
<td>Vacuum packed bacon</td>
<td>Cabbage odour</td>
<td>Methane thiol produced by metabolic activity</td>
<td>Erwinia spp., Erwinia carotovora (now Pectobacterium carotovorum)</td>
</tr>
<tr>
<td>Dry cured meats</td>
<td>‘Bone taint’</td>
<td>Metabolic activity and the production of volatile compounds</td>
<td>Pectobacterium carotovorum subsp. Pectobacterium carotovorum subsp. atrosepticum, Pectobacterium chrysanthemi (note all previously Erwinia spp.)</td>
</tr>
<tr>
<td>Meat and poultry</td>
<td>Off-odours</td>
<td>Metabolic activity and the production of volatile compounds</td>
<td>Various Enterobacteriaceae, especially Serratia spp. and Proteus spp.</td>
</tr>
<tr>
<td>Fish (especially lightly</td>
<td>Off-flavours and</td>
<td>Reduction of trimethylamine oxide (TMAO) and formation of trimethylamine (TMA).</td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>preserved and vacuum</td>
<td>off-odours</td>
<td>Metabolic activity and the production of various volatile compounds</td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>packaged fish)</td>
<td></td>
<td></td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>Fish and some other</td>
<td>Histamine</td>
<td>Decarboxylase activity (histidine to histamine)</td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>products (e.g. cheese)</td>
<td></td>
<td></td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>Meats</td>
<td>Cadaverine</td>
<td>Decarboxylase activity (lysine to cadaverine and ornithine or arginine to putrescine)</td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>Putrescine</td>
<td>Metabolic activity and enzymatic breakdown</td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proteus Enterobacter, Escherichia</td>
</tr>
<tr>
<td></td>
<td>Slime formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food</td>
<td>Type of spoilage</td>
<td>Cause</td>
<td>Bacterial genera or species involved</td>
</tr>
<tr>
<td>-----------------------</td>
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<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Hard cheeses (e.g. cheddar)</td>
<td>Gas (early blowing)</td>
<td>Fermentation of lactose and production of gas</td>
<td><em>E. coli</em> and other members of the Enterobacteriaceae</td>
</tr>
<tr>
<td>Hard cheeses (e.g. cheddar)</td>
<td>Off-flavours</td>
<td>Metabolic activity and the production of volatile compounds</td>
<td>Various Enterobacteriaceae</td>
</tr>
<tr>
<td>Coleslaw</td>
<td>Gas production and off-odours (strong faecal smell)</td>
<td>Fermentation and metabolic activity</td>
<td><em>Klebsiella, Enterobacter, Hafnia</em> and <em>Citrobacter</em></td>
</tr>
<tr>
<td>Beer spoilage</td>
<td>Suppress fermentation Off-odours (characteristic parsnip-like or fruity odour)</td>
<td>Metabolic activity and the production of volatile compounds (increased level of dimethyl sulphide, dimethyl disulphide, diacetyl and fusel oils)</td>
<td><em>Obesumbacterium proteus</em></td>
</tr>
<tr>
<td>Shell eggs</td>
<td>Bacterial rots</td>
<td>Enzymatic (proteolytic) breakdown of albumin and blackening the yolk (black rot)</td>
<td><em>Proteus, Enterobacter</em> (black rot), <em>Serratia</em> (red rot)</td>
</tr>
</tbody>
</table>
Members of the Enterobacteriaceae are also associated with spoilage of cheeses. In hard cheeses such as cheddar, *E. coli* and other Enterobacteriaceae can be responsible for off-flavours caused by the production of various volatile compounds (Morales *et al.*, 2004) and early blowing caused by gas production from the fermentation of lactose (ICMSF, 1998). In cottage cheese, *Proteus, Escherichia* and *Enterobacter* are associated defects including a condition termed ‘slimy curd’ (Cousin, 1982; Jay, 2000).

### 22.9.2 Meat, poultry and associated products

In fresh meats Enterobacteriaceae form a small proportion of the spoilage flora. Under refrigeration conditions growth of some bacteria is retarded whereas psychrotrophic strains, including some Enterobacteriaceae, can multiply and may eventually cause spoilage. Fresh meats have an ultimate pH of *ca* 5.6 and contain sufficient carbohydrates, including glucose, to support growth of bacteria up to levels of *ca* cfu/cm² (Gill, 1976). Initially the glucose is attacked but when the supply of simple carbohydrates available is exhausted, the bacteria begin to utilise free amino acids and related simple nitrogenous compounds. This generally occurs when the bacterial population reaches a maximum level and at this point noticeable odours and spoilage become apparent. Foul odours are generally associated with the breakdown of amino acids to yield hydrogen sulphide (H₂S), which is a product of sulphur-containing amino acids, and ammonia (NH₃) from many amino acids and indole from tryptophan (Jay, 2000).

In meat and poultry growth of Enterobacteriaceae is generally favoured by storage at 5 °C and above rather than 0 to 1 °C. In one study refrigerated poultry has been shown to permit only slow growth of some Enterobacteriaceae such as *Citrobacter* and *Proteus* and in pure culture *Proteus* has been shown to cause off-odour on chicken muscle stored at 5°C and this genus may therefore contribute to spoilage in competition with psychrotrophic bacteria (McMeekin and Patterson, 1975).

Packaging under modified atmospheres (MAP) provides an effective way of retarding growth of a range of bacteria and extending the shelf-life of certain foods, although Enterobacteriaceae are reported to be more numerous on MAP than on vacuum packed meat, especially pork (Dainty and Mackey 1992). Various gas combinations have been used but 60–80% oxygen and 20–40% carbon dioxide are common mixtures. While storing meats in reduced or oxygen-free atmospheres can help to retard the growth of some bacteria and extend product shelf-life, Enterobacteriaceae are less affected. On lamb chops stored in low-O₂ and O₂-free atmospheres, growth of Enterobacteriaceae is reported to be unaffected by the presence of CO₂ and consequently growth of these facultatively anaerobic bacteria increased from undetectable levels to finally constitute 10–30% of the flora on these products (Newton *et al.*, 1977).
Packing meat and poultry under vacuum is also used to extend shelf-life and is effective when used in combination with refrigerated storage. In CO₂-packaged chicken, stored at 3°C or −1.5°C, Enterobacteriaceae have been shown to be inhibited or retarded by the conditions that favoured growth of lactobacilli, whereas Enterobacteriaceae can predominate and cause putrid odours in vacuum packaged chicken (Gill et al., 1990). Similar findings have been reported for vacuum packaged beef and it has also been observed in this type of meat that Hafnia alvei can become the dominant Enterobacteriaceae at 4°C whereas at −1.5°C Serratia liquefaciens is dominant (Borch et al., 1996). In vacuum-packed bacon Enterobacteriaceae are responsible for putrefactive spoilage, characterised by production of H₂S and methanethiol (Gardner, 1983). The latter is an odiferous compound that has been described as ‘cabbage odour’ spoilage which has been reported to be caused by Proteus inconstans (Gardner and Patterson, 1975). In dry cured hams Enterobacteriaceae, particularly Serratia spp. and Proteus spp., may be a major cause of ‘bone taint’. This is characterised by a foul-smelling odour that may be caused by the volatile compounds that arise from the metabolism of these bacteria and can result in higher amounts of ketones, alcohols and esters being produced, which contribute to this type of spoilage (Garcia et al., 2000).

### 22.9.3 Fish and seafoods

Spoilage of fresh-water and salt-water fish and shellfish can be caused by a variety of bacteria, although fresh fish stored on ice is particularly susceptible to bacterial spoilage. Contributing factors of fish flesh include its high post-mortem pH (>6.0) and the non-protein nitrogen (NPN) fraction, which provides free amino acids and other nitrogen-containing compounds for bacterial growth (Gram and Huss, 1996). Two most important characteristics, which are characteristic of fish spoilage bacteria, are their ability to produce H₂S and to reduce trimethylamine oxide (TMAO), which is part of the NPN fraction of all marine and some fresh-water fish, to trimethylamine (TMA). With the exception of Erwinia and some species of Shigella, most of the species of Enterobacteriaceae can reduce TMAO to TMA (Barrett and Kwan, 1985). Despite this, the most common spoilage organisms associated with iced fresh fish are Gram-negative psychrophilic and psychrotrophic bacteria such as Pseudomonas spp. and Shewanella putrefaciens.

Although Enterobacteriaceae may be found among the spoilage flora of fish stored on ice (Papadopoulous et al., 2003; Chytiri et al., 2004) their role in spoilage of chilled wet fish is likely to be insignificant compared with other spoilage bacteria (Gram et al., 1987). However, in lightly preserved fish products containing preservatives or low levels of salt and typically stored chilled and packaged under vacuum, Enterobacteriaceae can become part of the dominant spoilage flora, especially if the fish is stored at temperatures above 10°C.
In herring extracts stored anaerobically at 15°C Enterobacter spp. and Proteus spp. have been shown to reduce TMAO to TMA which is assisted by H₂ produced from fermentation processes (Strøm and Larsen, 1979). Enterobacteriaceae have been shown to grow on herring fillets under aerobic conditions, especially in nitrogen when they have been shown to become part of the dominant microflora (Molin et al., 1983), whereas in 100% CO₂ their growth is retarded and this inhibition becomes more pronounced when the storage temperature is lowered to 0 to 4°C (Molin and Stenström, 1984). As well as TMA, Enterobacteriaceae can produce a range of other volatile compounds that can contribute to off-odours in fish. One such compound is disulphide dimethyl which, together with TMA, is produced by Enterobacteriaceae in cold-smoked salmon yielding off-odours (Joffraud et al., 2001).

