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Microorganisms in food ecosystems

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1. Introduction

Food microbiology is a branch of microbial ecology. The importance of ecological concepts in understanding the occurrence and growth of microorganisms in foods is well recognised by food microbiologists (Mossel, 1971; ICMSF, 1980; Boddy and Wimpenny, 1992; Montville, 1997). These ecological principles are the foundations upon which modern quality assurance (Mossel and Struijk, 1992; Mossel et al., 1998), predictive modelling (McMeekin and Ross, 1996a; Whiting and Buchanan, 1997a) and risk analysis (Notermans and Teunis, 1996; van Gerwen et al., 1997) strategies have been developed to prevent outbreaks of food spoilage and foodborne disease. They are also the bases for the functional use of microorganisms in the production of fermented foods and beverages, and for their use as probiotic and biocontrol agents. Guided by commercial objectives, food microbiology has evolved into a field of study with a strong focus on groups of microorganisms and groups of commodities (ICMSF, 1996, 1998a) but, increasingly, less focus on the microbiology of the ecosystem as a whole. This trend to compartmentalise our knowledge needs to be balanced against the ''big picture'' and, ironically, is occurring at a time when other branches of microbial ecology (e.g., waters, soil, phyllosphere) are strengthening the totality of their microbiological studies and advancing fundamental understanding of their ecosystems.

Food microbiologists must remain aware of the totality of the ecosystem. The growth, survival and activity of any one species or strain, whether it be an unwanted spoilage or pathogenic organism, or a desirable biocontrol or probiotic organism, will, in most cases, be determined by the presence of other species. This presentation will commence with an outline of what information is needed to describe the microbiology of food ecosystems, and then discuss some limitations and new directions in obtaining this knowledge. It will conclude by applying the total ecological concept to two commodities, cheese and wine, thereby demonstrating microbiological profiles that are far more diverse and complex than is generally recognised.

2. What ecological information is needed?

To effectively manage the growth and activities of microorganisms in foods, the following ''layers'' of information and understanding are needed:

- Reliable data about the diversity and taxonomic identity of the species and strains that contaminate and colonise the food at every stage of production – from the raw material to the time the product is consumed.
- Quantitative data that describe the growth cycle and changes in populations of these species and

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strains throughout the production and retailing chain.
- Information about the spatial distribution of microbial species throughout the product.
- Biochemical and physiological explanation of the colonisation process.
- Impact of the so-called intrinsic, extrinsic, processing and implicit factors on microbial growth, survival and biochemical activity.
- Correlations between the growth and activity of individual organisms and product quality and safety.

Obtaining this information is a challenging task and, most likely, the food microbiologist will need to collaborate with other specialists in chemistry, biochemistry, electron microscopy and sensory evaluation.

3. Limitations of methodology

The aim of microbiological analysis, whether it be to profile the diversity of species occurring in a product or to determine the presence or absence of specific pathogens, should be to obtain the ecological truth. Is this achievable with the current portfolio of analytical methods? Despite many innovations in recent years (Giese, 1995; Cox and Fleet, 1997), the examination of foods for total or specific microflora follows the basic operations of (i) maceration/blending of the sample, (ii) dilution of the homogenate, (iii) plating of dilutions onto appropriate agar media, and (iv) isolation and identification of colonies. Pre-enrichment and selective enrichment culture before plating will be needed to recover species present at low populations (e.g., less than 100–500 cells/g). While this basic approach has had long-term acceptance and general success, there are inherent limitations that are worthy of re-emphasis.

3.1. Maceration

Microbial cells occurring on the surface of products suddenly become exposed to a vastly different chemical environment on maceration for microbiological analysis. Tissue extracts generated by maceration could be toxic or inhibitory to some microbial species, thereby giving erroneous data about the ecological composition of the natural product. The assumption that maceration is an ecologically sound prelude to microbiological analysis requires more rigorous scrutiny, especially since it is already known that extracts of vegetables, herbs and species are toxic to some microorganisms (Tassou et al., 1995; Kyung and Fleming, 1997). This question becomes especially relevant when analysing heterogeneous products (e.g., mixed salads and pastas), and when attempting to increase the sensitivity of detection by macerating greater quantities of sample.

3.2. Dilution

To many microbiologists, the dilution stage is an innocuous or harmless operation with respect to an ecological outcome. It should facilitate the dispersion of cell clumps and should not affect cell viability. Many years ago, bacteriologists realised the important influence of diluent composition and the time span between dilution and plating on viable plate counts and, without rigorous trialling, more or less standardised this operation using 0.1% peptone as the general diluent (Straka and Stokes, 1957). For the isolation of yeasts and moulds, no such standardisation has yet occurred and diluents commonly used range from distilled water, saline, phosphate buffer and 0.1% peptone, with various outcomes (Mian et al., 1997). In an effort to standardise this operation, the International Commission on Food Mycology has undertaken an international collaborative trial on diluents used for the analysis of yeasts in foods. Although this work is still going on, it has shown that the response of yeasts to any one diluent was repeatedly inconsistent. It was concluded that, apart from diluent composition and timing between dilution and plating, other factors such as stage of cell life cycle, cell stress prior to dilution, degree of cell clumping and aggregation, shear forces during shaking, presence of contaminating metal ions, pH and temperature could all impact on the survival of the yeast cells during the dilution operation. These conclusions could also apply to bacteria and filamentous fungi. In essence, the dilution operation may not be ecologically innocuous, as frequently assumed.

