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Recent advances in cheese microbiology

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Abstract

Microorganisms are an essential component of all natural cheese varieties and play important roles during both cheese manufacture and ripening. They can be divided into two main groups; starters and secondary flora. The starter flora, *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* used either individually or in various combinations depending on the cheese variety, are responsible for acid development during cheese production. Starters may be either blends of defined strains or, as in the case of many cheeses manufactured by traditional methods, composed of undefined mixtures of strains which are either added at the beginning of manufacture or are naturally present in the cheese milk. During cheese ripening, the starter culture, along with the secondary flora promote a complex series of biochemical reactions which are vital for proper development of both flavour and texture. The secondary flora is composed of complex mixtures of bacteria, yeasts and moulds, and is generally specifically associated with particular cheese varieties. In many cheese varieties, the action of the secondary flora contributes significantly to the specific characteristics of that particular variety. The secondary flora may be added in the form of defined cultures, but in many situations is composed of adventitious microorganisms gaining access to the cheese either from ingredients or the environment. During cheese manufacture and ripening, complex interactions occur between individual components of the cheese flora. Environmental factors within the cheese also contribute to these interactions. Elucidation of such interactions would greatly add to our understanding of the cheese ripening process and would enable a more targeted approach to starter/adjunct selection for cheese quality improvement. In the past, research in this area was dependent on classical microbiological techniques, which are very time consuming, not suitable for handling large numbers of isolates and generally not suitable to studies at sub species levels. However, developments in this area have recently undergone a major revolution through the development of a range of molecular techniques, which enable rapid identification of individual isolates to species and strain level. Application of such techniques to the study of cheese microbiology should lead to major advances in understanding this complex microbial ecosystem and its impact on cheese ripening and quality in the coming years.

Keywords: Cheese microbiology; Starters; Secondary flora; Molecular methods

1. Introduction

Cheesemaking began about 8000 years ago and now there are in excess of 1000 cheese varieties world wide (Sandine & Elliker, 1970), each unique with respect to its flavour and form. Manufacture of most cheese varieties involves combining four ingredients: milk, rennet, microorganisms and salt, which are processed through a number of common steps such as gel formation, whey expulsion, acid production and salt addition, followed by a period of ripening. Variations in ingredient blends and subsequent processing has led to the evolution of all these cheese varieties. While variations in processing parameters such as cook temperature and curd handling techniques play a major role in determining the characteristics of each cheese type, the cheese microflora play a critical and pivotal role in the development of the unique characteristics of each cheese variety.

The primary objective of cheese manufacture originally was to extend the shelf life and conserve the nutritious components of milk. This is achieved either by acid production and/or dehydration. Production of lactic acid by the starter flora during cheese manufacture results in a decrease in the pH of the milk and this, in combination with cooking and stirring, promotes syneresis of the curd and expulsion of whey (Walstra, 1993). While all acid coagulated cheeses are consumed fresh, most rennet coagulated cheese undergo a period of ripening which can range from about three weeks for Mozzarella to two years or more for Parmesan and extra-mature Cheddar. Cheese ripening is a complex

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process involving a range of biochemical reactions. High densities of microorganisms are present in cheese throughout ripening and they play a significant role in the maturation process (Cogan, 2000). The microflora of cheese may be divided into two groups: starter lactic acid bacteria and secondary microorganisms. Starter lactic acid bacteria are involved in acid production during manufacture and contribute to the ripening process. Secondary microorganisms do not contribute to acid production during manufacture, but generally play a significant role during ripening. The secondary microflora comprise of non-starter lactic acid bacteria (NSLAB) which grow internally in most cheese varieties and other bacteria, yeasts and/or moulds, which grow internally or externally and are usually unique to specific cheese varieties or closely related types.

1.1. Techniques used to study microorganisms in cheese

The main objective of the cheese microbiologist is to develop a clear view of the cheese microflora and its evolution during ripening. It is important that the complete flora is monitored and that the individual components are accurately identified and characterised. A wide range of techniques are available which can be divided into three groups: (1) methods which depend on cultivation followed by phenotypic characterisation; (2) methods which depend on cultivation followed by molecular characterisation; and (3) methods which depend on molecular characterisation only. All of these methods have associated advantages and disadvantages.

Classical cheese microbiology depends exclusively on the first approach. Cheese samples are homogenised and plated on a range of media, followed by phenotypic characterisation of a selection of the resulting isolated colonies. While selective plating does result in a useful overview of the microbial flora, only strains which can grow under the selective conditions used will be monitored and phenotypic characterisation may be unreliable, as phenotypic characters are dependent on culture and environmental conditions used in the assay. This approach is also limited in its sensitivity and is not suitable for routine analysis to subspecies or strain level.

The application of molecular techniques to characterise microorganisms overcomes many of the problems associated with phenotypic characterisation. While the most frequently used methods involve the characterisation of nucleic acid or proteins, fatty acid analysis (Johnsson, Nikkilä, Toivonen, Rosenqvist, & Laakso, 1995; Rementzis & Samelis, 1996; Decallonne, Delmee, Wauthoz, El Lioui, & Lambert, 1991; Kämpfer & Kroppenstedt, 1996), cell wall and membrane antigenic profiling (Sharpe, 1970), and whole-cell pyrolysis (Magee, 1993) have also proven useful. Protein characterisation includes whole-cell protein profiling (Samelis, Tsakalidou, Metaxopoulos, & Kalantzopoulos, 1995; Pot, Ludwig, Kersters, & Schleifer, 1994; Tsakalidou et al., 1994; Dicks & van Vuuren, 1987), enzyme homology studies (Ardö & Jönsson, 1994; Sasaki, Bosman, & Tan, 1995; Lortal, Valence, Bizet, & Maubois, 1997) and multilocus enzyme electrophoresis (Zahner, Momen, Salles, & Rabinovitch, 1989). A wide range of methods based on nucleic acid analysis have been developed and the advantages and disadvantages associated with each of these have recently been reviewed (Farber, 1996; O'Sullivan, 1999). All these molecular approaches require pre-growth of the microorganisms on various media and as such there is a danger that only a sub-fraction of the overall community is being assayed. The development of culture independent methods for microbial analysis has revolutionised microbial ecology (for review see O'Sullivan, 1999) and its application to cheese microbiology should lead to major new insights into this complex microbial ecosystem.

1.2. Factors influencing growth of microorganisms in cheese

A number of physical parameters control the growth of microorganisms in cheese during ripening, including water content, salt concentration and pH. The extent of variation in these parameters is influenced by the cheese making process.

1.2.1. Moisture

All microorganisms require water for growth and one of the most effective ways of controlling their growth is to reduce the available water either through dehydration or addition of some water soluble component such as sugar or salt. An increase in the moisture content of cheese leads to increased susceptibility to spoilage. The concept of water activity, (a_w) developed by Scott (1957), has provided a basis for an increasing understanding of microbe/water relations in food. Water activity is directly proportional to the moisture content of the cheese and inversely to the concentration of NaCl and other low molecular weight compounds (Esteban & Marcos, 1989). a_w is a thermodynamic concept defined as the ratio between the vapour pressure of the water present in a system (p) and that of pure water (p_0) at the same temperature:

$$a_w = p/p_0 \quad 0 \leqslant a_w \leqslant 1.$$

During the first stages of cheese manufacture, a_w is ~ 0.99, which supports the growth and activity of the starter culture. However, after whey drainage, salting and during ripening the prevailing a_w levels (0.917–0.988; Rüegg & Blanc, 1981) are significantly lower than the optimal requirements of starter bacteria. It is thus likely that a_w contributes to the control of their metabolic activity and multiplication (Brown, 1976).