22.9.4 Fruit and vegetables
Strains of Erwinia are perhaps the most important bacterial agents responsible for spoilage of fresh vegetables post-harvest. The main type of spoilage by these bacteria is soft rots caused by pectic enzymes that break down pectins, resulting in a characteristic mushy appearance which is sometimes accompanied by a bad odour and water-soaked appearance. These enzymes are produced by a range of bacteria, notably Erwinia spp., but especially Pectobacterium (Erwinia) carotovora, although some pseudomonads such as Pseudomonas marginalis and to a lesser extent some coliform bacteria can also be responsible for soft rotting of vegetables. This type of spoilage can affect a wide range of vegetables including onions, garlic, asparagus, green beans, carrots, parsnips, celery, lettuce, cabbage, potatoes, spinach, Brussels sprouts, cauliflower, cucumbers, peppers, etc. (Jay, 2000). Certain species of Erwinia, many of which now belong to the genus Pectobacterium, also cause a rot in potatoes known as ‘black leg’ and they may even cause rot of pears despite the low pH (3.8 to 4.6) of this fruit, although the pH may be lower at the surface (Kraft, 1992). These bacteria are well adapted to growth on vegetables because, unlike many of the common bacteria, they are capable of fermenting many of the sugars and alcohols that exist in certain vegetables (Jay, 2000).

As well as rots, members of the Enterobacteriaceae, together with other bacteria and yeasts, can be responsible for gas production and blowing the lids off pre-packed mayonnaise-based salad products. Gas production can also occur in coleslaw, together with off-odours which are sometimes described as a strong faecal smell. Members of the Enterobacteriaceae involved in this type of spoilage include the genera that are typically found in soil and on plant material such as Klebsiella, Enterobacter, Hafnia and Citrobacter. The origin of these bacteria is often the cabbage component of the coleslaw (Sutherland et al., 1986).
22.9.5 Other foods
Enterobacteriaceae have been associated with the spoilage of a range of other foods and drinks, although many of these have not been extensively studied and their exact role in the spoilage of these products remains unclear. Examples include beer spoilage by *Obesumbacterium proteus*, spoilage of maple syrup by various members of the Enterobacteriaceae, spoilage of fresh cream desserts by *Serratia* spp. and other members of the Enterobacteriaceae, which is characterised by clotting cream (from acid production) and gas production (Sutherland *et al.*, 1986). In shell eggs Enterobacteriaceae are responsible for bacterial rots, with *Enterobacter* and *Proteus* associated with causing black rots and *Serratia* with red rots (ICMSF, 1998).

22.10 Production of biogenic amines including histamine by Enterobacteriaceae
Biogenic amines can be formed as a consequence of normal metabolic activity in plants, animals and microorganisms. These low molecular weight organic bases possess biological activity, sometimes causing illness if consumed at high levels, and are commonly produced by the decarboxylation of amino acids via different metabolic pathways (Halasz *et al.*, 1994). The formation of amines by bacteria is influenced by temperature, oxygen supply and pH as all these factors will affect growth and enzyme activity of the microorganisms of concern. Under favourable conditions such as those that can occur during spoilage of foods, increased decarboxylase activity in certain microorganisms can result in raised levels of biogenic amines. Not only can this serve as a useful indicator of spoilage but ingestion of large amounts of these compounds can present a food safety risk. The biogenic amine most frequently associated with foodborne intoxications is histamine.

Although some members of the Enterobacteriaceae are pathogens and others can cause spoilage, one aspect often overlooked is the histidine decarboxylase activity of certain members of this family. Bacteria that decarboxylate histidine, an amino acid abundant in the muscle of fish belonging to the families Scomberesocidae and Scombridae, such as mackerel and tuna, etc., can produce substantial amounts of histamine, commonly referred to as scombroid toxin. Fish belonging to other families for example Coryphaenidae (mahi-mahi) and Clupeidae (sardines and herrings) may also be affected, as well as other foods such as cheese.

In spoiled fish, or fish that has been temperature abused, multiplication of bacteria that possess histidine decarboxylase activity can result in the production of high levels of histamine. Consumption of fish or other foods containing high levels of histamine results in scombroid poisoning which is an allergic-type reaction to high levels of histamine. The most common reported symptoms include rash, flushed skin, facial swelling, nausea, vomiting, diarrhoea, dizziness
and headache. The victim may also report a peppery taste in the mouth, burning throat, stomach pain and itchy skin or palpitations. The onset of symptoms may be immediately or several hours after consumption of the contaminated food. Duration is typically a few hours although in certain cases it can be several days and require the administration of antihistamines.

Histidine decarboxylase activity is present in a number of bacteria although in incidents of scombroid poisoning involving fish, members of the Enterobacteriaceae are the dominant histamine-producing bacteria. Histamine-producing isolates identified in studies of temperature abused or spoiled fish include *Morganella morganii*, *Hafnia alvei*, *Citrobacter freundii*, *Proteus mirabilis*, *Klebsiella* spp., *Enterobacter* spp., including *Enterobacter aerogenes*, and *Serratia* spp. (Yoshinaga and Frank, 1982; Kim *et al.*, 2001). In these and other studies Enterobacteriaceae were reported to be responsible for producing large quantities of histamine and the main species were identified as *M. morganii*, *Ent. aerogenes* and *Klebsiella pneumoniae*. The latter, which has been implicated in an outbreak of scombroid fish poisoning (Taylor *et al.*, 1979), was identified as a histamine producer in the past, together with strains of *K. planticola*, *K. ornithinolytica* and *K. oxytoca*. However, genetic analysis and further studies with histamine-producing strains previously identified as *Klebsiella pneumoniae* or *K. oxytoca* has now revealed these to be *Raoultella planticola*, formerly classified as *Klebsiella planticola*, and this species, together with *Raoultella ornithinolytica*, which formerly belonged to the genus *Klebsiella*, represent histamine-producing strains, whereas true strains of *Klebsiella pneumoniae* and *K. oxytoca* have been shown to produce no histamine (Kanki *et al.*, 2002).

An investigation with 693 strains of Enterobacteriaceae belonging to different genera and species confirmed histidine decarboxylase activity in all strains of *M. morganii*, *Ent. aerogenes* and *Raoultella ornithinolytica* and in some strains of *Raoultella planticola* and *Citrobacter youngae* (Wauters *et al.*, 2004). The species associated with a high level of histamine formation in fish is *M. morganii* (Kim *et al.*, 2001), which has been shown to produce large amounts of histamine at low temperature (0–5 °C) following storage at elevated temperatures (10–25 °C) (Klausen and Huss 1987). This occurs because histamine production continues following growth at 25 °C, which is reported to be the optimum temperature for production of histamine and other amines in this bacterium (Kim *et al.*, 2000). In Indian anchovy prolific histamine formers have been identified as *M. morganii*, *Ent. aerogenes* and *Proteus vulgaris*, with the optimum temperature for histamine production of these strains reported to be 35 °C (Rodtong *et al.*, 2005).

Besides fish, another food implicated in histamine poisoning is cheese (Taylor *et al.*, 1982), although bacteria besides Enterobacteriaceae, notably lactobacilli, are often responsible for the raised levels of histamine in cheese (Halasz *et al.*, 1994). In addition to histidine members of the Enterobacteriaceae can decarboxylate a number of other amino acids to amines, for example tyrosine and lysine to tyramine and cadaverine, respectively, and arginine
and ornithine to putrescine. In a study of 104 cheese-associated Enterobacteriaceae species all strains were shown to decarboxylate at least two amino acids, with most strains producing high amounts of cadaverine from lysine (Marino et al., 2000). Putrescine, tyramine and histamine were produced by 96%, 68% and 58% of strains, respectively and a relationship between high concentrations of cadaverine and high numbers of Enterobacteriaceae was demonstrated. Producers of the highest amount of cadaverine (2279 ppm) and histamine (18 ppm) were Ent. aerogenes, for putrescine it was Ent. cloacae (218 ppm) and Cit. freundii (205 ppm) and for tyramine it was Arizona spp. (S. Arizonae) (121 ppm). However, it has been reported that biogenic amine production by bacteria in synthetic media may be strain dependent rather than related to specific species (Bover-Cid et al., 2001).

In fermented sausages the Enterobacteriaceae are responsible for cadaverine and putrescine production (Bover-Cid et al., 2001; Suzzi and Gardini, 2003). These bacteria have also been reported to produce significant amounts of these biogenic amines in chilled-stored pork and beef (Slemr, 1981) and chilled vacuum packed beef (Edwards et al., 1987). Consequently the presence of high levels of biogenic amines in certain foods can be used as potential indicators of spoilage.