3.3. Enrichment cultures

Enrichment cultures are widely used in food analyses to enhance the cell population and detectability of minority species, especially pathogens such as *Salmonella*, *Listeria monocytogenes*, *Escherichia coli* and *Campylobacter jejuni*. The goal is to amplify populations as low as 1 cell/25 g of product to minimum levels of 10^5–10^6 cells/ml of culture for routine detection by plating, ELISA or nucleic acid probe technologies. Failure of the enrichment culture to give this minimum population within the prescribed incubation time leads to a false-negative result. Given the serious consequences of false-negative data in managing food safety, it is surprising to find very few studies on the growth kinetics of these pathogens in commonly used enrichment media. These growth kinetics and achievement of a detectable population are determined by many factors such as medium composition, time and temperature of incubation, degree of aeration, interference from food components, competition from non-target flora in the food, the possible influences of bacteriophages, and extent of any sublethal injury. We have frequently experienced occasions where *Salmonella* or *L. monocytogenes* have not reached 10^5–10^6 cells/ml in approved enrichment media. Ecological surveys of foods for the presence of foodborne pathogens are almost invariably based on the use of enrichment culture (e.g., Bryan and Doyle, 1995; Uyttendaele et al., 1997, 1998) but the reliability and limitations of this methodology are rarely questioned (Hawa et al., 1984; Fleet et al., 1991).

3.4. Anaerobes

It is not unreasonable to expect that obligate anaerobes, in addition to facultative anaerobes, will contribute to the microflora of many foods and beverages. Attention must be given to good anaerobic methodology to successfully isolate these organisms (Anderson and Fung, 1983). The literature would suggest that food microbiologists have not been particular rigorous in meeting these requirements and it is likely that significant anaerobic microflora have been overlooked in the ecology of many products. An illustration of this point is the recent discovery of the strictly anaerobic bacterial species, *Pectinatus cerevisiphilus*, *P. frisingensis*, *Selenomonas lacticifex* and *Megasphaera cerevisiae*, in spoiled, packaged beer (Jespersen and Jakobsen, 1996).

3.5. Unknown and non-culturable species

It is now well-established that many natural ecosystems such as soil, water, sediments and sludge, harbour microbial populations that greatly exceed those measured by culturing on agar media. Indeed, it is estimated that plate culturing techniques reveal only 1–10% (or less) of the true microbial population in those environments (Amman et al., 1995; Head et al., 1998). This anomaly is explained by two phenomena: (i) the presence of unknown, novel species that are not culturable by existing methods, and (ii) the presence of known species that are metabolically active and viable but have entered a non-culturable state. Understanding these phenomena has evolved from the use of molecular methods that can detect the non-culturable species. The principal strategy is based on analysis of the total DNA extracted from the ecosystem. Using PCR technology, microbial ribosomal (r)-DNA in the extract is specifically amplified, cloned, and then sequenced. The sequence data are compared with sequences in r-DNA data bases to give genus or species identification. Another approach uses fluorescently labelled r-DNA probes that allow detection and spatial location of targeted species in situ (Amman, 1995). By combining these methods with denaturing gradient gel electrophoresis to separate PCR amplified r-DNA fragments, it is even possible to profile specific changes in the composition of complex microflora as the ecosystem evolves with time (Ferris and Ward, 1997; Muyzer and Smalla, 1998). To date, these molecular technologies have received almost no application in studying the microbiology of food ecosystems. Consequently, we may not fully know the microbial composition of some foods, especially fresh products such as vegetables and meats, or complex fermented products (e.g., mould-ripened soft cheeses, meat sausages, cocoa bean fermentations) where a diversity of species may be present.

Food microbiologists are, however, familiar with the viable but non-culturable (VBNC) phenomenon, where adverse conditions such as nutrient depletion,

low temperature and other stresses can cause healthy, culturable cells to enter a phase which does not produce colonies on media that normally support their growth. Such cells, nevertheless, remain metabolically active and capable of causing infection. Given appropriate conditions, they can recover from their debilitated state (McKay, 1992; McDougald et al., 1998; Kell et al., 1998). While their presence is not evident by colony culture, they are detectable by assay with fluorescent stains that can measure membrane, DNA, RNA and other physiological functions (Kepner and Pratt, 1994; McFeters et al., 1995). The VBNC state has been experimentally induced in most foodborne pathogens, including *Salmonella* (Joux et al., 1997). *C. jejuni* (Rowe et al., 1998), *Vibrio vulnificus* (Oliver, 1991), *Vibrio parahaemolyticus* (Jiang and Chai, 1996) and *E. coli* (McDougald et al., 1998), but there is debate as to whether it occurs in nutrient rich food environments. However, it is not inconceivable that VBNC forms could occur in bottled waters or on the surfaces of fresh fruits and vegetables where nutrients may be limiting. The VBNC state should not be confused with the concept of sublethal injury (Ray, 1986). The main difference between the two phenomena is that sublethally injured cells will not grow on selective media but grow on non-selective media, whereas the cells in the VBNC state will not grow on either type of media. Both types of cells are capable of repair and resuscitation to the healthy state.

3.6. Quantitative data

The economic and social consequences of microorganisms in foods depend not only on the species present but, most importantly, on their quantitative populations. It is the number of microbial cells that ultimately determines whether or not the product will cause an outbreak of disease or develop an off-flavour. Also, populations are not static and can change both qualitatively and quantitatively throughout the production and retail chain. In many products, sequential development of species and strains occurs, with each organism impacting upon the chemical composition of the ecosystem according to its biochemical reactivity and, importantly, its maximum population. Reliable, confident assessments of public health and spoilage risks (Whiting and Buchanan, 1997a,b; De Roever, 1998; ICMSF, 1998b) require quantitative ecological data that take into consideration the dynamic nature of microorganisms in food ecosystems. Unfortunately, the vast majority of ecological studies in food microbiology fall significantly short of providing this quantitative knowledge. Many studies simply describe the isolation and identification of the ''most predominant'' species at one point in the product's history, while others have provided semiquantitative data by reporting the frequency of isolation of specific organisms. Population changes are mostly described in reference to microbial groups (e.g., total plate count, coliforms, psychrotrophs, lactic acid bacteria) rather than data for particular species or strains. Ecological surveys for pathogens such as *Salmonella*, *L. monocytogenes* and *E. coli* continue to be reported as isolation frequencies (e.g., % sample positive) with population levels (cells/g) rarely being mentioned. Further advances in understanding and managing the microbial ecology of foods will require more quantitative knowledge. An obstacle here is methodology, especially for the pathogens where enrichment culture forms the basis of the analyses. For some years, we have been using a centrifugation-plating technique which avoids cultural enrichment and enables direct enumeration of cells down to a detection limit of 1 cell/25 g of sample (Hawa et al., 1984; Fleet et al., 1991). This strategy not only gives fast, quantitative data, but also reveals the failures of enrichment methods.