Lactic acid bacteria (LAB) generally have higher a_w minima than other cheese bacteria; the minimum a_w for *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Propionibacterium freudenreichii* ssp. *shermanii* are 0.93, >0.98, >0.96 and 0.96, respectively (Weber & Ramet, 1987). A depression in a_w during cheese ripening occurs due to water loss by evaporation, salt, and the hydrolysis of proteins to peptides and amino acids and triglyceride to glycerol and fatty acids; the hydrolysis of each peptide or ester bond requires one water molecule. Since significant proteolysis occurs in cheese, the unbound moisture level will decrease during maturation (Cogan, 2000). Control of moisture loss is effected by increasing the relative humidity in the ripening room or by packing the cheese in wax or plastic. There may be variation in the a_w values in different zones in cheese; for brine-salted hard and semi-hard cheeses values are usually higher toward the centre; Cheddar cheese is dry-salted and vacuum packed and, therefore, no loss of moisture will occur and no change in a_w since the salt is uniformly distributed in the cheese.

1.2.2. Salt

Salt and a_w are very much interrelated and the inhibition of starter and spoilage bacteria by salt mainly reflects the effect of salt in reducing a_w. The concentration required depends on the nature of the food, its pH and moisture content, but, generally, 10–100 g/kg is sufficient. The a_w depression which occurs, when salt (or any solute) dissolves in water, is the major inhibitory factor (Rüegg & Blanc, 1977). The relationship between salt concentration (x, g/kg of cheese) and a_w is described by Cogan (2000) as:

$$a_w = -0.0007x + 1.0042.$$

The correlation co-efficient (r^2) was 0.997. The salt concentration in cheese ranges from 0.7 to 7 g/100 g, corresponding to a_w values of 0.99–0.95, respectively. Many microorganisms could grow under such conditions, thus illustrating the contribution of other interacting factors to the inhibition of microbial growth. The concentration of salt dissolved in the cheese moisture (i.e. % S/M) rather than the actual concentration of salt added by dry- or brine-salting of the cheese determines the inhibitory effect of salt.

1.2.3. pH and organic acids

The optimum pH for the growth of most common bacteria is around neutral and growth is often poor at pH values <5.0. Due to the accumulation of organic acids, cheese curd post-manufacture has a pH ranging between 4.5 and 5.3; such low pH values will not allow the survival of acid-sensitive species. The real inhibitor is thought to be the undissociated form of the organic acid (Beuchat & Golden, 1989). The principal organic acids found in cheese are lactic, acetic, and propionic acids which have pK_as of 3.08, 4.75 and 4.87, respectively, such that lactic acid is the least and propionic acid the most effective inhibitor at the same concentration at the pH of cheese. However, lactate in cheese curd is invariably present at much greater concentrations than either of the other two acids, except in the case of Swiss cheese where the concentration of propionic acid may be higher than lactic acid in the ripened cheese (Steffen, Eberhard, Bosset, & Rüegg, 1993).

1.2.4. Ripening temperature

The microorganisms involved in cheese manufacture and ripening are either mesophilic or thermophilic having temperature optima of ~30°C or 42°C, respectively. The temperature at which cheese is ripened is a compromise between the need to promote ripening reactions and control growth of the desirable secondary flora, and the need to prevent the propagation of potential spoilage and pathogenic bacteria. Higher temperatures promote accelerated ripening (Folkertsma, Fox, & McSweeney, 1996), but the changes to body and flavour are often detrimental.

1.2.5. Redox potential

The redox or oxidation–reduction potential (E_h) is a measure of the ability of a chemical/biochemical system to oxidise (lose electrons) or reduce (gain electrons). An oxidised or reduced state is indicated by a positive or negative mV value, respectively. The E_h of milk is about +150 mV, while that of cheese is about −250 mV. While the exact mechanism of the reduction in E_h in cheese is not fully established, it is most probably related to fermentation of lactic acid by the starter during growth, and the reduction of small amounts of O_2 in the milk to water (Crow et al., 1995a). As a consequence of these reactions, the cheese interior is essentially an anaerobic system, which can only support the growth of obligatory or facultatively anaerobic microbes. In different microbial cultures, E_h may range from about +300 mV for aerobes to less than −400 mV for anaerobes (Brown & Emberger, 1980). The E_h of cheese is one of the major factors in determining the types of microorganisms which will grow in cheese; therefore, obligate aerobes, such as *Pseudomonas*, *Brevibacterium*, *Bacillus* and *Micrococcus* spp. are excluded from growth in the interior of the cheese. Bacteria which develop on the cheese surface are predominantly obligate aerobes.

1.2.6. Nitrate

Nitrate (NO_3^-) is added to the cheese milk as saltpetre (KNO_3) or $NaNO_3$, in the production of some cheeses, especially Dutch-type varieties, such as Edam and

Gouda to prevent growth of *Clostridium tyrobutyricum* which ferments lactate to butyrate, H_2 and CO_2:

$$2\,\text{Lactate} \rightarrow 1\,\text{Butyrate} + 2CO_2 + 2H_2.$$

The H_2 and CO_2 are responsible for the large holes present in the cheese while the butyrate is responsible for off-flavour formation. In these cheeses, the rate of NaCl migration is relatively slow and equalisation of the salt concentration throughout the cheese can take several weeks. In addition, the moisture content is high. Therefore, growth inhibition of spoilage microorganisms such as *C. tyrobutyricum* is necessary prior to achieving salt equilibrium and nitrate fulfils this function. During ripening nitrate is reduced to nitrite (the actual growth inhibitor) by the indigenous xanthine oxidase present in the milk/curd (Nieuwenhoff, 1977). Nitrite does not affect the growth of LAB, but does inhibit propionibacteria essential for eye formation in Emmental cheese and thus is not suitable for control of *C. tyrobutyricum* in cheese where growth of propionibacteria is required. Nitrite can react with aromatic amino acids in cheese to produce nitrosamines, many of which are carcinogenic. The reaction is pH dependent, occurring preferentially in the pH range of 2–4.5. Cheese has a higher pH and this slows down the reaction leading to the formation of nitrosamines. Most of the nitrate, which is added during cheesemaking is found in the whey or diffuses into the brine. By the time the cheese is ready for consumption, the levels of nitrite is usually well below the permissible level of 50 mg/kg as laid down in Dutch regulations.

1.3. Starter bacteria

The primary function of starter bacteria is to produce acid during the fermentation process; however, they also contribute to cheese ripening where their enzymes are involved in proteolysis and conversion of amino acids into flavour compounds (Fox & Wallace, 1997). Starter bacteria could be defined as isolates which produce sufficient acid to reduce the pH of milk to <5.3 in 6 h at 30–37°C. Starter bacteria are either added deliberately at the beginning of manufacture or may be natural contaminants of the milk, as is the case in many artisanal cheese varieties made from raw milk. They grow during manufacture and typically attain densities of 10^8 cfu/g within hours of the beginning of manufacture. Either mesophilic or thermophilic starter cultures are used, depending on the cheese being manufactured; mesophilic cultures are used in the production of Cheddar, Gouda, Edam, Blue and Camembert, while thermophilic cultures are used for high temperature (50–55°C) cooked hard cheeses such as Emmental, Gruyère, Parmesan and Grana. Starter bacteria encountered most often are members of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus*.