22.11 Prevention and control of spoilage by Enterobacteriaceae

Research has shown that growth of Enterobacteriaceae involved in spoilage is affected by the interaction between different factors, for example temperature, salt concentration and pH, and it is the combination of these that has a greatest effect on preventing growth compared with each factor individually (Davis et al., 1997). Storage temperature is perhaps one of the most important factors that can retard growth and prevent or delay spoilage. Freezing is particularly effective at preventing multiplication and spoilage and this has been used with great effect for the preservation of a wide variety of foods, including meats, fruits, vegetables and fish, etc. The presence of psychrotrophic Enterobacteriaceae, however, does mean that refrigeration may not always prevent growth and multiplication of some strains which could subsequently cause spoilage.

Irrespective of the food involved, preventing initial contamination by Enterobacteriaceae, avoiding post-process contamination and preventing their growth are all important primary factors that can prevent subsequent spoilage by these bacteria. Ensuring proper hygiene during food production and implementing effective cleaning procedures are critical elements that contribute to preventing spoilage by Enterobacteriaceae. The adoption of quality assurance (QA) approaches and the proper implementation and maintenance of Hazard Analysis Critical Control Point (HACCP) systems has become an essential
part of preventing microbiological contamination, including the prevention of bacterial spoilage. Formal microbiological risk assessment (MRA) has also become a useful tool for improving food safety and quality as part of HACCP and other risk-based approaches used by food manufacturers (Brown and Stringer, 2002). Although HACCP and MRA are generally associated with ensuring food safety, the principles and impact of these systems should give the added benefit of reducing the risk from food spoilage organisms.

In heat-processed foods such as UHT products, spoilage is normally uncommon and often the result of post-process contamination. As Enterobacteriaceae are not particularly heat resistant, preventing post-process contamination and ensuring correct performance of equipment as well as monitoring and verifying the effectiveness of a process or treatment are vital. In these situations Enterobacteriaceae provide an important function as indicator organisms. Compared with many other bacteria the Enterobacteriaceae are not always the predominant spoilage flora in certain foods, however, given the right conditions they can multiply to become either the dominant population responsible for spoilage or a major contributor to flavour and other defects together with other spoilage bacteria. Refrigeration provides a method of preventing spoilage by a large proportion of Enterobacteriaceae in some foods, particularly meat and poultry and in milk and dairy products. However, if the storage temperatures increase or a product is subjected to temperature abuse at temperatures above 10°C multiplication of a wider range of Enterobacteriaceae can occur, with rapid onset of spoilage. Growth of psychrotrophic bacteria is more difficult to control, except by ensuring proper cleaning and disinfection of equipment and the processing environments.

Various alternative methods for preventing spoilage by Enterobacteriaceae have been investigated. Besides adjusting the intrinsic and extrinsic factors such as altering gaseous environments by MAP, adjusting the pH and the use of preservatives and other processing aids, alternative approaches have included the competitive exclusion by other bacteria such as lactic acid bacteria and the lactoperoxidase system in milk (Cousin, 1982). High-pressure treatment of foods appears to show potential although it has limitations. While it has been shown to increase the shelf-life of squid when used at 400 megapascal (MPa), pressures above this affected sensory characteristics and Enterobacteriaceae appear to possess a relatively high piezotolerance (Paarup et al., 2002). Chemical treatments, although effective, are not always permitted under current legislation for certain foods such as raw meats and they are often considered undesirable by consumers. In one study vacuum packaging did not suppress the growth of Enterobacter spp. which was contributing to the psychrotrophic spoilage of minced beef, whereas the addition of 0.5% ascorbic acid, which reduces pH and oxidation-reduction potential (Eh), resulted in significant inhibition (von Holy and Holzapfel, 1988). In British fresh sausage sulphur dioxide, added as sodium metabisulphite, has been shown to
inhibit a range of Enterobacteriaceae at pH 7, including salmonellae (15–109 ppm), *Serratia liquefaciens*, *Serratia marcescens* and *Hafnia alvei*, which were more resistant, requiring 185–270 ppm (Banks and Board, 1982).

### 22.12 Future trends

The Enterobacteriaceae is a large genetically diverse group of bacteria that includes some important human and plant pathogens, and others that are widely distributed in nature. With the introduction and greater application of molecular-based methods it is inevitable that our understanding of these bacteria will improve and that taxonomy will change. Even now, with the use of more advanced genetic typing methods, this family has undergone recent changes, including assignment of species to different genera, the creation of new species and subspecies and even the introduction of new genera to accommodate these changes. The application of DNA arrays will also improve our knowledge of gene expression in these bacteria. While this approach is currently being used to provide greater understanding of various pathogens and their role in pathogenesis and survival, it has the potential to provide us with a better understanding of their role in food spoilage.

Although much of the focus in the past has been directed towards food safety and the prevention of contamination by food pathogens, there is now greater realisation that food spoilage can also facilitate the growth of pathogens by altering the intrinsic factors in a food and there is also the huge economical loss to both the food industry and to the consumer. Moreover, food spoilage is a diverse and complex area of microbiology that often involves interaction between many factors and groups of microorganisms. A topic starting to receive attention is the role of quorum sensing, in which bacteria appear to be able to modulate a number of cellular functions (genes) through signalling mechanisms. This phenomenon is thought to play a role in a number of foodborne pathogens, including *E. coli* O157, *Yersinia enterocolitica* as well as Enterobacteriaceae involved in spoilage (Smith *et al.*, 2004). In Gram-negative bacteria, including members of the Enterobacteriaceae, *N*-acylhomoserine lactones appear to act as signalling molecules and these may therefore play a role in bacteria associated with food spoilage (Gram *et al.*, 1999; Bruhn *et al.*, 2004; Smith *et al.*, 2004). Moreover, with a greater understanding of quorum sensing and the genes and signalling molecules involved it may be possible to prevent spoilage by interfering with this process. This novel approach to preventing or delaying spoilage could therefore become an alternative intervention strategy and the subject of future research.

Besides preventing spoilage, future research will undoubtedly continue into improving methods of detection, not only of the spoilage organisms but also the compounds they produce, some of which can give early indications of food spoilage and deterioration. Molecular techniques will enable rapid
and specific detection of spoilage bacteria or the genes encoding spoilage-related enzymes. Amines in Enterobacteriaceae can be detected by gas liquid chromatography (Ghenghesh and Drucker 1989), and detection of volatile organic compounds and bacterial spoilage now can be done within minutes rather than days, using techniques such as proton transfer reaction mass spectrometry (Mayr et al., 2003). Electronic nose, an instrument consisting of an array of non-specific electronic chemical sensors that can recognise odour patterns, has also been used to determine spoilage in vacuum packed beef (Blixt and Borch 1999). This approach is likely to improve further and be used to detect spoilage in a wider range of foods.

22.13 Sources of further information

The following provide useful sources of additional information on the Enterobacteriaceae:

- DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (approved lists of up-to-date bacterial nomenclature). http://www.dsmz.de/bactnom/bactname.htm

22.14 References


Enterobacteriaceae


Food spoilage microorganisms


Food spoilage microorganisms


23

Other spoilage bacteria

G. Betts, Campden and Chorleywood Food Research Association, UK

23.1 Introduction

The main groups of bacteria responsible for spoilage of different foods have been described in other chapters, for example, *Pseudomonas*, Enterobacteriaceae and lactic acid bacteria. These organisms all grow readily on general all-purpose plate count agars, and in the majority of cases, the total plate count from spoiled foods will be similar to any specific count for the spoilage groups mentioned. For example, in modified atmosphere packed (MAP) sliced meat products, the count of lactic acid bacteria at the end of shelf-life is likely to be equal to that of the total plate count.

There are, however, other genera of bacteria that are responsible for spoilage of particular food groups. Many of these are psychrotrophic or psychrophilic, growing best at a temperature of 20–25°C. The use of a general enumeration method at 30–37°C may not allow for the isolation of these groups.

The spoilage bacteria considered in this chapter can be divided into Gram-positive organisms and Gram-negative organisms. The Gram-negative group contains the following genera: *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, *Photobacterium* and *Psychrobacter*. Many of these are closely related in terms of their physiological characteristics and growth requirements. They are therefore of interest in the same food groups which are predominantly chilled proteinaceous foods, particularly fish, meat and poultry. The Gram-positive group contains the genera *Brevibacterium*, *Brochothrix*, *Corynebacterium* and *Micrococcus*. As with the Gram-negative group, these spoilage organisms also tend to be associated with chilled proteinaceous and dairy foods.

This chapter contains information on the growth and survival characteristics of these other spoilage organisms and the food groups from which they have
been isolated. For some of these genera, the available information is limited. The taxonomic status of many of these species is constantly changing and species are often moved to different or new genera. Information on the current validated or approved species in each genus at the time of writing this chapter is given based on data from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Anon., 2005) and Euzéby (2005).

23.2 Gram-negative organisms

23.2.1 Achromobacter

General characteristics and taxonomy

Natural habitat
Achromobacter occur in water and soils. Some species are common inhabitants of the intestinal tracks of animals. They have been known to cause opportunistic infections in humans.

Characteristics
Achromobacter are non-motile, non-pigmented aerobic rods associated with spoilage of meats. They are catalase and oxidase positive (Table 23.1).