4. Eco-biochemistry and physiology

Microbial growth in food ecosystems requires biochemical and physiological explanation to understand how specific microorganisms impact on food quality and to be able to develop processes for managing this growth. Fleet (1992) and Huis in't Veld (1996) have provided general discussion of these topics while more specific accounts have been reported for microorganisms associated with dairy (Champagne et al., 1994; Frank, 1997), meat (Dainty and Mackey, 1992; Gram and Huss, 1996; Jackson et al., 1997), vegetable (Lund, 1992; Brackett, 1997) and wine (Fleet, 1997, 1998) ecosystems. Excellent progress has been made in understanding the biochemical reactions associated with microbial growth

in many foods and in explaining how food properties regulate the growth response. However, much of our knowledge is derived from pure culture studies of microbial isolates in laboratory media, and more specific attention is needed to describe the unique reactions of the in-situ environment which will simultaneously harbour microbial cells in a diversity of physiological states-growing, non-growing, dead and autolysed.

In most food ecosystems, the in-situ environment will mean association of microbial cells with a solid substrate either through attachment or entrapment or both. In essence, the cells are immobilised and localised in high densities. Not much is known about the specific biochemical and physiological properties of high densities of microorganisms associated with solid systems. However, studies with microbial cells that have been specifically immobilised in alginate, polyacrylamide and other substrates clearly demonstrate that their properties of growth, survival, tolerances of extremes, biochemical activity and even cell composition can be significantly different from those of cells growing freely in liquid culture (Rehm and Omar, 1993; Norton and D'Amore, 1994). Thus, the in-situ solid phase associations found in food ecosystems could induce unique biochemical and physiological reactions. Because of the physical closeness of cells in these environments, cell–cell signalling and other communication mechanisms could also influence these responses.

For many foods, the in-situ association of microbial cells can extend over a long time-frame, thereby providing good opportunity for these cells to adapt to specific stresses of the environment and to adapt to an existence which, for the greater part, is essentially a resting or non-proliferating state. The growing or exponential state probably presents only a short time span throughout the total association with the ecosystem and, indeed, cyclical movement between exponential and non-proliferating stages is likely to occur, depending on nutrient availability and environmental factors. Nutrient limitation and end-product accumulation will induce transition to the non-proliferating phase. While the availability of carbon and nitrogen substrates may not be an issue in many foods, there could be limiting concentrations of sulphur, phosphorus, trace metals and vitamins (Boddy and Wimpenny, 1992). Also, nutrient availability must be considered in relation to the concepts of nutrient uptake and transport into the cells. It is well known in the fermentation of alcoholic beverages by yeasts that sugar transport into the yeast cells can be a significant rate-limiting reaction (Bisson, 1993; Fleet, 1998). The physiology and molecular biology of substrate transport will emerge as important issues in explaining the growth responses of microorganisms in food ecosystems.

Environmental factors (pH, temperature, water activity, preservative concentrations) that limit growth have been described for many organisms, especially the foodborne pathogens (ICMSF, 1996). Nevertheless, there are species in many important groups of microorganisms such as lactic acid bacteria (Stiles and Holzapfel, 1997), pseudomonads (Palleroni, 1993) and yeasts (Praphailong and Fleet, 1998) where this information is not complete and more studies are needed. These limiting values for microbial growth are the bases of predictive and risk assessment strategies now used in quality assurance programs (McMeekin and Ross, 1996a,b; van Gerwen et al., 1997). The uncertainties of the in-situ response can diminish confidence in these initiatives. A key issue, here, is the adaptive reactions of microorganisms on exposure to in-situ stresses. Through a range of molecular mechanisms, they can change their limits of tolerance to environmental pressures (Watson, 1990; Hall et al., 1995; Galinski, 1995; Rallu et al., 1996; Bearson et al., 1997). The problem is compounded by the fact that in-situ food environments rarely present a single stress, and microbial cells may be simultaneously exposed to a combination of stresses (e.g., low pH, low temperature; high NaCl concentration, low temperature, low pH; ethanol, acetic acid). The effect of the combined stresses on growth and survival may be additive or synergistic (interactive) where the outcome is significantly greater (or less in some cases) than the additive response (Gould and Jones, 1989; Gould, 1992; Leistner and Gorris, 1995). Further complications are introduced by the changing state of the ecosystem which, in many cases, is not static. The severity of the stress can change with time as will the physiological state of the microbial cells. Cells in the stationary phase of growth are, generally, more tolerant of stresses than exponential phase cells (Watson, 1990; Kolter et al., 1993). A related but overlooked outcome of the stress response is the changing chemical composition of the microbial cells

(e.g., changes in protein and lipid composition, increased concentrations of some amino acids, glycerol, trehalose) which could impact on sensory and other acceptability criteria for the food.

As noted already, weakly or non-proliferating cells in a resting or stationary phase of growth, probably dominate in many foods (e.g., consider the 10^6–10^8 cfu/g for many vegetable salads; the 10^6–10^8 cfu/g in many soft cheeses). These cells are still metabolically active and can conduct biochemical reactions that are distinctively different from cells growing exponentially (Kolter et al., 1993; Rees et al., 1995). The flavour-impacting reactions that occur during the production of many fermented foods and beverages are generally the consequences of microbial activity under non-proliferating conditions.