Both mesophilic and thermophilic cultures can be subdivided into mixed (undefined) cultures in which the number of strains is unknown, and defined cultures, which are composed of a known number of strains. Undefined or mixed-strain mesophilic cultures are mainly composed of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*, which may include citrate metabolising strains, for flavour production. A survey of 113 isolates from a commonly used undefined culture indicated that a range of strains having different plasmid profiles, acid producing capabilities and phage sensitive ranges were present, confirming that such cultures are indeed composed of mixed strains (Lodics & Steenson, 1993). Daly (1983) reviewed the use of mesophilic cultures in the dairy industry. Defined mesophilic cultures are now used for the manufacture of most Cheddar cheese world wide. Thermophilic starters are composed of either single or multiple strains of *S. thermophilus* and thermophilic lactobacilli such as *Lb. delbrueckii* ssp. *delbrueckii*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. delbrueckii* ssp. *lactis* or *Lb. helveticus*. Undefined or mixed thermophilic cultures are used in the small-scale production of Gruyère, Emmental and Grana cheeses of France, Switzerland and Italy. These cultures are generally produced, by incubating whey from the previous day's production, overnight at 40–45°C. The flora is composed primarily of *S. thermophilus*, though enterococci, lactococci and leuconostocs may also be present, and several species of lactobacilli, including *Lb. helveticus*, *Lb. delbrueckii*, *Lb. acidophilus*, and *Lb. fermentum*. A similar natural whey starter containing thermophilic LAB is used to make Parmesan (Parmigiano Reggiano), a hard, cooked cheese from Northern Italy. This starter consists primarily of undefined strains of *Lb. helveticus* (75%) and *Lb. delbrueckii* ssp. *bulgaricus* (25%); however, their precise composition is uncontrolled and significant variations at strain level are likely to occur (Coppola et al., 1997; Nanni et al., 1997). Varieties such as Pecorino Sardo (from Sardinia) and Majorero (from Spain) are manufactured without the deliberate addition of any starter cultures and rely on lactic acid bacteria, which are naturally present in the cheese milk to reduce the pH during manufacture.

A recent survey involved the characterisation of 4379 bacterial isolates from 35 European artisanal dairy products, including 25 cheeses (Cogan et al., 1997). The study indicated that 38% of the isolates were *Lactococcus*, 17% *Enterococcus*, 14% *S. thermophilus*, 12% mesophilic *Lactobacillus*, 10% *Leuconostoc* and 9% thermophilic *Lactobacillus*. Acid production by the isolates varied considerably. Of the *Lactococcus* and mesophilic *Lactobacillus* isolates, only 8% and 2%, respectively, produced sufficient acid to reduce the pH of milk to <5.3 in 6 h at 30°C. In contrast, 53%, 32% and 13% of the *S. thermophilus*, thermophilic *Lactobacillus* and *Enterococcus* isolates, respectively, reduced the pH

to 5.3. This indicates that most of the mesophilic bacterial isolates from artisanal cheeses do not produce sufficient acid, when tested individually, to make cheese and as such do not conform to the definition of "starter" bacteria. It is possible, that if these isolates are prt$^-$ that they may contribute to acid production when used in combination with other typical starter strains, this however has not been investigated. Many of the thermophilic isolates were good acid producers and, hence, suitable as starter cultures. The presence of enterococci as a natural part of the flora of some artisanal products has provoked much debate, as there is some evidence that they may be associated with clinical infections in immuno-compromised patients or patients undergoing surgery (Boyce et al., 1992). The fact that some strains are also vancomycin resistant is also a cause for concern (Noskin, 1997). Despite these considerations, they are found in high densities in many cheeses, particularly those made around the Mediterranean, and are felt to have a very positive role in flavour development.

The LAB present in starter cultures and some of the biochemical characteristics, which can be used to differentiate them, are shown in Table 1. The taxonomy of starter LAB has recently undergone significant revision, due to the reclassification of some species following detailed genotypic characterisation (Schleifer & Klipper-Bälz, 1987). Former species appellations are included in Table 1 to facilitate interpretation of the earlier literature. Cell morphology, isomer and amount of lactic acid produced and citrate metabolism capabilities (mesophilic cultures only), ability to grow at 10°C and 45°C, utilisation of glucose, galactose and lactose can readily distinguish between the species listed. *Leuconstoc* spp. is differentiated from the other starter bacteria by their ability to metabolise sugars by the phosphoketolase pathway and their poor ability to grow in milk. This is probably due to the lack of a proteinase system to degrade milk proteins and release growth substrates. This can be overcome by addition of 0.3% (w/v) yeast extract as a stimulant; if lactose cannot be catabolised, 1% (w/v) glucose may also be necessary.

Production of homogeneous, high quality Cheddar cheese requires uniform lactose fermentation and proteolysis. As the rate and extent of both these processes are dependent upon temperature and salt concentration, the latter should be as uniform as possible (Thomas & Pearce, 1981). The rate of lactose fermentation by starters in Cheddar is dependent on the S/M levels in the fresh cheese (Turner & Thomas, 1980). At low S/M levels, all the lactose (4%) was utilised within eight days, while starter metabolism was curtailed at 6% S/M such that lactose concentrations in the cheese remained high for several weeks post-manufacture. This residual lactose was utilised by non-starter lactic acid bacteria (NSLAB), but there was no strong

Table 1
Taxonomy and some distinguishing characteristics of the LAB found in starter cultures (from Cogan & Hill, 1993)[a]

Organism	Old name	Shape	Reduction of litmus in milk before coagulation	Lactic acid produced in milk (%)[b]	Isomer(s) of lactate produced	Metabolism of citrate	NH_3 from arginine	Growth			Fermentation[c]		
								10°C	40°C	45°C	Glu	Gal	Lac
Streptococcus thermophilus	Unchanged	Cocci	–	0.6	L	–	–	–	+	+	+	–	+
Lactobacillus helveticus	Unchanged	Rods	–	2.0	DL	–	–	–	+	+	+	+	+
Lactobacillus delbrueckii ssp. *bulgaricus*	*Lb. Bulgaricus*	Rods	–	1.8	D	–	–	–	+	+	+	–	+
Lactobacillus delbrueckii ssp. *lactis*	*Lb. lactis*	Rods	–	1.8	D	–	–/+	–	+	+	+	+/–	+
Lactococcus lactis ssp. *cremoris*	*Str. cremoris*	Cocci	+	0.8	L	–	–	+	–	–	+	+	+
Lactococcus lactis ssp. *lactis*	*Str. lactis*	Cocci	+	0.8	L	+/–	+	+	+	–	+	+	+
Leuconostoc lactis	Unchanged	Cocci	–	<0.5	D	+	–	+	–	–	+	+	+
Leuconostoc mesenteroides ssp. *cremoris*	*Leuc. cremoris*	Cocci	–	0.2	D	+	–	+	–	–	+	+	+

[a] +/–, majority of strains positive; –/+, majority of strains negative.
[b] These are approximate values; individual strains vary.
[c] Glu, glucose; Gal, galactose; Lac, lactose.

correlation between residual lactose and growth of NSLAB, indicating that they use other energy sources besides lactose and/or other factors for growth. According to Martley and Lawrence (1972) strains of *L. lactis* ssp. *lactis* survived much better than *L. lactis* ssp. *cremoris* strains in the presence of 4–5% NaCl. One of the main roles of starter bacteria is to provide a suitable environment, with respect to redox potential, pH and moisture content in the cheese, which allows enzyme activity from the rennet and starter, and growth of secondary flora to proceed favourably. The temperature during manufacture and the S/M must be controlled to ensure that the activity of the starter is sufficient to allow the required pH to be reached within one day of manufacture.