Taxonomy
The genus has not been formerly recognised since the 1970s; however, the name Achromobacter is still considered to be a convenient tag to put on all non-pigmented non-motile Gram-negative rods that do not easily fit into another genus (Collins et al., 2004). Former Achromobacter species were placed into Acinetobacter, Alcaligenes or Moraxella. This proved to have some difficulties as although Achromobacter shared some similarities with the three above-mentioned genera there were some anomalies. For example, many Achromobacter species were similar to Acinetobacter except they are oxidase positive and Acinetobacter species are oxidase negative (Kraft, 1992). Busse and Auling (1992) give a good description of the similarities between Alcaligenes and Achromobacter species.

According to Kersters (1992), several bacterial species misnamed as Achromobacter belong to the genus Deleya. This species comprises Gram-negative, non-fermentative halotolerant organisms, which require up to 7% salt for growth. They are often isolated from salty foods, for example, Achromobacter halophilus from salted salmon and A. turbidus and A. viscosum from salted aubergine (eggplant).

There are currently six species and two subspecies of Achromobacter on the list of prokaryotic names with standing in nomenclature (Euzéby, 2005): Ach. denitrificans; Ach. insolitus; Ach. piechaudii; Ach. ruhlandii; Ach. spanius; Ach. xylosoxidans; Ach. xylosoxidans subsp. denitrificans; Ach. xylosoxidans subsp. xylosoxidans.
Table 23.1  Main characteristics of Gram-negative spoilage organisms

<table>
<thead>
<tr>
<th>Genus</th>
<th>Rods/cocci</th>
<th>Size (µm)</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Respiration</th>
<th>Growth temperature</th>
<th>Associated foods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at 5°C</td>
<td>Optimum (ºC)</td>
</tr>
<tr>
<td>Achromobacter</td>
<td>R</td>
<td>No data available</td>
<td>+ve</td>
<td>+ve</td>
<td>Aerobic</td>
<td>Yes</td>
<td>No data available</td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>R</td>
<td>0.9–1.6 × 1.5–2.5</td>
<td>+ve</td>
<td>–ve</td>
<td>Strictly aerobic</td>
<td>Yes</td>
<td>33–35</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>R</td>
<td>0.5–1.0 × 0.5–2.6</td>
<td>+ve</td>
<td>+ve</td>
<td>Aerobic (some strains facultative)</td>
<td>Yes</td>
<td>20–37</td>
</tr>
<tr>
<td>Moraxella subgenus</td>
<td>R</td>
<td>1.0–1.5 × 1.5–2.5</td>
<td>+ve</td>
<td>+ve</td>
<td>Aerobic</td>
<td>Yes</td>
<td>33–35</td>
</tr>
<tr>
<td>Moraxella subgenus</td>
<td>C</td>
<td>0.6–1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branhamella Flavobacterium</td>
<td>R</td>
<td>0.5 × 1.0–3.0</td>
<td>+ve</td>
<td>+ve</td>
<td>Aerobic</td>
<td>Yes</td>
<td>30</td>
</tr>
<tr>
<td>Photobacterium</td>
<td>R</td>
<td>0.8–1.3 × 1.8–2.4</td>
<td>+ve</td>
<td>+ve</td>
<td>Facultative</td>
<td>Yes</td>
<td>18–25</td>
</tr>
<tr>
<td>Psychrobacter</td>
<td>R</td>
<td>0.9–1.3 × 1.5–3.8</td>
<td>+ve</td>
<td>+ve</td>
<td>Facultative</td>
<td>Yes</td>
<td>20</td>
</tr>
</tbody>
</table>
Relevant foods
Achromobacter (more probably Acinetobacter in current terminology) were found to dominate in breaded shrimps, and pork sausages (Kraft, 1992). Isolates formerly placed in the Achromobacter group have been isolated from spoiled eggs (Board, 2000) and are present on marine and fresh fish from temperature and tropical zones (Gram and Huss, 2000). They have also been found in raw milk and in many sources of bottled, ground and spring waters (Warburton and Austin, 2000).

Growth and survival characteristics
Achromobacter species are psychrotrophic and therefore grow well under refrigeration conditions although exact data on their minimum growth characteristics are not available. They are inhibited by 100 to 200 mg/kg nitrite at pH 6.0 (Gould, 2000).

Enumeration, detection and isolation
Bacteria in the Achromobacter group will grow on general all-purpose growth medium such as nutrient agar (NA) or plate count agar (PCA). As with all the psychrotrophic Gram-negative organisms it may be advisable to use a reduced incubation temperature for enumeration of this group.

23.2.2 Acinetobacter

General characteristics and taxonomy

Natural habitat
Acinetobacter are found in soil and water and are common inhabitants of the intestinal tracts of many animals.

Characteristics
Acinetobacter are Gram-negative rods of approximately 0.9–1.6 µm in diameter and 1.5–2.5 µm in length. During the stationary phase of the growth curve, the shape becomes more spherical than rod shaped.

Acinetobacter are strictly aerobic organisms and use oxygen as the terminal electron acceptor. All strains can grow well between 20 and 30°C. Most strains have an optimum of 33 to 35°C, although, some fail to grow at 37°C. Many strains can grow at 5°C. It is recommended that an incubation temperature of 30°C is used and in some cases the use of a lower temperature may be advisable in addition to 30°C (Kampfer, 2000), for example, in the range 5 to 12°C, in order to isolate psychrophilic strains.

Acinetobacter strains are catalase positive and oxidase negative and form smooth white to cream colonies, which are non-pigmented.

Taxonomy
The genus Acinetobacter was in the family Neisseriaceae until 1991 when it
was moved to a new family, Moraxellaceae, along with *Moraxella*, *Psychrobacter* and other related bacteria.

There is some difficulty in differentiation of *Acinetobacter* at the species level. The genus was first proposed by Brisou and Prevot (1954) and while identification to genus level is unambiguous, further differentiation to species level based on phenotypic properties can be complex and time consuming (Towner, 1992).

Traditionally, a microbial species has been defined as a group of organisms with a high degree of phenotypic similarities, but now it is generally accepted that species should include strains with at least 70% DNA relatedness (Towner, 1992). There are currently 17 different genomic species of *Acinetobacter* on the list of prokaryotic names with standing in nomenclature (Euzéby, 2005): *Ac. baumannii*; *Ac. baylyi*; *Ac. bouvetii*; *Ac. calcoaceticus*; *Ac. gerneri*; *Ac. grimontii*; *Ac. haemolyticus*; *Ac. johnsonii*; *Ac. junii*; *Ac. Iwoffii*; *Ac. parvus*; *Ac. radioresistens*; *Ac. schindleri*; *Ac. tandoii*; *Ac. tjernbergiae*; *Ac. towneri*; *Ac. ursingii*.

**Relevant foods**

*Acinetobacter* are widespread in the environment, for example, they have been isolated from soil, water and sewage and a variety of foods including milk, vegetables, chicken. They belong to the typical psychrophilic spoilage flora of chilled foods and can grow well under refrigerated conditions. *Acinetobacter johnsonnii* and *Acinetobacter Iwoffii* are the species predominantly isolated from spoiled foods such as bacon, eggs and fish (Kampfer, 2000).

**Meat**

Despite being strictly aerobic, *Acinetobacter* species have been isolated from vacuum packaged (VP) beef following 6 weeks’ storage at 2°C (Sakala *et al.*, 2002) although the percentage of isolates belonging to this genus were low. Of the 1493 strains identified, only 3 were *Acinetobacter*, 6 were *Psychrobacter* and 13 were *Pseudomonas*. The majority were lactic acid bacteria and other Gram-positive organisms.

In aerobically stored mince beef stored at 4°C for 8 days, *Acinetobacter* spp. grew more rapidly than other psychrotrophes such as *Pseudomonas putrefaciens* (Chung *et al.*, 2002). However, the meat had a more acceptable sensory score and fewer volatile compounds when it contained *Acinetobacter* rather than *P. putrefaciens*. The authors concluded that it was the type of bacteria present which was more important for production of off-odours due to lipid oxidation than the number of organisms present. In this case, a low number of *P. putrefaciens* had a greater spoilage potential than a large number of *Acinetobacter*.

**Poultry**

On commercially processed poultry products, *Acinetobacter* and *Aeromonas* were the primary isolates recovered from carcasses taken from the processing
line. Strains most frequently isolated were *A. radioresistens* and *A. baumanii*. During chill storage of prepared carcasses, there was a significant increase in the population of bacteria on these carcasses, with *Pseudomonas* growing more rapidly than other species.

Fish

Kim *et al.* (2000) found that *Acinetobacter, Pseudomonas, Aeromonas* and *Flavobacterium* were predominant on catfish fillets and those treated with 0.7% hydrogen peroxide, ascorbic acid (0.5%) + salt (1%). *Acinetobacter* and *Moraxella* were also predominant on catfish samples treated with 10 ppm ozone. No *Acinetobacter* were isolated from fillets treated with 10% brine.

In a study of four fish processing plants (two producing cold-smoked salmon, one producing semi-preserved herrings and one producing caviar) a total of 1009 organisms were isolated. The cold smoking sites were dominated by *Pseudomonas*, Enterobacteriaceae, Coryneforms and *Acinetobacter* (Bagge-Ravn *et al.*, 2003). In shellfish (mussels, prawns, scallops and oysters) treated with high pressure at 300 to 600 MPa for 2 minutes at 20°C and stored for up to 28 days at 2°C, psychrotrophic coliforms and pseudomonads were inactivated, while it appeared that *Acinetobacter* were better able to survive. The range of bacteria present in shellfish decreased after pressure treatment but the main bacteria isolated from treated shellfish were *Bacillus, Acinetobacter/Moraxella* and lactic acid bacteria which made up 96% of the isolated organisms (Linton *et al.*, 2003).