The non-proliferating phase is eventually followed by cell death and cell autolysis. Consequently, most ''long-term'' food ecosystems will harbour a substantial population of dead, autolysed cells. Autolysis is characterised by extensive loss of cellular structure and function, and enzymatic breakdown of cell proteins, nucleic acids, lipids and polysaccharides (Charpentier and Feuillat, 1993; Hernawan and Fleet, 1995; Kang et al., 1998). These degradation products become part of the ecosystem, serving as nutrients or antagonists for other microorganisms and, also, they impact on food sensory properties. Microbial autolysis is an important in-situ phenomenon, the biochemistry, physiology and significance of which have been very much overlooked in studies on the microbial ecology of foods.

5. Spatial heterogeneity

With few exceptions, most foods present an environment that is heterogeneous in physical structure and chemical composition. Consequently, microbial growth throughout the ecosystem is likely to be spatially heterogeneous. That is, different locations within the same food product could have significantly different microflora. Thus, the microbial ecology on the surfaces of cut or damaged vegetables and fruits will be different to that of uncut or undamaged product which have intact cuticle and waxy layers (Brackett, 1997). Similarly, the microbial populations on the outer surfaces of meat and dairy products will be different from those of the inner parts of the foods. Electron microscopy has revealed the potential for microbial cells to attach to food surfaces, become entrapped within the food structure and to grow as microcolonies and biofilms (Mattila and Frost, 1988; Marcellino and Benson, 1992; Morris et al., 1997; Parker et al., 1995, 1998). Cells within microcolonies and biofilms have increased resistance to processing conditions, as well as altered biochemical behaviour (Zottola and Sasahara, 1994; Costerton et al., 1995). Consequently, it is relevant to know their occurrence and location.

More significantly, it is important to know where particular species are located throughout the product. Until recently, it was not possible to obtain such information. The combined uses of fluorescence microscopy and confocal scanning laser microscopy with fluorescently labelled antibodies or nucleic acid probes now enable the in-situ localisation of specific organisms (Stringer et al., 1995). Using these technologies, it has been possible to demonstrate that *E. coli* 0157:H7 does not necessarily attach to the outer surfaces of radish sprouts or lettuce leaves and can become associated with cut surfaces and the inner parts of stomata and other tissue (Itoh et al., 1998; Seo and Frank, 1999). Similarly, it has been possible to pin-point the location of the yeast-like fungus, *Aureobasidium pullulans* on leaf surfaces (Li et al., 1997). Further application of these new methods will significantly advance and refine our in-situ knowledge of food ecosystems.

6. Microbial interactions

With the exception of highly processed products, most foods harbour a mixture of microorganisms which includes different species of bacteria, yeasts and filamentous fungi as well as strains within these species. In addition, bacteriophages and killer yeasts with virus like particles will also constitute part of the microflora. In the natural pursuit of survival, growth and dominance, interactions will occur between these different strains and species, the outcome of which will determine the population levels of any particular organism at any given time during the production and retailing time frame. Ecological theory describes the range of interactive associations

as competitive, amensalism or antagonism, commensalism, mutualism and parasitism or predation (Fredrickson, 1977; Boddy and Wimpenny, 1992) and these could occur both within and between different microbial groups (e.g., bacteria–bacteria; yeast–yeast; bacteria–yeast; bacteria–fungi, etc.). There are many examples of these types of interactive associations scattered throughout the food microbiology literature, but only a few will be mentioned here.

Antagonism is probably the best known microbial interaction in food ecosystems because it can be applied as a natural biocontrol strategy to enhance food quality and safety. The production of bacteriocins and their use to control spoilage and pathogenic bacteria have been extensively studied in recent years (Barnby-Smith, 1992; Muriana, 1996; Montville and Winkowski, 1997). While this is a classic example of ''bacteria–bacteria'' interaction, there are circumstances where bacteriocins will inhibit yeasts (Dielbandhoesing et al., 1998). The production of killer toxins by yeasts (Young, 1987; Wickner, 1993; Shimizu, 1993) is somewhat analogous to bacteriocin production by bacteria. These toxins are extracellular proteins or glycoproteins that disrupt cell membrane function in susceptible yeasts. While these antagonistic interactions were originally thought to be species-specific, there is now clear evidence that they occur across species in different yeast genera (Palpacelli et al., 1991; Llorente et al., 1997) and, indeed, they can kill various filamentous fungi (Walker et al., 1995). Moreover, there is no doubt that killer interactions between yeasts naturally occur in food ecosystems. A less recognised form of antagonism is the production of cell wall lytic enzymes. Examples include the production of β-$(1 \rightarrow 3)$-glucanases by bacterial and yeast species that destroy the $\beta(1 \rightarrow 3)$-glucans in the cell walls of fruit spoilage fungi such as *Penicillium expansum* and *Botrytis cinerea* (Wisniewski et al., 1991). Another less familiar form of microbial interaction that could be significant in food systems is the ability of yeasts and bacterial cells to agglutinate and aggregate. Most species of Enterobacteriaceae and some lactic acid bacteria will agglutinate *Saccharomyces cerevisiae* by reaction with the surface mannoproteins of the yeast (Mirelman et al., 1980).

In addition to antagonism, commensalistic microbial interactions frequently occur in food environments. Examples include the degradation of complex proteins and carbohydrates by some species to produce simple substrates for the growth of other species, utilisation of organic acids by yeasts and moulds to favour the growth of bacteria, the autolytic release of nutrients by dead cells, and the production of vitamins, specific amino acids, carbon dioxide and other micronutrients by some species that will assist the growth of other species (Boddy and Wimpenny, 1992).