Starters make the only significant contribution to the microbial biomass of LAB in the young curd. This relatively high starter biomass represents considerable biocatalytic potential for cheese ripening reactions, which could be modulated through autolysis of the starter cells. The major autolytic activity in lactococci is due to a muraminidase (Mou, Sullivan, & Jago, 1976; Niskasaari, 1989). Autolysis of starter cells may be influenced by the NaCl concentration and associated salt in moisture values of cheese (Bie & Sjostrom, 1975), and varying manufacturing conditions, such as elevated cook temperatures (Lowrie, Lawrence, & Peberdy, 1974). Should the starter reach too high a population or survive too long, flavour defects such as bitterness, which mask or detract from cheese flavour are produced. In a study by Wilkinson, Guinee, O'Callaghan, and Fox (1994) in which starter viability and release of intracellular starter enzymes into cheese was monitored, it was concluded that *L. lactis* ssp. *cremoris* strains had different autolytic patterns. Higher levels of free amino acids were produced by the most autolytic strain (AM2) relative to the least autolytic strain (HP). Crow et al. (1995a) reported that intact starter cells fermented lactose, removed oxygen and probably initiated a number of flavour reactions, while peptidolytic processes were accelerated by autolysed cells. In young curd, the ratio of intact to lysed cells was deemed important in the control of Cheddar cheese ripening (Crow, Martley, Coolbear, & Roundhill, 1995b). The end-products of peptidolysis, amino acids, accumulate faster following starter autolysis, and are major precursors of the compounds required for cheese flavour production (Sandine & Elliker, 1970; Urbach, 1995). These reactions provide not only important flavour compounds, but also precursors for further flavour-generating reactions in cheese. Autolysis of *Lb. helveticus* has been reported in Grana cheese (Botazzi, Battistotti, Vescovo, Rebecchi, & Bianchi, 1992) and in experimental Swiss type cheese (Valence, Richoux, Thierry, Palva, & Lortal, 1998). The application of strains of *S. thermophilus* and/or *Lb. helveticus* as starter adjuncts in the manufacture of Cheddar cheese is now quiet common. Kiernan, Beresford, O'Cuinn, and Jordan (2000) demonstrated that *Lb. helveticus* autolysed very rapidly in Cheddar cheese and resulted in significantly higher levels of free amino acids and improved the flavour of the cheese.

1.4. Secondary flora

1.4.1. Non-starter lactic acid bacteria (NSLAB)

NSLAB are mesophilic lactobacilli and pediococci, which form a significant portion of the microbial flora of most cheese varieties during ripening. They are not part of the normal starter flora; they generally do not grow well in milk (Cogan et al., 1997), and do not contribute to acid production in the cheese vat. Lactobacilli are traditionally divided into three groups on the basis of being either (I) obligatory homofermentative, (II) facultatively heterofermentative, or (III) obligatory heterofermentative (Kandler & Weiss, 1986). The NSLAB lactobacilli regularly encountered in cheese are members of the facultatively heterofermentative group (II), and are thus sometimes referred to as facultatively heterofermentative lactobacilli (FHL). Many species of mesophilic lactobacilli have been isolated from cheese, but those most frequently encountered are *Lb. casei*/*Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* and *Lb. curvatus* (Jordan & Cogan, 1993; Coppola et al., 1997; Fitzsimons, Cogan, Condon, & Beresford, 1999). *Pediococcus acidilactici* and *Pe. pentosaceus* are the most frequently encountered pediococci in cheese.

The NSLAB flora of many cheese varieties has been reported in the literature in recent years. The bacterial flora of ripened semi-hard cheeses, Jarlberg and Norvegia from Norway and Herrgard, Greve and Gouda from Sweden, manufactured from pasteurised milk was found to be dominated (76%) by mesophilic lactobacilli (Linberg, Christiansson, Rukke, Eklund, & Molin, 1996), predominantly *Lb. paracasei*/*Lb. casei* and *Lb. rhamnosus*. The NSLAB populations of many traditional Greek cheeses are dominated by *Lb. plantarum*. In Feta and Teleme cheeses 47.8% and 65.8% of the lactobacilli isolated were identified as *Lb. plantarum* (Tzanetakis & Litopoulou-Tzanetaki, 1992). *Lb. plantarum* and *Lb. paracasei* comprised 18.4% and 15.8% of the flora of Kefalotyri cheese ripened for four months (Litopoulou-Tzanetaki, 1990). *Lb. plantarum* (56.9%) and *Lb. paracasei* (37.2%) were the main species isolated from Tenerife goat's milk cheese. *Lb. plantarum* was present in high proportion in two day-old cheese but decreased during ripening, whereas *Lb. paracasei* present initially at low densities increased during ripening and was the dominant *Lactobacillus* species in 60 day-old cheese (Zarate, Belda, Perez, & Cardell, 1997). *Lb. plantarum* predominated during cave ripening (16–120

days) of Cabrales cheese (Nunez, 1978), while *Lb. plantarum* and *Lb. brevis* were the main lactobacilli identified in Afuega'l Pitu cheese (Cuesta, Fernández-García, González de Llano, Montila, & Rodríguez, 1996). The most common *Lactobacillus* species identified in Majorero cheese was *Lb. plantarum*, although *Lb. paracasei*, *Lb. brevis* and *Lb. fermentum* were also present in small amounts (Fontecha et al., 1990). Most of the lactobacilli isolated from Mahon cheese were also identified as *Lb. plantarum* (Ramos, Barneto, Suarez, & Inigo, 1982). *Lb. paracasei* and *Lb. plantarum* were the most frequently isolated NSLAB from Arzua cows' milk cheese (Centeno, Cepeda, & Rodriguez-Otero, 1996), a similar population was observed in Armada cheese (Tornadijo, Fresno, Bernardo, Martin Sarmiento, & Carballo, 1995). Few data are available on the NSLAB flora from Portuguese cheese; however, lactobacilli predominated in Serpa, while *Lb. paracasei*, and *Lb. plantarum* were prevalent in Serra da Estrela cheese (Roserio & Barbosa, 1996). *Lb. paracasei* was the dominant *Lactobacillus* isolated from the Italian cheese Montasio. *Lb. paracasei* was not detected immediately post manufacture, but grew to levels of 10^7 cfu/g during the first month of ripening and remained at that level up to 120 days (Lombardi, Cattelan, Martina, & Basso, 1995). Prolonged ripening times are a specific feature of Parmigiano Reggiano. The evolution of the bacterial flora of this cheese was studied up to 24 months of ripening. *Lb. paracasei*, *Lb. rhamnosus* and *P. acidilactici* were the dominant NSLAB flora. During ripening densities decreased from $\sim 10^8$ cfu/g at five months of ripening to $\sim 10^4$ cfu/g at 24 months (Coppola et al., 1997). *Lb. plantarum* and *Lb. paracasei* were identified in Casu Axedu (Ledda, 1996) and Fontina (Cocconcelli, 1996a) cheeses. *Lb. fermentum* and *Lb. plantarum* were identified in Toma cheese (Cocconcelli, 1996b).