Drinks

Soft drinks are also susceptible to spoilage by *Acinetobacter* species. Six bottles of soft drink spoiled by white filamentous growth were found to be contaminated with *Ac. johnsonii*. The samples of drink had a pH of 3.6 and were stored at ambient temperatures. Battey and Schaffner (2001) modelled the spoilage of cold-filled ready to drink beverages by a cocktail of *Ac. calcoaceticus* and *Gluconobacter oxydans*. One of the most important factors preventing growth was pH. There was no growth at a pH of 2.8 while at pH 3.8 growth occurred in all samples irrespective of the presence of preservative or sugar. At pH 3.3, the ability of the cocktail to grow was dependent on the total preservatives and ºBrix. Generally, growth did not occur when the total amount of preservatives (potassium sorbate and sodium benzoate) exceeded 500 ppm.

**Growth and survival characteristics**

*Acinetobacter* appear to be able to tolerate low pH and have been reported to grow at pH 3.3 in soft drinks (Cantoni and Lacumin, 2003).

Reported ranges of growth temperatures for *Acinetobacter–Moraxella* spp. in relation to growth and poultry are <1°C to 34°C (Mead, 1982). These organisms can therefore grow readily at refrigeration temperatures, e.g. 5°C.
Enumeration, detection and isolation

Acinetobacter can be isolated on a wide range of commercially available growth media such as brain heart infusion agar, NA and tryptone soy agar (TSA).

There are some selective media that have been used for these organisms. Kampfer (2000) gives details of Hotlin's selective media (agar 10 g l\(^{-1}\); casein pancreatic digest 15 g l\(^{-1}\); peptone 5 g l\(^{-1}\); NaCl 5 g l\(^{-1}\); dessiate ox bile 1.5 g l\(^{-1}\); fructose, 5 g l\(^{-1}\); sucrose 5 g l\(^{-1}\); mannitol 5 g l\(^{-1}\); phenylalanine 10 g l\(^{-1}\); phenol red 0.02 g l\(^{-1}\); pH 7.0) after autoclaving filter sterilised antibiotics are added as follows: vancomycin 0.01 g l\(^{-1}\); ampicillin, 0.061 g l\(^{-1}\); cefsulodin, 0.03 g l\(^{-1}\).

Samples are incubated overnight at 37°C. Red colonies are tested for oxidase reaction and phenylalanine deamination. Addition of three to five drops 10% aqueous ferric solution is added to incubated cultures. A positive reaction results in the development of a deep green colour as the ferric salt reacts with phenylpyruvic acid formed from deamination of the phenylalanine. A negative reaction results in no colour change.

Colonies that are negative for both oxidase and phenylalanine deamination are considered to be presumptive Acinetobacter species. Identification at the genus level can be made on some basic biochemical and metabolic characteristics (Kampfer, 2000):

- gram-negative coccoid rods;
- oxidase negative;
- catalase positive;
- non-motile in hanging drop preparation;
- negative nitrate reduction;
- hydrolysis of tween;
- negative for D-glucose fermentation.

In addition, identification to the genus level can be done using commercially available kits such as API 20NE.

Rapid enumeration of spoilage organisms is important in determining the likelihood of food spoilage. The use of electrical conductivity measurements such as impedance methods is one way in which total bacterial counts can be achieved more rapidly than plate counts. The principle of this technique is that microbial growth causes changes to the electrical conductivity of the growth medium. This change in conductivity is measured by the instrument and can be equated to microbial numbers. Some studies have shown that bacterial species differed widely in their impedance characteristics. Some bacteria show a high impedance effect, while others, including Acinetobacter, did not cause any change to the impedance of the medium (Schulenburg and Bergann, 2000). Therefore for foods where Acinetobacter dominate or where they have an important role in the spoilage of the food, the use of a rapid enumeration technique such as impedance may not be appropriate.
Control in foods
Acinetobacter are fairly tolerant to some preservative factors. They can grow at chill temperatures and at low pH (pH 3.3). They appear to be able to withstand exposure to 0.7% hydrogen peroxide and 10 ppm ozone. They are not tolerant to high levels of salt, e.g. 10% brine and are not resistant to heat.

23.2.3 Alcaligenes

General characteristics and taxonomy

Natural habitat
Alcaligenes are found in soil and water and are common inhabitants of the intestinal tracts of some animals.

Characteristics
Alcaligenes are Gram-negative, aerobic bacteria although some strains are capable of anaerobic respiration in the presence of nitrite or nitrate. They are catalase and oxidase positive. They are short rods or cocci with dimensions 0.5–1.0 µm in diameter by 0.5–2.6 µm in length and the cells occur singly. The colonies are non-pigmented.

Taxonomy
There are currently 14 species and 7 subspecies of Alcaligenes on the list of prokaryotic names with standing in nomenclature (Euzéby, 2005): Al. aestus; Al. aquamarinus; Al. cupidus; Al. defragrans; Al. denitrificans; Al. denitrificans subsp. denitrificans; Al. denitrificans subsp. xylosoxydans; Al. eutrophus; Al. faecalis; Al. faecalis subsp. faecalis; Al. faecalis subsp. homari; Al. faecalis subsp. parafaecalis; Al. latus; Al. pacificus; Al. paradoxus; Al. piechaudi; Al. ruhlandii; Al. venustus; Al. xylosoxidans; Al. xylosoxidans subsp. denitrificans; Al. xylosoxidans subsp. xylosoxidans.

Relevant foods
One of the main features of Alcaligenes is their use in the area of biotechnology. They produce a plastic-like polymer, which serves as a model for the industrial production of biodegradable materials.

As far as the food industry is concerned, they are recognised as potential contaminants of dairy produce, meat and poultry. Alcaligenes have been associated with rancidity in butter caused by their ability to degrade butyric acids and off-odours in milk due to breakdown of fat and proteins (Collins et al., 2004). They have been found in bottled and ground spring waters (Warburton and Austin, 2000). They are common contaminants of eggs where they produce no apparent spoilage attributes (Board, 2000). They are found on marine and fresh fish (Gram and Huss, 2000) and on raw vegetables (Nygen-thé and Carlin, 2000).
Growth and survival characteristics
General growth characteristics for this genus are shown in Table 23.1.

Enumeration, detection and isolation
Alcaligenes can be enumerated on standard plating media, although the incubation temperature may need to be reduced from the usual 30/37°C in order to allow maximum isolation of these organisms. For detection/identification, an isolated colony should be streaked onto blood agar and MacConkey agar at 22–22°C for 24 to 48 hours. White colonies should be re-streaked onto agar slopes and tested for the following reactions (Table 23.2) which are positive for Alcaligenes species (Collins et al., 2004).

23.2.4 Flavobacterium

General characteristics and taxonomy
Natural habitat
Flavobacterium are found widely in the environment in soils and water and have been isolated from a wide range of chilled foods such as milk, raw meats and wild and farmed fresh-water fish (Gonzalez et al., 2000).

Characteristics
Flavobacterium are Gram-negative rods 0.5 × 1.0–3.0 μm. They are catalase and oxidase positive (Table 23.1). Generally the colonies are yellow to orange in colour although some non-pigmented colonies do occur.

Taxonomy
The genus was first named in 1923 to describe bacteria forming yellow (Flavus) or orange pigmented colonies in culture media. Many species that were not genetically linked were assigned to the genus on the basis of this phenotypic attribute. Recent taxonomic studies based on molecular techniques have shown that many Flavobacterium species should really be assigned to different or new genera such as Cytopaga, Bergeyella or Weeksella. The

<table>
<thead>
<tr>
<th>Test</th>
<th>Typical reaction for Alcaligenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>+ve</td>
</tr>
<tr>
<td>Catalase</td>
<td>+ve</td>
</tr>
<tr>
<td>Hugh and Leifson medium</td>
<td>Alkaline reaction</td>
</tr>
<tr>
<td>Arginine broth</td>
<td>Not hydrolysed</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Not liquefied</td>
</tr>
<tr>
<td>Bromocresol purple broth</td>
<td>Alkaline</td>
</tr>
</tbody>
</table>
Other spoilage bacteria currently contains 56 species according to the approved lists (Anon., 2005; Euzéby, 2005).

Relevant foods

*Flavobacterium* spp. have been isolated from many chilled foods. They occur in significant numbers in many types of fresh and frozen fish species including halibut, sole cod, salmon and trout and seem to be particularly significant in milk and dairy products (García-López *et al*., 2000a). Because of their lipolytic properties they are able to grow in margarines and emulsions. *Flavobacterium maloloris* has been associated with spoilage of butter where it has been shown to decompose the protein component of the butter to produce isovaleric acid which gives a putrid cheesy odour. Other dairy products such as cheddar cheese, cream, butters, rice pudding and milk-based canned goods are also susceptible to spoilage by *Flavobacterium*.