Greater consideration needs to be given to the role of bacteriophages in food environments. Most studies concern their ability to destroy starter cultures of lactic acid bacteria used in milk fermentations, but this is a very specialised case (Frank, 1997). By analogy to other ecosystems, such as the marine environment (Arman and Kott, 1996; Wichels et al., 1998), foods are likely to harbour an enormous diversity of bacteriophages that could have a significant impact on the in-situ bacterial ecology. Generally, bacteriophages occur in the same habitats as their bacterial hosts (Sandmeier and Meyer, 1995). There are only a few reports on the isolation of bacteriophages from foods, including phages for *Pseudomonas* spp. from refrigerated meat (Greer and Dilts, 1990), *Leuconostoc oenos* from wines (Davis et al., 1985a, *Propionibacterium* spp. from cheese (Gautier et al., 1995) and *V. vulnificus* from oysters (De Paola et al., 1998). We have readily isolated phages against *Enterococcus durans* from cheese and against *Pseudomonas fluorescens*, *Ps. viridiflava* and *Ps. corrugata* from broccoli. Phage activity would certainly cause bacterial cell lysis and nutrient release in ecosystems and probably accounts for the variable population data often obtained from food samples.

7. Diversity in the microbial ecology of foods – case studies

When the ecological principles outlined in the previous sections are applied to specific food commodities, two conclusions become apparent: (i) the microbial ecology of most foods is more diverse and complex than generally thought; and (ii) there remain many gaps in knowledge and understanding. These points will be illustrated by reference to two products: cheese and wine.

8. Cheese

Cheese manufacture is a vast, economically important industry that produces a diverse range of products. It is an excellent example to consider in the context of microbial ecology, since it embraces all of the practical interests in food microbiology-fermentation, spoilage, safety, biocontrol, probiotics and, also, the issues and controversies surrounding the use of genetically modified microorganisms in food production.

8.1. Cheese processing

The basic steps in cheese manufacture are: (i) collection and processing of the milk which includes pasteurisation in most, but not all, cases; (ii) conversion of the milk into a cheese curd by the action of proteolytic enzymes and by fermentation with lactic acid bacteria that are usually added as a starter culture but, in some cases, are allowed to develop naturally, (iii) processing of the curd by heating, cutting, addition of sodium chloride and moulding, (iv) maturation (ripening) of the curd, generally, by storage under controlled humidity and temperature for periods ranging from a few weeks to many months; and (v) packaging and retailing (Chapman and Sharpe, 1990; Olson, 1995; Banks, 1998; Stanley, 1998a,b). To many microbiologists, the microbial ecology of the process is, principally, fermentation of the milk by lactic acid bacteria. Unfortunately, this is a gross underestimation of the total picture. Rather, it is the microbiological and biochemical changes that occur during maturation where distinctive and unique cheese character is developed and where the important issues of spoilage and safety emerge.

8.2. Microbiology of milk fermentation

Most research on cheese microbiology has focussed on the role of lactic acid bacteria in fermentation of the milk. Today, this process is largely accomplished by the inoculation of commercially produced starter cultures of *Lactococcus lactis* and, in some cases, *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* spp. *bulgaricus* (Law, 1982; Choisy et al., 1987; Stanley, 1998a,b). Since these species are added to milk at initial populations of 10^6 cfu/ml or more, the milk is pasteurised in most cases, and since the time of action is relatively short (1–4 h), the ecology of this fermentation is rather unremarkable. The fermentation starts as an aqueous system, but the curd which develops harbours very high populations (10^9–10^{10} cfu/g) of entrapped, essentially immobilised lactic acid bacteria in a non-proliferating phase of growth. The main ecological issue is the intervention of bacteriophages which destroy the lactic acid bacteria and disrupt the fermentation (Johnson and Steele, 1997). It is important that the fermentation commences rapidly and produces sufficient lactic acid, bacteriocins and other antagonistics to prevent the growth of spoilage and pathogenic bacteria (Marshall and Tamine, 1997). Most research is now directed toward understanding the biochemistry, physiology and molecular biology of the fermentation, especially the metabolism of lactose into lactic acid and the production of components that contribute to cheese flavour and texture (Marshall, 1992; Cogan, 1995; Marshall and Tamine, 1997; Johnson and Steele, 1997). This research is providing the basis for developing genetically modified starter cultures that have improved fermentation performance (Gasson, 1997).

8.3. Microbiology of maturation

It has been known for more than 50 years that many cheeses, especially the soft and semi-soft varieties, contain high populations (10^6–10^9 cfu/g) of microorganisms that are not the lactic acid bacteria added as starters to ferment the milk. These microorganisms have been loosely referred to as the secondary or adventitious microflora, and it is generally considered that they positively contribute to the maturation process (Law, 1982; Chapman and Sharpe, 1990; Stanley, 1998a,b). In some cases, microorganisms are deliberately added as part of the maturation process. Well known examples are use of the filamentous moulds *Penicillium camamberti* and *P. roqueforti* and the yeast like mould, *Geotrichum candidum*, in the maturation of Camembert, Brie and blue-veined cheeses, and use of *Propionibacterium shermanii* during maturation of Swiss, Emmenthal and Gruyére cheeses (Choisy et al., 1987). However, even in these cases, the maturation microflora comprises a complex mixture of wild bacteria, yeasts and

bacteriophages as well as any added organisms. Such complexity develops whether the cheese is produced from pasteurised or non-pasteurised milk, or whether or not starter cultures are used to ferment the milk (Gripon, 1987; Reps, 1987; Kaminarides et al., 1992; Macedo et al., 1993; Cuesta et al., 1996; Valdes-Stauber et al., 1997; Coppola et al., 1997; Giraffa et al., 1998). These organisms originate as natural contaminants of the process-coming from the milk, added proteolytic enzymes, brine (NaCl) solutions, surrounding air and contact with equipment. When conditions within the curd become favourable, they initiate growth.