Swiss-type cheeses include Emmental, Gruyère, Appenzeller, Maasdamer, Jarbergost and Comté. These cheeses may be manufactured from raw, thermized or pasteurised milks. Gilles, Turner, and Martley (1983) reported that NSLAB grew to cell densities in excess of 10^6 cfu/g in Swiss-type cheese during the warm room incubation period. The evolution of NSLAB in Swiss-type mini-cheeses made from raw and pasteurised milks was reported (Demarigny, Beuvier, Dasen, & Duboz, 1996, Beuvier et al., 1997). NSLAB levels at the end of ripening were higher in raw milk cheese (10^8 cfu/g) than in cheese made from pasteurised milk (10^6 cfu/g). The NSLAB population of young cheese was composed primarily of *Lb. paracasei*, *Lb. plantarum* and *Lb. brevis*, but as the cheese ripened *Lb. paracasei* became the dominated member of the NSLAB flora. A recent report on industrially produced Emmental cheese made from thermised milk indicated that 56–88% of the isolates were members of the *Lb. paracasei* group (Thierry, Salvat-Brunaud, Madec, Michel, & Maubois, 1998).

A study of UK-manufactured Cheddar after six and nine months of ripening indicated that *Lb. paracasei*/*Lb. casei* and *Lb. plantarum* were the dominant species; however, *Lb. curvatus*, *Lb. brevis*, *Lb. helveticus*, *Lb. fermentum*, *Lb. bifermentans*, *Lb. buchneri*, *Lb. parabuchneri*, *Lb. farciminis* and *Lb. kefir* were also isolated (Williams & Banks, 1997). Studies on the NSLAB populations of eight week-old commercial Irish Cheddar revealed that the NSLAB flora consisted of 55% *Lb. paracasei*/*Lb. casei*, 28% *Lb. plantarum* and 14% *Lb. curvatus* (Jordan & Cogan, 1993). A similar study was undertaken by Fitzsimons et al. (1999) with mature Irish Cheddar. Phenotypic characterisation of 331 isolates from 14 premium quality and three sensorially defective cheeses indicated that 96.4% of the isolates were *Lb. paracasei*, 2.1% *Lb. plantarum*, 0.3% *Lb. curvatus*, 0.3% *Lb. brevis* and 0.9% were unidentified. In the latter study, RAPD was used to identify the dominant NSLAB strain groups in nine cheeses; an average, of seven strains per cheese were found.

The data for many of the cheeses studied indicate that the dominant species change during ripening, with *Lb. paracasei* predominating later in ripening (Jordan & Cogan, 1993; Lombardi et al., 1995; Demarigny et al., 1996; Beuvier et al., 1997; Zarate et al., 1997; Fitzsimons et al., 1999). A study of the *Lactobacillus* community and the dynamics of the constituent populations during a 39 week ripening period for Cheddar cheese revealed temporal changes in the species and strains of NSLAB. A mixture of *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*, and unidentified strains was found up to six weeks maturation; thereafter only *Lb. paracasei* strains were isolated. Evidence for the appearance, disappearance and recurrence of different strains of *Lb. paracasei* was observed during ripening (Fitzsimons, Cogan, Condon, & Beresford, 2001).

The energy source used by NSLAB for growth has not yet been clearly defined, since at the time of most active growth of NSLAB, lactose has usually been exhausted (Turner & Thomas, 1980). NSLAB can transform the L isomer of lactate to the D isomer (Thomas & Crow, 1983) but it is unlikely to act as an energy source. Citrate is present in young Cheddar cheese at ~ 8 mmol/kg and some investigators have suggested that it maybe a potential energy source for NSLAB (Jimeno, Làzaro, & Sollberger, 1995a). However, one study in Cheddar indicated that there was very little if any utilisation of citrate in three of four cheeses investigated (Jordan & Cogan, 1993), implying that, at least in some cases, high densities of NSLAB can be attained in the absence of citrate utilisation. A recent report (Palles, Beresford, Condon, & Cogan, 1998) indicated that both growing and non-growing cells of *Lb. casei* ATCC 393 and *Lb. plantarum* DPC 1919 could metabolise citrate in the presence or absence of a fermentable sugar at pH 5.3, the pH at which Cheddar

cheese is ripened. However, the data indicated that citrate was not used as an energy source by NSLAB.

Starter cells are present at initial densities of approximately 10^8–10^9 cfu/g in most cheese varieties but levels decrease during ripening due to the hydrolytic activity of their own autolysin enzymes (Thomas & Batt, 1969). It was demonstrated in a model buffer system (Thomas, 1987) that many strains of NSLAB could grow on the products released from starter cells undergoing autolysis. Cell densities of *Pe. pentosaceus* 1220 increased in this system from 10^2 to 10^6 cfu/mL when *L. lactis* ML_3 was added at 10^{10} cfu/mL. Addition of ribose to the system resulted in a further 20-fold increase in cell densities, suggesting that ribose could be used as a carbon source. However, many strains of NSLAB isolated from mature Cheddar cheese were found to be unable to ferment ribose (Fitzsimons et al., 1999), suggesting that other carbon sources must also be used by NSLAB for growth in cheese. Mesophilic lactobacilli have been shown to possess some glycoside hydrolase activity (Williams & Banks, 1997) and in model systems can utilise sugars from glycoproteins of the milk fat globule membrane as an energy source (Fox, McSweeney, & Lynch, 1998; Diggin, Waldron, McGoldrick, Cogan, & Fox, 1999). Fenelon, O'Connor, and Guinee (2000) reported that the growth rate of NSLAB up to six months of ripening decreased as the fat content was reduced. The reduced growth rate in the lower fat cheese may be associated with the decreased MNFS (Lane, Fox, Walsh, Folkertsma, & McSweeney, 1997; Fenelon & Guinee, 1999) content of the cheese, which would depress a_w (Lawrence & Gilles, 1980) and restrict microbial growth (Rüegg & Blanc, 1981).

The source of NSLAB in cheese has been the focus of much debate as they are found in cheeses made from both raw and pasteurised milk. In the case of traditional EU cheeses made from raw milk the main source is likely to be the cheese milk. However, cheeses manufactured from pasteurised milk, such as Cheddar, also contain NSLAB. The likely source of NSLAB in these cheeses is either through post-pasteurisation contamination or failure of pasteurisation to fully inactivate NSLAB (Turner, Lawrence, & LeLievre, 1986, Martley & Crow, 1993). In a survey of 21 NSLAB cultures, which included typical Cheddar cheese isolates the most heat resistant strain, *Lb. casei* NCDO161, suffered a 3.5 log reduction when heated to 72°C for 15 s, and the majority of the other cultures were reduced by 6 log cycles (Turner et al., 1986). This would suggest that NSLAB would be inactivated by pasteurisation. However, another study indicated that small numbers of NSLAB may survive pasteurisation in an injured state, revive during cheese ripening and subsequently grow in the cheese (Jordan & Cogan, 1999). It has been demonstrated that NSLAB can grow rapidly in Cheddar with a generation time of 8.5 days in cheese ripened at 6°C (Jordan & Cogan, 1993); thus, low levels of contamination will result in NSLAB rapidly becoming a significant proportion of the total cheese flora.