*Flavobacterium* are found on a wide range of fresh meat and poultry products. Some 6% of the isolates grown on TSAYE (TSA plus yeast extract) from samples of poultry carcasses were *Flavobacterium* and 12% of isolates from the immersion chiller water. No *Flavobacterium* were found in the scald tank water, so these organisms might be sensitive to the temperature of 53.5 °C used (Geornaras *et al*., 1996).

*Flavobacterium* have also been found in many stages of the production of bottled waters. They have been found in ground and spring waters, municipal treated waters in parts of the filtration systems such as activated carbon filters, reverse osmosis filters and water softeners as well as in the final bottled water (Warburton and Austin, 2000). Traditional alcoholic beverages produced from grains (maize and sorghum) have been found to contain a range of organisms including *Acetobactor, Alcaligenes* and *Flavobacterium* spp. The organisms present gave a vinegary flavour and off-odour to the products. No levels were given for the amount of *Flavobacterium* present but the data demonstrate this organism was able to survive in these beverages which had an initial pH of 3.4 to 3.6 and an ethanol content of 3.0% (Sanni *et al*., 1999).

Growth and survival characteristics

pH

The levels of *Flavobacterium* in a biofilm on stainless steel were only reduced by 83% after exposure to a solution of water at pH 11 for 20 minutes (Bremer *et al*., 2002).

Chlorine

Biofilms containing *Flavobacterium* and *Listeria monocytogenes* were treated with various solutions of chlorine. A 4 log reduction in levels of *Flavobacterium* on stainless steel was achieved by exposure to a 200 ppm solution adjusted to pH 6.5 for 20 minutes (Bremer *et al*., 2002).
Preservatives
Minimum inhibitory levels of nitrite at pH 6.0 are 100 to 200 mg/kg for most species of Flavobacterium, Achromobacter and Micrococcus (Gould, 2000).

Mahmoud et al. (2004) looked at the effect of essential oil compounds on the shelf-life of carp. Oils tested included, thymol, carvacrol, cinnamaldehyde and eugonal. A wide range of microflora (genera) was isolated from the fish and Flavobacterium was found to be the dominant organism in the skin and gills along with Vibrionaceae. Flavobacterium was particularly sensitive to the antimicrobials tested and showed an inhibition zone of 42 mm in the presence of 2% carvacrol compared with 8 mm for Alcaligenes spp. and 19.8 for Moraxella spp.

Enumeration, detection and isolation
Flavobacterium are easy to isolate in standard plating medium such as PCA, NA or tryptone soy agar (TSA) where they produce circular convex colonies with entire or wavy edges (García-López, 2000a). Incubation conditions of 30°C for 48 hours or 7°C for 10 days can be used.

23.2.5 Moraxella

General characteristics and taxonomy
Natural habitat
These organisms are found in marine environments and are present on the mucous membranes of humans and other animals.

Characteristics
Moraxella are in the family Moraxellaceae along with Acinetobacter, Psychrobacter and other related groups. They are aerobic, although with some strains show weak facultative abilities. Moraxella are catalase positive and have an optimum growth temperature of 33–35°C. Cells of this genus often show pleiomorphic characteristics in growing cultures particularly in conditions of reduced oxygen and elevated temperatures.

Taxonomy
The genus Moraxella is unusual in that it contains two distinct groups or subgenera. The first group is the classic rod-shaped organisms and these are in the subspecies Moraxella. The second subgroup, the Branhamella, contains coccoid organisms. The rods are short and plump and are typically 1.0–1.5 µm by 1.5–2.5 µm. They occur in pairs or short chains. The cocci are 0.6 to 1.0 µm in diameter and occur singly or in pairs.

Moraxella species are named by both the genus and subgenus group, hence Moraxella [Branhamella] caviae is abbreviated to M. [B.] caviae. There are currently 19 species of Moraxella on the list of prokaryotic names
with standing in nomenclature (Euzéby, 2005), 4 of which are subgenera Branhamella and 6 are subgenera Moraxella: M. anatipestifer; M. [M.] atlantae; M. boevrei; M. [M.] bovis; M. canis; M. caprae; M. [B.] catarrhalis; M. [B.] caviae; M. [B.] cuniculi; M. equi; M. [M.] lacunata; M. lincolnii; M. [M.] nonliquefaciens; M. oblonga; M. [M.] osloensis; M. [B.] ovis; M. [M.] phenylpyruvica; M. saccharolytica; M. urethralis.

Relevant foods
Moraxella have been isolated from a variety of chilled proteinaceous foods such as meat and fish as well as from a range of dairy products. These organisms form the predominant flora on marine fin fish (e.g. cod, sole, halibut, hake) as well as a large proportion of the psychrotrophic flora of tropical marine fish and fresh-water fish (e.g. bass, perch, salmon). Moraxella have also been associated with meat products, although their significance may be small as these organisms constitute a minor part (<1%) of the spoilage microflora (Santos et al., 2000).

However, previous reports (Gennari et al., 1992) found that members of the Moraxellaceae including Moraxella-like organisms were present at levels of $10^3–10^5\text{g}^{-1}$ in fresh meat and up to levels of $10^8–10^9\text{g}^{-1}$ in spoiled meat.

Enumeration, detection and isolation
Moraxella can grow in standard enumeration media such as TSA or PCA incubated at 33–35°C (Santos et al., 2000).

23.2.6 Photobacterium

General characteristics and taxonomy

Natural habitat
Photobacterium are common in the marine environment and on the surfaces and in the intestinal contents of marine animals. Some species are bioluminescent and are found as symbionts in specialised luminous organs of fish.

Characteristics
Photobacterium are oxidase positive and motile by means of a polar flagellum (Kaysner, 2000). Photobacterium are able to grow in aerobic and anaerobic environments (Molin, 2000). They are Gram-negative rods approximately 0.8–1.3µm by 1.8–2.4µm (Table 23.1).

Taxonomy
The genus Photobacterium belongs to the family Vibrionaceae, along with Vibrio, Aeromonas and Plesiomonas. There are currently 12 species and 2 subspecies of Photobacterium on the list of prokaryotic names with standing
in nomenclature (Euzéby, 2005): *Ph. angustum*; *Ph. damsela*; *Ph. damsela* subsp. *damsela*; *Ph. damsela* subsp. *piscicida*; *Ph. fischer*; *Ph. histaminum*; *Ph. iliopiscarium*; *Ph. indicum*; *Ph. leiognathi*; *Ph. lipolyticum*; *Ph. logei*; *Ph. phosphoreum*; *Ph. profundum*; *Ph. rosenbergii*.

**Relevant foods**

*Photobacterium phosphoreum* and *Shewanella putrefaciens* are the specific spoilage organisms in VP ice-stored fish. They both produce similar quantities of trimethylamine (TMA) which is the metabolite responsible for the fishy odour in fish. *Ph. phosphoreum* does not cause such foul odours as it is unable to produce volatile sulphide compounds. In a study by Dalgaard *et al.* (1997), *Ph. phosphoreum* was found in all marine fish and grew to high numbers in most products. None of the samples showed any signs of sensory spoilage until the levels of *Ph. phosphoreum* exceeded $10^7 \text{cfu g}^{-1}$.

As well as being a common spoilage organism of fish, this bacterium has also been associated with food poisoning due to histamine production. Kanki *et al.* (2004) report an incidence of histamine fish poisoning (HFP) from dried sardine in Japan 2002 where *Ph. phosphoreum* was found to be the causative organism.

**Growth and survival characteristics**

***Modified atmospheres***

*Photobacterium phosphoreum* is resistant to carbon dioxide and has been identified as the organism responsible for spoilage in VP and MAP cod stored at 0°C (Dalgaard *et al*., 1993). They are also the dominant organisms in salmon stored at 2°C in 60% CO$_2$/40% N$_2$ (Emborg *et al*., 2002), although, recent studies have shown that histamine production by these organisms is strongly inhibited in product packed in 40% CO$_2$/60% O$_2$ (Emborg *et al*., 2005). The rate of growth of this organism has been reported to be increased under anaerobic conditions which may contribute to its ability to spoil MA packs (Sivertsvik *et al*., 2002).

***High pressure***

Paarup *et al.* (2002) looked at the effects of high pressure on the bacteriological changes in VP squid. They found that all colonies isolated from the fresh squid prior to treatment were *Ph. phosphoreum* (equivalent to $10^4 \text{g}^{-1}$) as were all colonies isolated from spoiled untreated squid and that treated at 200 MPa (equivalent to $10^7 \text{g}^{-1}$). *Ph. phosphoreum* was not detected from spoiled samples of squid treated at 300 or 400 MPa, although they were present in treated unspoilt samples. Isolates of *Ph. phosphoreum* from spoilt squid did not have luminous properties while those from unspoilt samples did. The authors suggested that the non-luminous strains were able to produce more spoilage metabolites than luminous strains.
Enumeration, detection and isolation

The optimal temperature for growth of Photobacterium is generally 18 to 25 °C and therefore it is likely that general enumeration techniques at 30°C will underestimate the numbers of these organisms present. Photobacterium require sodium to grow and so do not grow well in media containing insufficient salt (sodium chloride). Dalgaard et al. (1997) stated that some microbiological methods recommended for examination of fish products by national and international authorities are inappropriate for heat-labile, psychrotrophic organisms like Photobacterium.