Over the years, there has been significant progress in identifying the main species that comprise the maturation microflora, although the data are far from complete and are extremely variable, even for the one type of cheese. The bacteria associated with maturation are diverse and include: (i) the so-called non-starter lactic acid bacteria that comprise various species of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Enterococcus* (Giraffa et al., 1997); (ii) micrococci and staphylococci (e.g., *Micrococcus varians*, *Staphylococcus xylosus*) (Choisy et al., 1987; Bhowmik and Marth, 1990; Vivier et al., 1994); (iii) corynebacteria and brevibacteria (e.g., *Brevibacterium linens*) (Valdes-Stauber et al., 1997); (iv) propionibacteria (e.g., *Propionibacterium shermanii*, *P. freudenreichii*), and (v) various Enterobacteriaceae (de Boer and Kuik, 1987; Nooitgedagt and Hartog, 1988). Occasionally, spoilage species of clostridia (*Clostridium tyrobutyricum*), coliforms and *Bacillus*, and pathogenic species of *Salmonella*, *L. monocytogenes* and *E. coli* can develop during maturation (Chapman and Sharpe, 1990; ICMSF, 1998a; Altekruse et al., 1998). Process failure generally accounts for such problems but natural ''biocontrol'' of these adverse species by bacteriocins produced by other microflora (e.g., *Enterococcus* spp., Giraffa et al., 1995; *B. linens*, Eppert et al., 1997) is an important but underestimated function of the maturation process. There are suggestions that the yeast *Debaryomyces hansenii* can inhibit the growth of spoilage clostridia (Fleet, 1990).

Yeasts have emerged as significant organisms in the maturation process, although their precise role is not understood. Predominant species include *D. hansenii*, *Yarrowia lipolytica* and *Kluyveromyces marxianus*, which are often, but inconsistently, present in cheeses at populations of 10^6–10^9 cfu/g (Fleet, 1990; Jakobsen and Narvhus, 1996; Roostita and Fleet, 1996). We have found cheese isolates of *D. hansenii* to possess killer activity, the action of which is enhanced by the presence of NaCl (Kolak and Fleet, unpublished data; Llorente et al., 1997).

While the significance of bacteriophages in milk fermentation is well recognised, their potential impact on the bacterial ecology of maturation has not been considered. Gautier et al. (1995) have isolated bacteriophages of *P. freudenreichii* from Swiss cheese and we have isolated phages active against *E. durans* durans from blue-veined cheeses but, apart from these two observations, there appears to be no other reports on this topic.

The main intrinsic factors affecting the growth, survival and biochemical activities of the maturation flora are the moisture content, salt (NaCl) concentration and pH of the curd as well as availability of oxygen. These properties are not uniform throughout the curd and change with time. For example, in many cheeses the salt content is initially higher at the outer surface but progressively equilibrates as it diffuses into the inner parts of the curd. Although microorganisms are distributed throughout the curd (Marcellino and Benson, 1992; Parker et al., 1998), substantially higher populations are located on the outer surface because of the availability of oxygen. Thus, oxidative microorganisms (brevibacteria, micrococci, *D. hansenii*, *Y. lipolytica*, moulds) are more prevalent on the curd surface, while fermentative species (lactic acid bacteria, *K. marxianus*) are more predominant within the curd.

Some key biochemical reactions of the maturation microflora are fermentation of residual lactose, utilisation of lactic acid and enzymatic degradation of curd proteins and lipids, but these activities are moderated by the intrinsic properties of the curd, especially pH. The *Penicillium* spp. as well as yeasts such as *D. hansenii* and *Y. lipolytica* are particularly strong utilisers of lactic acid, especially at the surface of the curd, causing its pH to significantly increase over time. This decrease in acidity is also assisted by the strong proteolytic activity of many of the species present. Thus, the pH of the curd can increase from less than pH 5.0 at the beginning of maturation to values near neutrality during maturation and retailing. Accordingly, bacterial species that could not grow in the original curd because of low

pH, can now grow. Such species could include spoilage clostridia and pathogenic strains of *E. coli* and *L. monocytogenes*. Also, this bacterial growth is assisted by the greater availability of nutrients such as amino acids originating from proteolysis, and glycerol formed during lipolysis. Autolysis of yeasts and moulds is considered to be another important source of nutrients for bacterial growth (Fleet, 1990). Microbial autolysis, generally, is emerging as a most significant reaction in cheese maturation. Its occurrence is not restricted to yeasts and moulds, but also extends to lactic acid bacteria. It should be recalled that the curd harbours high populations of the starter lactic acid bacteria (e.g., *L. lactis*) that, generally, are not salt tolerant and quickly die off after brining. Autolysis of these cells and release of their intracellular contents, including active enzymes, will profoundly impact on the chemical and physical properties of the curd during maturation (Fox et al., 1996). A further consideration in relation to autolysis would be the altered composition of microbial cells when exposed to the acidic, high-salt stresses of the curd. In response to the salt stress, yeasts accumulate significant concentrations of glycerol whereas bacteria are likely to produce amino acids (Witter and Anderson, 1987). These substances will be released during cell autolysis.

8.4. Summary

Most cheeses harbour a diversity of wild microflora which evolves in a successional process throughout maturation and retailing. The subtleties of cheese character, as well as cheese shelf-life and safety, are uniquely determined by the composition and evolution of this flora, yet this ecology remains poorly described. Obtaining this information is important so that processes can be managed to encourage growth of the desired species and prevent the growth of undesirable species. This challenging task is complicated by the very complexity of the microflora, itself, and the limitations of cultural methodologies in analysing complex ecosystems. Molecular ecological techniques will be needed to help unravel the microbiological mysteries of cheese and, indeed, are likely to reveal the presence of a greater diversity of organisms. The dynamics of growth, survival and biochemical activity of this microflora will reflect an array of stress reactions in response to the changing conditions of salt and pH, but will be moderated by the phenomenon of cell immobilisation within the curd. Cell–cell interactions will play an important role in shaping the ecological profile, and current thinking must be expanded beyond the concept of bacteriocins to include the influences of bacteriophages, killer yeasts and cross-group (e.g., yeast–bacteria, bacteria–fungi etc) responses.