The microbial flora of cheese, particularly those made from raw milk, is very complex. It is thus likely that many interactions occur between strains of NSLAB and other bacteria in the cheese. The study of these interactions is difficult because of the complex microbiological ecosystem found in cheese (Martley & Crow, 1993). However, some progress has been made in this area. It has been reported that *Lb. casei*, *Lb. rhamnosus*, and *Lb. plantarum* could inhibit propionic acid bacteria and enterococci in cheese (Jimeno et al. 1995a; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996). The mechanism of the inhibition, however, remains obscure. Inhibition is normally observed only in cheese or cheese juice; indeed, in some culture media stimulation is observed (Jimeno, Sollberger, & Làzaro, 1995b). This implies that the inhibition is not mediated by an inhibiting substance, such as a bacteriocin, but may be through competition for limiting metabolites.

The role of NSLAB in Cheddar cheese flavour development has been a contentious issue for many years. In the last decade, there have been a number of studies on the effect of adjunct lactobacilli on Cheddar flavour development, with most authors reporting increased levels of proteolysis and enhanced flavour intensity (El Soda, Desmazeaud, Aboudonia, & Kamal, 1981; Puchades, Lemieux, & Simard, 1989; Lemieux, Puchades, & Simard, 1989; Broome, Krause, & Hickey, 1990; Trépanier, Simard, & Lee, 1991; McSweeney et al., 1994; Johnson, Etzel, Chen, & Johnson, 1995; Lane & Fox, 1996; Lynch et al., 1996). It would appear that selection of the adjunct strain is crucial, since certain strains of *Lb. casei* ssp. *casei* and *Lb. casei* ssp. *pseudoplantarum* produced high quality Cheddar, while other strains of these species resulted in cheese with acid and bitter flavour defects (Lawrence & Gilles, 1987). The level of gas production caused by inoculated or adventitious heterofermentative lactobacilli was reduced on addition of homofermentative lactobacilli to Cheddar during production (Laleye, Simard, Lee, & Holley, 1990).

1.4.2. Propionic acid bacteria (PAB)

PAB grow in many cheese varieties during ripening, and are the characteristic microflora associated with Swiss-type cheeses such as Emmental, Gruyère, Appenzell and Comté. PAB are Gram positive short rod-shaped bacteria which metabolise lactate by the following pathway (Steffen, 1973):

$$3\,\text{Lactate} \rightarrow 2\,\text{Propionate} + \text{Acetate} + CO_2 + H_2O.$$

Two major groups within the genus are recognised, the "cutaneous" and the "classical or dairy" PAB. The classical PAB are the most important with respect to

cheese microbiology, and five species are currently recognised: *P. freudenreichii*, *P. jensenii*, *P. thoenii*, *P. acidipropionici* and *P. cyclohexanicum* and one *P. coccoides* proposed (Vorobjeva, 1999). Recently, a number of molecular methods for detection and identification of classical PAB have been reported (Riedel, Wingfield, & Britz, 1998; Rossi, Torriani, & Dellaglio, 1998, 1999).

In cheese manufactured from raw milk, sufficient "wild" PAB were present. However, with the advent of pasteurisation PAB are now added to the cheese milk at the beginning of manufacture to ensure that they are present at approximately 10^3 cfu/g of cheese post manufacture (Vorobjeva, 1999). During cheese ripening, the temperature is increased from 18°C to 22°C for a short time to initiate propionic acid fermentation, with a resultant increase in levels of PAB from 10^8 to 10^9 cfu/g cheese (Steffen et al., 1993). Swiss-type cheeses undergo a propionic acid fermentation in 20–30 days post-manufacture and the propionic and acetic acids produced contribute to the development of characteristic flavours of these cheeses, while the CO_2 evolved is responsible for the large eyes produced. Subsequent to the development of sufficient eyes, the cheese is stored at a lower temperature to retard further growth and metabolism of the PAB. It was demonstrated that growth of PAB in a milk-based medium was poor (Baer, 1995); however, growth could be stimulated following proteolysis by rennet and starter bacteria. It was recently reported (Piveteau, Condon, & Cogan, 2000) that growth of PAB in milk or whey did not occur unless the initial cell density were $>10^6$ cfu/mL. The growth inhibition was reported to be due to a heat stable inhibitor(s) present in whey. Pre-growth of some lactic acid bacteria, used as starter cultures in Swiss-type manufacture, in the milk medium removed the inhibition, which explains how PAB develop in Swiss-type cheese from low densities even though they are inhibited in milk.

Spontaneous autolysis of *P. freudenreichii* in synthetic media was demonstrated by Lemée, Lortal and van Heijenoort (1995); however, in Swiss cheese no evidence of autolysis of *P. freudenreichii* during ripening as measured by release of intracellular enzymes was detected by Valence et al. (1998). In Grana cheese, where PAB can result in late blowing, damaged cells of *P. freudenreichii* were detected by scanning electron microscopy, suggesting that in that particular cheese environment autolysis did occur (Cappa, Bottazzi, Bosi, & Parisi, 1997). Bacteriophage infection of *P. freudenreichii* during growth in Swiss-type cheese was recently demonstrated (Gautier, Rouault, Sommer, Briandet, & Cassin, 1995), such infections may contribute to PAB lysis during cheese ripening.

Interactions between PAB and other bacteria play a significant role during cheese ripening. Alekseeva, Anischenko, Schlegel, Ott and Vorobjeva (1983) reported that nine out of twenty two strains of LAB tested were antagonistic for PAB. *L. lactis* ssp. *lactis* had the greatest inhibitory effect, while *L. lactis* ssp. *cremoris*, *S. thermophilus* and *Lb. helveticus* were compatible with *P. freudenreichii* and *P. shermanii*. Jimeno et al. (1995a) reported that *Lb. rhamnosus* and *Lb. casei* inhibited the growth of *P. freudenreichii* in hard Swiss-type cheese. Piveteau et al. (2000) studied the interaction between 14 LAB, including strains of *Lb. helveticus*, *Lb. acidophilus*, *Lb. lactis*, *S. thermophilus* and *Lc. lactis* and four strains of PAB, either *P. freudenreichii* or *P. acidipropionici*, in whey. Stimulation or inhibition was judged by the effect on growth rate and final cell biomass. No inhibition was observed and growth of the four PAB strains was stimulated by *Lb. helveticus* and *S. thermophilus*. The consequences of such stimulatory and inhibitory interaction between PAB and other cheese microorganisms needs to be considered in more detail due to the impact of such interactions on cheese quality.