Any technique that uses pour plate procedures with molten agar at 45°C has the potential to cause heat injury to any Photobacterium present. In addition Dalgaard et al. (1997) found that standard PCA that did not contain NaCl (1%) was inappropriate.

23.2.7 Psychrobacter

General characteristics and taxonomy

Natural habitat

Psychrobacter are found in a variety of marine and terrestrial environments, including foods, soil, sea water, sea ice and air (García-López and Maradona, 2000).

Characteristics

Psychrobacter are coccobacilli being 0.9–1.3 µm in diameter and 1.5–3.8 µm in length. They are Gram-negative and catalase and oxidase positive. Most strains are psychrotrophic and grow at 5°C with an optimum temperature around 20°C.

Psy. immobilis does not generally grow at 37°C and those strains that can, do not normally grow at 5°C. They are aerobic but some strains can grow under anaerobic conditions provided there is a suitable electron acceptor.

Taxonomy

The taxonomic status of the Psychrobacter and other members of the Moraxellaceae appears to have been under constant review over the last few years. Originally Psychrobacter was part of the Neisseriaceae until phylogenetic studies demonstrated their relatedness to Moraxella and Acinetobacter, hence the proposal of the new family Moraxellaceae. Of the entire group, Moraxella are closest to the Psychrobacter.

This genus was created in 1986 and originally only contained one species: Psy. immobilis. Since then, new species have been described and the genus currently contains 26 species according to the approved lists (Anon., 2005; Euzéby, 2005): Psy. adeliensis; Psy. alimentarius; Psy. aquaticus; Psy. aquimaris; Psy. arenosus; Psy. celer; Psy. cibarius; Psy. faecalis; Psy. fozi;
Psy. frigidicola; Psy. glacincola; Psy. immobilis; Psy. jeotgali; Psy. luti; Psy. marincola; Psy. maritimus; Psy. namhaensis; Psy. nivimaris; Psy. okhotskensis; Psy. pacificensis; Psy. phenylpyruvicus; Psy. proteolyticus; Psy. pulmonis; Psy. salsus; Psy. urativorans; Psy. vallis.

Relevant foods
Although they are present on fresh proteinaceous foods such as meat, poultry and fish, Psychrobacter and Acinetobacter have a low spoilage potential because they lack the important food spoilage biochemical attributes such as proteolysis and production of H$_2$S (Gennari et al., 1992). They are able to form acid from carbohydrates aerobically but do not produce trimethylamine (TMA) or indole. They do show lipase and lecithinase activity (García-López and Maradona, 2000).

They are dominant on poultry on its arrival at the processing plant (Mead, 2000) and account for about 3% of the microflora on Swedish fresh fillets stored at 2–8°C (Molin, 2000). Salt cured cod has been shown to contain high levels of Psychrobacter. Over 90% of the total viable count comprised Psychrobacter species which were able to hydrolyse lipids but not degrade proteins and could survive in the presence of up to 25% salt (Bjorkevoll et al., 2003).

Enumeration, detection and isolation
Members of the Moraxellaceae (Psychrobacter, Acinetobacter and Moraxella) grow well on general growth media such as PCA at 25°C. Selective media have been suggested, for example, NA with 1 µg ml$^{-1}$ crystal violet and 0.1% bile salts incubated at 25°C for 3 days. Psychrobacter and Acinetobacter are convex, opaque and light blue. Extended incubation at 5°C was also found to improve the colony coloration (Gennari et al., 1992).

23.3 Gram-positive organisms
23.3.1 Brevibacterium

General characteristics and taxonomy
Natural habitat
Brevibacterium are found in dairy products, fresh and salt water, marine organisms, insects and decaying organic matter.

Characteristics
Brevibacterium are irregular rods arranged singly or in pairs. They often orientate at angles to give a V shape. They are 0.6–1.2µm in diameter by 1.5–6µm in length. They are strictly aerobic and the colonies are often pigmented with yellow or purple coloration. Their optimum growth temperature is 20 to 35°C (see Table 23.3).
### Table 23.3 Main characteristics of Gram-positive spoilage organisms

<table>
<thead>
<tr>
<th>Genus</th>
<th>Rods/cocci</th>
<th>Size</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Respiration</th>
<th>Growth temperature</th>
<th>Associated foods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>at 5°C</td>
<td>Optimum</td>
<td>at 37°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brevibacterium</td>
<td>R</td>
<td>0.6–1.2 ×</td>
<td>+ve</td>
<td>−ve</td>
<td>Aerobic</td>
<td>Yes</td>
<td>20–35</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td>1.5–6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brochothrix</td>
<td>R</td>
<td>0.6 ×</td>
<td>+ve</td>
<td>+ve</td>
<td>Facultative</td>
<td>Yes</td>
<td>20–25</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>R</td>
<td>0.5–0.8 ×</td>
<td>+ve</td>
<td>−ve</td>
<td>Facultative</td>
<td>No data available</td>
<td>30–37</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>C</td>
<td>0.6–2.0</td>
<td>+ve</td>
<td>+ve</td>
<td>Aerobic</td>
<td>Yes</td>
<td>25–37</td>
</tr>
<tr>
<td></td>
<td>Pairs tetrads or clusters</td>
<td>1.5–8.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Taxonomy
There are currently 35 species of *Brevibacterium* according to approved lists of bacterial names (Anón., 2005; Euzéby, 2005).

**Relevant foods**
*Brevibacterium* are of interest to the food industry because they produce amino acids such as glutamic acid which is of use in the production of flavour enhancer such as monosodium glutamate. They also produce important enzymes used in cheese ripening. *Brevibacterium linens* is the type strain and has a growth temperature range of 8–37°C and an optimum of 21–23°C (Weimer, 2000). *Brevibacterium* have also been isolated from wheat samples (Legan, 2000).

**Growth and survival characteristics**

**pH**
The type strain *Brev. linens* grows over a wide pH range from 5.5 to 9.5. It is sensitive to acidic conditions and in cheese ripening, this organism relies on the growth of yeasts and moulds which metabolise the lactate from the milk to CO₂ and water.

**Water activity**
The salt tolerance varies considerable between strains. For example, *Brev. linens* strains vary in tolerance from 0 to 20% salt with an average of around 5% (Weimer, 2000).

**Control in foods**

**Heat**
*Brevibacterium* are not resistant to heat so should be readily inactivated by typical pasteurisation treatments e.g. 70°C for 2 minutes.

**Enumeration, detection and isolation**
Brevibacteria grow well on most universal nutrient media and elective media and they can be isolated either by direct plating or streaking/plating following an enrichment procedure (Holzapfel, 1992). One such enrichment procedure utilises the organisms’ ability to produce methanethiol from L-methionine and involves incubation at 20–25°C in a mineral-mix medium (Holzapfel, 1992). No strictly selective agar media are available but generally TSA supplemented with 4.0% NaCl is used with incubation at 20–25°C for 5–7 days prior to further identification (Holzapfel, 1992).
23.3.2 **Brochothrix**

*General characteristics and taxonomy*

**Natural habitat**

*Brochothrix* is found in soil and water and is a common inhabitant of the intestinal tract of animals.

**Characteristics**

*Brochothrix* are Gram-positive facultatively anaerobic rods that can occur either singly or in chains. The dimensions are approximately 0.6–0.7 µm in width by 1–2 µm in length. They have a growth range of 0 to 30 °C with an optimum around 20–25 °C. The colonies are non-pigmented. They produce lipases and proteases that contribute to food spoilage but the production of these are optimal at higher temperatures. Lipases are not produced in detectable amounts at temperatures below 20 °C and the production of proteases was lower at 6 °C than at 10 °C (Braun and Sutherland, 2004).

**Taxonomy**

There are currently 2 species of *Brochothrix* according to approved lists of bacterial names (Anon., 2005; Euzéby, 2005): *B. campestris* and *B. thermosphacta*. These species are biochemically similar and are both present in soil and grass (Holley, 2000).

**Relevant foods**

*Brochothrix thermosphacta* has been isolated from a wide range of food types including beef, lamb, pork, fish, frozen vegetables and dairy products. This organism is often associated with the spoilage of fresh and cured meats where it produces diacetyl and a range of fatty acids from the aerobic metabolism of glucose which give rise to sour, musty, acidic or sweaty odours. Although *B. thermosphacta* may not be the most dominant organism, these sensory defects may be observed once this organism has reached 10⁵ cfu g⁻¹ (Holley, 2000).

Vermeiren *et al.* (2005) found that sensory changes in broth and cooked ham occurred at a lower level of *B. thermosphacta* than other typical meat spoilage organisms. *Brochothrix* grew faster than any of the lactic acid bacteria in a broth system, but in a model cooked ham, *Leuconostoc mesenteroides* grew faster than *B. thermosphacta*. Higher levels of the lactic acid bacteria (10⁷ to 10⁸ g⁻¹) were needed than *B. thermosphacta* (10⁶ g⁻¹) to achieve noticeable changes in sensory characteristics.

There were low numbers of *Brochothrix* present in fresh raw beef which was untreated or treated with hot water and/or a lactic acid spray, and stored at 4, 10 or 25 °C. This organism was detected only periodically in a limited number of samples (Koutsomanis *et al.*, 2004).