9. Wine

Almost 150 years ago, Louis Pasteur showed that wine was the product of an alcoholic fermentation of grape juice by yeasts. This section will show that the microbial ecology of the process now extends far beyond a simple alcoholic fermentation and involves complex interactive contributions from yeasts, filamentous fungi, lactic acid bacteria, acetic acid bacteria, other bacterial groups, and even bacteriophage (Fleet, 1993). It will demonstrate how recent approaches of quantitative ecological analyses and molecular methods have been significant in advancing this knowledge.

9.1. Wine processing

The basic operations in winemaking are: (i) crushing of the grapes and extraction of the juice; (ii) alcoholic fermentation of the juice by yeasts; (iii) optional malolactic fermentation (MLF) of the wine by lactic acid bacteria; (iv) bulk storage and ageing of the wine in cellars; (v) packaging and retailing. The production of fortified wines (ports, sherries) and sparkling wines involves additional specialized operations. Microbial growth and activity can be significant at all stages of wine production (Boulton et al., 1994; Fleet, 1997, 1998).

9.2. Microbiology of grapes

Grapes represent a principal source of microorganisms in wine production but, surprisingly, their ecology remains poorly researched and diminished by studies that have used inadequate sampling and cultural (enrichment) methods (Martini et al., 1996). Nevertheless, it is generally agreed that the surfaces of mature sound grapes harbour microbial populations at levels of 10^3–10^5 cfu/g consisting mostly of

yeasts and various species of lactic acid bacteria and acetic acid bacteria. Contrary to many early reports, the principal wine yeast, *Saccharomyces cerevisiae*, is not prevalent (<50 cfu/g) on sound grapes, thereby raising questions about its true origins in wine fermentation (Martini et al., 1996). Many intrinsic and extrinsic factors affect the occurrence and growth of microorganisms on the surfaces of grape berries, including rainfall, temperature, grape variety, berry maturity, location of berry in the bunch, physical damage due to bird, insect and mould attack, and the application of agrichemicals such as fungicides and insecticides. Moreover, the outer surface of the berry is covered by a waxy, cuticular layer which will affect the adherance of microbial cells and their ability to colonize the surface (Hardie et al., 1996). Scanning electron micrographs suggest localized colonization of the surface by yeast cells, especially where the surface layers are damaged (Belin, 1972). Unfortunately, there are no definitive studies on how microorganisms contaminate and colonize the surfaces of grapes. However, damaged grapes quickly develop populations of 10^6–10^8 cfu/g and harbour high populations of filamatous fungi (e.g., *Botrytis cinerea*) and acetic acid bacteria. These grapes have altered chemical composition and fungal enzymes that adversely affect wine flavours and colour, and can negatively impact on yeast growth during alcoholic fermentation and the growth at lactic acid bacteria during MLF (Donèche, 1993). Control of fungal growth on grapes is a key issue in wine making. Mycotoxin production and carryover into wine is a possibility that has not been adequately addressed. Fungicide residues on grapes can impact on the yeast ecology of alcoholic fermentation (Mlikota et al., 1996). Genetically modified grapes with anti-fungal levels of glucanase and chitinase (Giannakis et al., 1998) are sound in principle, but these enzymes also destroy wine yeasts. Biocontrol of grape fungi with selected, antagonistic species of yeasts is an interesting initiative (Suzzi et al., 1995).

9.3. Microbiology of alcoholic fermentation

The alcoholic fermentation is dominated by the growth of yeasts because of their ability to rapidly develop at the low pH (3.0–3.5) of the juice, and produce ethanol that inhibits the growth of filamentous fungi and bacteria. Many qualitative ecological studies over the past 100 years have shown that *S. cerevisiae* predominates in almost every wine fermentation, and collectively, they have given a misleading impression that this is the only species of relevance. The important contribution of other species to the overall fermentation has become evident only in recent years when more quantitative studies of yeast growth were undertaken (Fleet et al., 1984; Heard and Fleet, 1985, 1988).

The first 2–4 days of the fermentation are characterized by the growth of various species of *Kloeckera/Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia* and *Kluyveromyces* which achieve populations of about 10^7 cfu/ml before progressively dying off according to their tolerance of accumulating concentrations of ethanol. By this time, they have utilized sufficient sugars and amino acids in the juice, and generated sufficient amounts of end-products to have an imprint on wine character. *S. cerevisiae* also grows during these early stages but because of its unique and implicit tolerance of ethanol, continues to grow and predominate as the only species during the mid-to-final phases of the fermentation. The application of molecular techniques to the study of this ecology has revealed even further complexity (Querol and Ramon, 1996). It is now evident that each of the yeast species may be represented by several strains, and that successive strain evolution and death is characteristic of the ecological profile (Schutz and Gafner, 1993; Sabate et al., 1998). Strains with killer activity are commonly isolated from wine fermentations and also contribute to the changing profile (Shimizu, 1993). This profile and, hence, wine quality, can be moderated by a range of intrinsic, extrinsic and processing factors including grape juice composition, pesticide and fungicide residues, addition of sulphur dioxide, and degree of juice clarification and temperature. The temperature of fermentation, in particular, can have a profound impact. Low temperatures (10–15°C) increase the ethanol tolerance of *Kloeckera/Hanseniaspora* and *Candida* species to a point that they do not die off and become dominant contributors along with *S. cerevisiae* (Heard and Fleet, 1988). Red wines are fermented in contact with grape skins during the early stages and present the interesting possibility of biofilm development at the solid–liquid interface.

The origins of the yeasts responsible for the fermentation have attracted significant controversy. Many wines are produced by traditional, natural fermentation, where the yeasts originate from the grapes and winery equipment (e.g., crushers, pumps, hoses, fermentation tanks). For many years, it was believed that grapes were the principal source of *S. cerevisiae* that dominated the fermentation. It is now evident that this species largely originates from winery equipment. This equipment accumulates a residential microflora that is dominated by strains of *S. cerevisiae* because of selection through its ethanol tolerance. Using pulsed-field gel electrophoresis and restriction analysis of DNA, it has been possible to type the strain profile of these *S. cerevisiae* and demonstrate the specificity of their winery association and carry over into fermentations from one vintage to the next (Constanti et al., 1997; Sabate et al., 1998).