1.4.3. Smear bacteria

Smear-ripened cheeses are produced in relatively large amounts in many European countries and are characterised by the development of a smear of bacteria and yeast on the surface of the cheese during ripening. Two sources of smear bacteria are used. In the first one, old or ripened cheese is washed with a brine-solution, which is then used to inoculate the surface of the young cheese. This will also inoculate the young cheese with undesirable bacteria, including potential pathogens, if they are also present on the old smear. This is termed the old–young smearing method and is traditionally used in Germany. In the second method, the young cheese is deliberately inoculated with one or more combinations of *Brevibacterium linens*, *Geotrichum candidum* or *Debaryomyces hansenii* after salting.

The pH of the surface of the cheese is ~5.0 and the cheese is generally ripened at temperatures of 12–16°C at a relative humidity of >90%. These conditions result in rapid development of the smear, which is also aided by spreading the microcolonies, which develop on the surface, by wiping the surfaces with a cloth dipped in brine every two or three days. The microbiology of the smear is complex and is poorly understood. It is generally believed that yeasts grow during the first few days of ripening, oxidising the lactate completely to CO_2 and H_2O. The yeasts also deaminate amino acids to the corresponding ketoacid and NH_3, which causes the pH on the surface to increase to a point where the bacteria can grow. The rise in pH can be relatively fast, e.g., the pH of the surface of Tilsit cheeses increases from an initial level of ~5 to >7.5 in the first 10 days of ripening (Eliskases-Lechner & Ginzinger, 1995). In this cheese, the salt tolerant bacteria increase from 10^4 to 10^9 cfu/cm^2 in the first three weeks of ripening after which their

densities remain constant for the next five weeks. The yeast counts were much lower and increased from 10^3 to 5×10^7 cfu/cm^2 within two weeks after which they gradually decreased.

It is generally believed that *B. linens* is the major bacterium growing on the surface of smear-ripened cheeses. However, recent studies have indicated that several micrococci (*M. luteus*, *M. lylae*, *Kocuria kristinae* and *K. roseus*), staphylococci (*St. equorum*, *St. vitulus*, *St. xylosus*, *St. saptrophyticus*, *St. lentus* and *St sciuri*) and coryneform bacteria (*Arthrobacter citreus*, *A. globiformis*, *A. nicotianae*, *B. imperiale*, *B. fuscum*, *B. oxydans*, *B. helvolum*, *Corynebacterium ammoniagenes*, *C. betae*, *C. insidiosum*, *C. variabilis*, *Curtobacterium poinsettiae*, *Microbacterium imperiale* and *Rhodoccoccus fascians*) are also found on the surface of these cheeses (Eliskases-Lechner & Ginzinger, 1995; Valdes-Stauber, Scherer, & Seiler, 1997; Irlinger, Morvan, El Solh, & Bergere, 1997; Irlinger & Bergère, 1999). The stage of ripening at which these bacteria were isolated is not clear. Recent data shows that a progression of bacteria occurs in the smear; staphylococci are the major organisms found early in ripening (within four days) and are replaced by coryneform bacteria on day 16 of ripening (Brennan, unpublished).

The classification of coryneform bacteria (*Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Rhodococcus* spp.) is very confusing and a polyphasic approach needs to be taken. Traditional phenotypic tests, must be accompanied by chemotaxonomic procedures (including determination of the sugar and amino acids present in the cell-wall, the presence or absence of mycolic acids, and the number and types of menaquinones) and 16S rRNA gene sequencing. Whether the bacteria go through a rod/coccus transformation during growth must also be examined. Such studies have shown that many of the coryneform bacteria isolated from cheese have been misclassified. For example, *Caseobacter polymorphus*, originally isolated from Limburger and Meshanger cheese, is now considered to be *C. variabilis* (Collins, Smida, & Stackebrandt, 1989), *B. ammoniagenes* has been reclassified as *C. ammoniagenes* (Collins, 1987) and methanethiol producing coryneforms, isolated from Cheddar cheese and milk, have been identified as *B. casei* (Collins, Farrow, Goodfellow, & Minnikin, 1983). In addition, the genus *Aureobacterium* has been amalgamated with *Microbacterium* (Takeuchi & Hatano, 1998).

Generally, smear cheeses are soft or semi-soft cheeses but there are some exceptions such as Beaufort, Comté and Gruyère, which are hard cheeses. Yeasts of the genera *Candida*, *Debaryomyces*, *Kluyveromyces* and *Rhodotorula* developed initially on the surface of Gruyère and Beaufort cheeses attaining densities of 10^9 cfu/g of smear within the first three weeks of ripening, after which they decrease to approximately 10^5 cfu/g of smear for the remainder of the ripening period. Bacteria grew to densities of 10^{11} cfu/g of smear and remained at that level throughout ripening (Accolas, Melcion, & Vassal, 1978). The bacterial community was composed primarily of highly-salt tolerant (15–20% NaCl) coryneform bacteria which were later identified as *Brachybacterium alimentarium* and *Br. tyrofermentans* based on chemotaxonomic criteria and 16S rRNA gene sequences (Schubert et al., 1996).

The role of these bacteria in the ripening of the cheese has not been studied to a great extent but many of them produce proteinases, peptidases and lipases which produce amino acids and fatty acids which are the precursors of many of the flavour compounds in the cheese. *B. linens* is also responsible for the production of methanethiol which is considered to be responsible for the "smelly sock" odour of many of these cheeses, although, recently, several strains of *G. candidum* have also been shown to produce sulphur compounds including methanethiol in model cheese systems (Berger, Khan, Molimard, Martin, & Spinnler, 1999). Except where deliberate inoculation of *B. linens* is practised, the other bacteria, which grow on the cheese, are more than likely adventitious contaminants, which grow well in the high salt concentrations and relatively high pH of the cheese surface. Their source has not been identified but it is likely that the major sources are the brine and the wooden shelves on which the cheese rests during ripening.

1.4.4. Mould

Moulds are important in the ripening of a range of cheeses. Mould ripened cheeses are divided into two groups: those which are ripened due to the presence of *Pencillium roqueforti* which grows and forms blue veins within the cheese, such as Roquefort, Gorgonzola, Stilton and Danish blue, and those which are ripened with *Pen. camemberti* which grows on the surface of the cheese, such as Camembert and Brie. Moulds are associated with a range of other cheese varieties also, however, the moulds and their impact on ripening in these cheeses are less well understood. The surface of the French cheeses, St. Nectaire and Tome de Savoie, is covered by a complex fungal flora containing *Penicillium*, *Mucor*, *Cladosporium*, *Geotrichum*, *Epicoccum* and *Sporotrichum*, while *Penicillium* and *Mucor* have been reported on the surface of the Italian cheese Taleggio and *Geotrichum* on that of Robiola (Gripon, 1993). *Mucor* is sprayed on the surface of the Norwegian cheese Gammelost (Oterholm, 1984). *Pen. roqueforti* has also been reported to develop within Gammelost cheese; in such situations, *Pen. roqueforti* is introduced to the cheese at piercing (Gripon, 1993). Interior- or surface mould-ripened cheeses have different appearances and the high biochemical activities of these moulds produce

the typical aroma and taste. The morphological and physiological properties of *Pen. roqueforti* and *Pen. camemberti* were reviewed by Moreau (1980).