Rabbit meat produced in large abattoirs was found to contain high levels
of *Brochothrix* as well as *Pseudomonas* and lactic acid bacteria, although *Brochothrix* was not as prevalent on meat produced in small abattoirs. (Rodriguez-Calleja *et al.*, 2004). Samples of leg joints from supermarkets were also found to have high levels of *Brochothrix*, particularly on over wrapped samples where a build-up of CO$_2$ was thought likely to enhance growth of this organism and inhibit growth of *Pseudomonas*.

As well as fresh and marine fish, certain shellfish contain *Brochothrix*. In VP shucked mussels, the levels of this organism were low at the beginning of storage but had increased to $10^4$ g$^{-1}$ during 12 days’ storage at 4°C. The levels were slightly lower at $3.3 \times 10^3$ g$^{-1}$ for mussels treated in ozonated water for 90 minutes prior to packing.

**Growth and survival characteristics**

**pH**

*Brochothrix thermosphacta* can grow over the pH range 5.0–9.0 with an optimum of 7.0 (Holley, 2000).

**Water activity**

This organism can tolerate 6.5% salt with some strains capable of growth up to 10% salt and grow in products with an $a_w$ as low as 0.94 at temperatures of 20–25°C.

**MAP**

*Brochothrix thermosphacta* grows in MAP foods. It can tolerate up to 50% CO$_2$ provided some oxygen is present.

**Preservatives**

This organism can tolerate 100 ppm nitrite at >pH 5.5 and 5°C aerobically in the presence of 2–4% salt (Holley, 2000).

**Enumeration, detection and isolation**

*Brochothrix* will grow on general-purpose medium but can be selectively isolated using streptomycin thallous acetate actidione (STAA) agar. This contains a base of the following per litre: peptone 20 g; agar, 13 g; glycerol 15 g; yeast extract 2 g; K$_2$HPO$_4$ 1 g; MgSO$_4$ \cdot 7H$_2$O 1 g. After autoclaving at 121°C for 15 minutes, the base is cooled to 50°C and 10 ml of STAA supplement is added. STAA contains per 10 ml: streptomycin sulphate 0.5 g; cycloheximide 0.05 g; thallous acetate 0.05 g.

STAA is used for the enumeration of *B. thermosphacta* as a spread plate technique. A known volume of up to 0.5 ml is spread on the surface of prepoured STAA plates and incubated at 22°C for 48 hours. After incubation, the colonies are tested for their oxidase reaction. The plates are flooded with oxidase reagent (NNN – tetramethyl-$p$-phenylenediamine dihydrochloride 1 g in 100 ml sterile distilled water), left for 15 seconds. The oxidase negative colonies are *Brochothrix*, i.e. they show no purple coloration.
**Control in foods**
This organism is fairly tolerant to chill temperature, and reduced $a_w$. It is however fairly sensitive to heat and should be inactivated by a heat treatment of $63^\circ\text{C}$ for 5 minutes (Holley, 2000). It also requires a small level of oxygen to be present so its growth could be inhibited in MAP products where oxygen scavengers are used to reduce the level of $\text{CO}_2$ to below 0.2%.

### 23.3.3 Corynebacterium

**General characteristics and taxonomy**

**Natural habitat**
*Corynebacterium* are widely spread in the environment and can be isolated from soil, water, plant material and animals.

**Characteristics**
*Corynebacterium* are Gram-positive, slender, non-spore-forming rods. Some species may be slightly curved or have club ends. Cell dimension can be from 0.3 to 0.8 $\mu$m in diameter and 1.5 to 8.0 $\mu$m in length. *Corynebacterium* are facultatively anaerobic and catalase positive (Table 23.3).

**Taxonomy**
There are currently 88 species and 11 subspecies of *Corynebacterium* according to approved lists of bacterial names (Anon., 2005; Euzéby, 2005).

**Relevant foods**
*Corynebacterium* have been isolated from raw grated beetroot (Lopez Osornio and Chaves, 1997). They are prevalent in poultry slaughtering premises where they cross-contaminate equipment and surfaces from the skin follicles. *Corynebacterium* were dominant in the reception area of the slaughterhouse, along with *Micrococcus* and *Staphylococcus* and were found in both chillers (Ellerbroek, 1997).

Smear-ripened cheese has been shown to be dominated by *Corynebacterium casei* (50.2% of isolates), *C. mooreparkense* (20%) and *C. flavescens* (Brennan et al., 2002). In the cheeses, the *Corynebacterium* was able to out-compete *Brev. linens*. The *Brevibacterium* was inoculated onto half of the cheese samples but was not isolated from the inoculated or uninoculated cheese after ripening.

**Enumeration, detection and isolation**
*Corynebacterium* have been shown to be isolated using total viable count methodology using an incubation at 30 $^\circ\text{C}$ for 48 h prior to further identification
of isolated colonies (Ellerbroek, 1997; Lopez Osornio and Chaves, 1997). Milk plate count agar containing 5% w/v NaCl has been used to isolate bacteria including *Corynebacterium* spp. from cheese (Brennan *et al*., 2002).

**Growth and survival characteristics**

Water activity

*Corynebacterium mooreparkense* and *C. casei* grew below pH 4.9 in the presence of 8% NaCl.

### 23.3.4 *Micrococcus*

**General characteristics and taxonomy**

Natural habitat

*Micrococcus* are found in soils and fresh water, and frequently on the skin of humans and other animals.

Characteristics

*Micrococcus* are aerobic, Gram-positive cocci ranging in size from 0.5 to 2.0 μm in diameter. They occur in pairs, tetrads or clusters but not in chains. They are catalase positive and often oxidase positive although this reaction may be weak (see Table 23.3). They form pigmented red or yellow colonies and have an optimum growth temperature of 25 to 37°C. *Micrococcus* are halotolerant and grow in 5% salt.

Taxonomy

There are currently 10 species of *Micrococcus* on the list of prokaryotic names with standing in nomenclature (Euzéby, 2005): *M. agilis*; *M. antarcticus*; *M. halobius*; *M. kristinae*; *M. luteus*; *M. lylae*; *M. nishinomiyaensis*; *M. roseus*; *M. sedentarius*; *M. varians*.

Relevant foods

*Micrococcus* are involved in the ripening of different cheeses due to their proteolytic and lipolytic activities (García-López *et al*., 2000b). *Micrococcus* are also dominant in certain types of cured meats, e.g. dry cured hams and fermented sausages. They dominate in these environments due to their tolerance of low $a_w$. They contribute to the appearance of the products because of their ability to reduce nitrate to nitrite to form the classic pink pigmentation of cured meats. They also contribute to the flavour through the activity of their proteolytic enzymes.
Growth and survival characteristics

Water activity
Micrococcus can tolerate 10% NaCl.

23.4 Conclusions

A wide range of spoilage bacteria are present on proteinaceous foods stored chilled or at ambient conditions. A major group is the Gram-negative psychrotrophic organisms that include Achromobacter, Acinetobacter, Moraxella, Psychrobacter and Alcaligenes. These are all very similar in their growth and biochemical characteristics and make up a large proportion of the total viable count or total psychrotrophic count of chilled food products. Many of them do not cause distinctive spoilage attributes in their own right but contribute to the general breakdown of fats and proteins. Some of them can be considered to be specific spoilage organisms (SSOs) as in the case of Photobacterium phosphoreum which produce the distinctive off-odours in fish spoilage along with other important SSOs such as Shewanella putrefaciens.

With respect to the Gram-positive spoilage organisms mentioned here, most of them contribute to the characteristics of many foodstuffs rather than causing spoilage, they are involved in ripening of cheeses or contribute to the colour and flavour of fermented meats in the case of Micrococcus. Only Brochothrix is a specific spoilage organism of VP or MAP meat products and, even then, the potential for this organism to be present varies from study to study. Where it is present, it is able to produce sensory spoilage at lower levels than other spoilage flora present in the same conditions.

Many of these organisms grow at low temperatures and some of them have a degree of tolerance to low pH or elevated salt levels. However, none of them is particularly resistant to heating and should be eliminated by typical pasteurisation treatments.

23.5 Future trends

Currently, the food industry routinely uses total microbial counts or counts of indicator groups such as Enterobacteriaceae as an indication of sensory quality of a product. Yet it is generally accepted that the two measures do not always correlate. Often, a product that is acceptable with regard to sensory quality may have exceeded the microbiological criteria, suggesting that those organisms present are not responsible for spoilage. In contrast, some products that have a relatively low total count may be unacceptable with respect to product quality, suggesting lower levels of specific organisms may be
responsible for producing large levels of spoilage metabolites or in fact that the spoilage is due to non-microbial product deterioration.

To use just total microbial counts and counts of major groups may not be an appropriate measure of product quality. For example, in a meat product tested at 30 °C for total microbial count, it is likely that groups such as lactic acid bacteria will dominate the population whereas the Gram-negative psychrotrophic bacteria which may be more relevant to spoilage of these foods are not adequately enumerated. Use of a total viable count at lower temperatures may be preferable.

Predictive models as described in other chapters of this book have been used increasingly over the past few years in many aspects of food quality and shelf-life. Models exist for the main spoilage groups and the development of new models for some of these other spoilage organisms may be of relevance for future shelf-life determination.

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