Because of their predominance, strains of *S. cerevisiae* have been commercialized as starter cultures for the inoculation and induction of wine fermentation. Inoculated fermentations tend to proceed more rapidly and predictably than their natural counterparts and are now practiced by many winemakers. There is a general assumption that the inoculated strain will overwhelm and suppress the growth of the natural flora and dominate the fermentation. Various quantitative and molecular ecological studies have now shown that these assumptions are not necessarily correct: the yeasts naturally present continue to contribute to the fermentation, and indeed there are many examples where the inoculated *S. cerevisiae* did not even dominate the fermentation (Heard and Fleet, 1985; Fleet, 1997).

With respect to biochemistry, it is pertinant to note that the greater part of the fermentation occurs after the yeast cells have entered the stationary phase (Bisson, 1993) and when a substantial proportion of the ethanol-sensitive yeast species have died and entered autolysis (Charpentier and Feuillat, 1993).

9.4. Malolactic fermentation

Almost 100 years ago, it was observed that many wines underwent a natural secondary fermentation about 2–4 weeks after completion of the alcoholic fermentation. This fermentation has been called the malolactic fermentation (MLF) and its ecology and biochemistry have been extensively studied (Davis et al., 1985b; Wibowo et al., 1985; Henick-Kling, 1995). It is conducted by acid and ethanol tolerant strains of lactic acid bacteria that survive the alcoholic fermentation or come from winery equipment. They are generally present at low or non-detectable levels (<10–100 cfu/ml) in the wine but, over 2–4 weeks, grow to populations of 10^7–10^8 cfu/ml. The most notable feature of this growth is the stoichiometric decarboxylation of L-malic acid to L-lactic acid and carbon dioxide, causing a deacidification of the wine and an increase in pH by about 0.3–0.5 unit. L-malic acid is a major component of most wines and originates from the grape. For high acid wines, such as those with an initial pH of 3.0–3.5, this deacidification gives a most desired improvement in wine sensory quality, but for less acid wines (e.g., pH 3.5–4.0), the deacidification depreciates sensory quality and, also gives the wine a final pH of 4.0 or higher. which makes it more prone to bacterial spoilage. Wines that have not undergone the MLF at the winery have a probability that this reaction will occur in the bottle. In such cases, the wine becomes gassy and cloudy and is spoiled.

Leuconostoc oenos is the main species that conducts MLF, and it is uniquely found in the winery environment. Molecular taxonomic studies have confirmed its uniqueness and this organism is now considered to be a new genus and species, *Oenococcus oeni* (Dicks et al., 1995). Other species of lactic acid bacteria, namely *Pediococcus parvulus*, *P. pentosaceus*, *P. damnosus*, and various *Lactobacillus* spp, can also conduct the MLF but, in addition, they give unpleasant off-flavours, and their growth is undesirable. Unlike *Leuc. oenos*, these species do not grow in wines below pH 3.5, so that control of pH is an effective mechanism for preventing their occurrence. There are interesting microbial interactive factors that affect the growth and ecology of lactic acid bacteria during MLF and these include the action of bacteriophages, bacteriocin production by the different strains of bacteria, autolysis of yeasts which provide nutrients for bacterial growth, but also the production of inhibitory substances by some strains of yeasts associated with the alcoholic fermentation (Fleet, 1997, 1998).

The MLF is a key process in modern winemaking. Its successful completion within a practical timeframe or its complete prevention have emerged as

major challenges. The unpredictability of naturally occurring MLF has led to the commercial availability of cell concentrates of *Leuc. oenos* for inoculation into wines to induce this reaction, but even this strategy can fail. Other innovations include the use of bioreactors charged with high densities (10^9–10^{10} cfu/ml) of immobilized cells of *Leuc. oenos*, and the development of genetically engineered yeasts with malolactic activity (Fleet, 1998).

9.5. Wine spoilage

Even after alcoholic and malolactic fermentations, wines may contain sufficient nutrients to support the growth of a range of spoilage yeasts and bacteria. Generally, these species will be tolerant of the combined effects of low pH (3.0–4.0) and high ethanol concentrations (10–15% w/v). Yeasts include oxidative species of *Pichia* and *Candida*, and fermentative species of *Zygosaccharomyces*, *Saccharomycodes* and *Brettanomyces/Dekkera*. Lactic acid bacteria include species of *Lactobacillus* and *Pediococcus* as mentioned already. The acetic acid bacteria, *Acetobacter pasteurianus* and *A. aceti*, are well known for their ability to oxidize ethanol at the wine–air interface to give vinegary (acetic acid) spoilage, but their involvement is probably more complex, since these species are frequently isolated from the middle of barrelled wines where little oxygen is present (Drysdale and Fleet, 1988). The application of molecular taxonomic methods will probably reveal the occurrence of novel species of acetic acid bacteria in wines. The potential for acid-tolerant, ethanol-tolerant species of *Bacillus* and *Clostridium* to grow in wines should not be underestimated. Various species of *Actinomyces*, *Streptomyces* and filamentous fungi can grow within the cracks and pores of wooden barrels and lenticels of corks, producing metabolites that give overpowering, deleterious taints when leached into the wine (Fleet, 1997, 1998).

9.6. Summary

Despite major advances in understanding the microbiology of wine fermentations, there are many areas where further information is required. The microbial ecology of the grape, especially the issue of fungal contamination and control, needs systematic study since it impacts on the rest of the process. It is now clear that yeast species other than *S. cerevisiae* are significant in alcoholic fermentation and more data are needed about their ecological and biochemical contributions, especially at the strain level. Bacteria associated with winemaking demonstrate unique tolerances to the harsh stresses of the wine environment and require more thorough study of their taxonomy and physiology using molecular methods. These studies are likely to reveal the presence of novel species.

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