For production of Roquefort cheese a water suspension of *Pen. roqueforti* spores is added to the milk just prior to setting, or spores are dusted onto the curd. Following whey drainage and salting, the cheese is pierced and ripened. Gas production by heterofermentative LAB and yeasts, results in curd-openness, which was deemed important by Devoyod, Desmazeaud, Assenat, and Auclair (1972) for the subsequent development of *Pen. roqueforti* and hence good flavour. It has been suggested that the production of methyl ketones by *Pen. roqueforti* are inhibitory to further mould growth, and may be a factor in preventing excessive mould development in blue veined cheese (Girolami & Knight, 1955).

A white felt, of *Pen. camemberti* develops on the surface of Camembert cheese 6–7 days post-manufacture. *Pen. camemberti* metabolises the lactate to CO_2 and H_2O, which results in deacidafication of the cheese surface within three weeks. The outer part of Camembert undergoes considerable modification of texture and the curd, which is firm and brittle at the beginning of ripening, later becomes soft. The surface flora establishes a pH gradient from the surface (basic) to the interior (acidic) due to consumption of lactic acid and NH_3 production. The increase in pH and breakdown of α_{s1}-casein by rennet are responsible for the softening of the curd (Gripon, 1993), which gradually extends towards the centre, and is visible in a cross-section of the cheese.

1.4.5. Yeast

Yeasts are found in a wide variety of cheeses; however, in most cases, their role in cheese ripening is unclear (Fleet, 1990). The low pH, low moisture content, low temperature and high salt levels of ripening cheese favour the growth of yeast.

Yeasts, which metabolise lactate in the presence of relatively high salt concentrations, grow during the early days of ripening of smear-ripened cheeses and aid in deacidifying it (Eliskases-Lechner & Ginzinger, 1995; Wyder & Puhan, 1999). The density of yeast in the smear either remains constant or decrease slightly during ripening. Many commercial smear preparations contain yeast species such as *G. candidum*, *Candida utilis*, *Debaryomyces hansenii*, *Kluyveromyces lactis*.

Fox, Guinee, Cogan, and McSweeney (2000) summarised the yeasts found in several different cheeses. *D. hansenii* was by far the dominant yeast and occurred in virtually all cheeses including Weinkase, Romadour, Limburger, Tilsit, Roquefort, Cabrales, Camembert and St. Nectaire. The next most important species were *K. lactis*, *Yarrowia lipolytica* and *Trichospora beigelii*. *D. hansenii* is also the dominant species in Danish Blue (van den Tempel & Jakobsen, 1998) and is one of two dominant yeasts in the French cheese Reblochon (the other is *G. candidum*) (Bärtschi, Berthier, & Valla, 1994). Whether a progression in the species of yeast occurs during ripening is not clear since, in many of these studies, the stage of ripening at which the yeasts were isolated was not defined. This point was addressed by van den Tempel and Jakobsen (1998) who found that *D. hansenii*, *Zygosaccharomyces* spp., *Y. lipolytica*, and *Cn. rugosa* were the dominant species in Danish Blue ripened for 1 and 14 days while only *D. hansenii* and *Cn. rugosa* were found after 28 days of ripening. These yeasts showed significant lipolytic activity on tri-butyrin agar but no proteolytic activity in casein agar, implying that their major role is in lipolysis and deacidification.

Brines are a potent source of yeasts and populations in Danish Blue brines ranged from 1.9×10^4 to 2.3×10^6 cfu/g depending on the dairy (van den Tempel & Jakobsen, 1998). The brines had a fairly typical composition ($\sim$22% NaCl, pH 4.5) and were held at 19°C. *D. hansenii* was the dominant yeast in three of the brines and *Cn. glutosa* in the fourth.

Yeasts contribute positively to flavour and texture development (Roostita & Fleet, 1996). In Roquefort cheese, some surface ripening is attributable to proteolytic activity of a surface slime composed in part by yeast, which is scrubbed off prior to packaging (Kinsella & Hwang, 1976; Kanauchi, Yoshioka, & Hammamoto, 1961). Gas production by heterofermentative LAB, particularly leuconostoc, was stimulated by yeast, resulting in curd-openness, and was deemed important by Devoyod et al. (1972) for the subsequent development of *Penicillium* and hence good flavour. The degree of piercing was found to be important by Galzin, Galzy and Bret (1970) as it increased the internal oxygen content and allowed the active multiplication of yeast capable of oxidising lactic acid resulting in deacidification. Lactococcal starters are used in the manufacture of Camembert and after curd production (pH 4.5–4.6) yeast grow on the surface (Lenoir, 1963; Schmidt & Lenoir, 1978, 1980), *Saccharomyces cerevisiae*, *K. lactis* and *D. hansenii* being the most common species. In the interior of the cheese, lactococci are dominant, the yeast population is about 1% of that on the surface. Marcellino and Benson (1992) documented the sequential appearance of microorganisms on the rind and in the curd of a St. Nectaire-type cheese over a two month period which is made without the deliberate inoculation with starter. Initially, the cheese surface is devoid of microbes and a rind has not formed. Multilateral budding yeast (*Debaryomyces* and *Torulopsis*) and *L. lactis* ssp. *cremoris* predominate up to the second day post-manufacture. Within the first four days, rapid growth of filamentous fungi and yeast, including *Mucor* and *G. candidum* occurs. Rind thickening proceeds up to two months as fungal hyphae penetrate into the curd.

From day 20, until the end of ripening coryneforms (*Arthrobacter* and *Brevibacterium*) can be seen near the surface of the cheese rind among fungal hyphae and yeast cells.

The involvement of yeast in the maturation process of Cheddar cheese is unclear. Most studies on the microflora of this cheese do not report on the presence of yeast, however, this is probably due to a lack of specific examination for yeast. Fleet and Mian (1987) reported that almost 50% of Australian Cheddar cheeses sampled contained 10^4–10^6 cfu/g. A study of South African Cheddar by Welthagen and Vijoen (1999) indicated that in 42 cheeses examined all contained yeast. The levels varied from $<10^2$ to $>10^7$ cfu/g, however, 88% of the cheeses had $<10^5$ cfu/g, a level deemed necessary to influence flavour development. Monitoring of yeast growth during ripening indicated that their density increased from 10^2 to 10^3 cfu/g over the first 30 days of ripening, from 30 to 40 days of ripening yeast counts increased to 10^6 cfu/g, before decreasing again. The reason for this growth pattern is not clear.

G. candidum has properties of both yeast and moulds and nowadays is considered to be a yeast. It is generally believed that *G. candidum* is found in all smear and mould-ripened cheeses although recent literature does not support this conclusion (Fox et al., 2000). The reason for this is that some workers probably considered it to be a mould and did not report its presence in the various cheeses in which yeast isolates were studied. *G. candidum* is one of the two dominant yeasts found in Reblochon cheese (the other is *D. hansenii*) and increases very rapidly from $\sim 10^3$ cfu/g on day 1 to $\sim 10^7$ cfu/g on day 8, after which it remains relatively constant up to 36 days of ripening (Bärtschi et al., 1994).

2. Summary

Cheese is a very involved microbial ecosystem, and a very complex microflora develops in most cheese varieties. The microflora plays a major role in cheese ripening, and selection of suitable strains would enable the cheese maker to control or modify flavour development. However, due to the complexity of the flora and the interactions which occur between individual components of it and the cheese environment, strain selection for flavour improvement is not always very obvious. The advent of molecular techniques to study cheese microflora will lead to a major increase in our understanding of this ecosystem and this knowledge will be harnessed to control cheese ripening.

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