20

Non-starter lactic acid bacteria (NSLAB) and cheese quality

T. P. Beresford, Dairy Products Research Centre, Ireland

20.1 Introduction

A wide diversity of microorganisms is associated with cheese and they are critical to the development of quality products. They contribute during both manufacture and ripening and are often responsible for the unique characteristics of flavour, aroma, appearance and texture associated with particular cheese varieties. Cheese microflora may be conveniently divided into two main groups, consisting of (a) starter and (b) secondary flora, on the basis that starters produce lactic acid during cheese manufacture while secondary flora do not. Secondary flora in turn may be considered to consist of four groups including:

- Propionic acid bacteria
- Moulds
- Smear flora
- Non-starter lactic acid bacteria (NSLAB).

NSLAB are unique among cheese flora in that they are adventitious micro-organisms that grow in all cheese types studied to date. The best studied members of the NSLAB complex are mesophilic lactobacilli; however, *Pediococcus*, *Enterococcus* and *Leuconostoc* should also be considered part of the complex on the basis that they are members of the lactic acid group of bacteria and generally do not produce significant amounts of lactic acid during manufacture. With the exception of *Leuconostoc* species, which are responsible for 'eye' formation ensuing from CO_2 production and flavour development resulting from production of diacetyl and acetate, the contribution of NSLAB to

cheese quality is not clearly defined. However, there is mounting evidence, in particular for mesophilic lactobacilli and to a lesser extent enterococci, that they do influence cheese quality. The application of molecular techniques and systematic approaches to strain selection is resulting in greater knowledge of NSLAB populations in cheese and identification of adjuncts with the potential to influence cheese quality.

20.1.1 Cheese microflora

The microflora of most natural cheese varieties is complex and consists of a wide range of microorganisms including bacteria, moulds and yeast. They form an essential component of the cheese and are required during both cheese manufacture and ripening. The microflora may be conveniently divided into two main groups, starters and secondary flora. These two groups are distinguished on the basis that starter flora are responsible for fermentation of the milk sugar lactose, primarily to lactic acid, during cheese manufacture, while both groups contribute to the development of flavour and texture during the ripening process, either directly due to their metabolic activity or through release of their enzymes into the cheese matrix (Cogan, 2000).

Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus and *Lactobacillus delbruckii* are the primary species of starter bacteria used in cheese manufacture. Depending on the cheese variety, individual species or blends of two or more species either are added to the cheese milk at the beginning of manufacture or may be naturally present in the milk.

The secondary flora is composed of mixtures of bacteria, yeast and moulds. Specific mixtures are often, though not exclusively, associated with particular cheese varieties, where their action contributes to the specific characteristics of that variety. The secondary flora may be added in the form of defined cultures, but in many situations are composed of adventitious microorganisms gaining access to the cheese either from ingredients or from the environment. They can be divided into four primary groups which include:

- Propionic acid bacteria such as *Propionibacterium freundenreichii* which grow internally in Swiss-type cheeses, for example Emmentaler, Gruyère and Comté, where they are responsible for ‘eye’ formation
- Moulds such as *Penicillium roqueforti* which grows internally in blue-veined cheeses, for example Roquefort, Gorgonzola and Stilton, or *Penicillium camembertia* which grow externally on Camembert and Brie type cheeses
- Bacteria and yeast which grow on the surface of smear ripened cheeses such as Tilsiter, Munster or Saint Paulin
- NSLAB which grow internally in many cheese varieties during ripening and consist primarily of mesophilic lactobacilli, though *Pediococcus, Enterococcus* and *Leuconostoc* may also be considered to form part of the NSLAB complex.

20.2 Bacteria comprising the NSLAB complex

A useful definition of NSLAB is 'lactic acid bacteria found in cheese which do not form part of the starter culture, i.e. do not contribute to acid production during the cheese manufacturing process'. Thus, as outlined above, the NSLAB complex is comprised of four main groups of bacteria:

- Mesophilic lactobacilli
- Pediococci
- Enterococci
- *Leuconostoc*.

Mesophilic lactobacilli are probably the most commonly encountered and best studied members of this complex. Lactobacilli are a genetically diverse group of organisms. They are Gram-positive, catalase-negative and generally non-motile, with complex nutritional requirements. Their cell shape can vary from long and slender, sometimes bent rods, to short, often coryneform coccobacilli; chain formation is common. They can grow in the temperature range 2–53ºC and are aciduric with an optimal pH usually 5.5–6.2. They have been traditionally divided into three groups on the basis of being either (I) obligatory homofermentative, (II) facultatively heterofermentative, or (III) obligatory heterofermentative (Kandler and Weiss, 1986). Members of the facultatively heterofermentative lactobacilli (Group II) are most often encountered in cheese as part of the NSLAB flora and are sometimes referred to as facultatively heterofermentative lactobacilli (FHL). Most Group II isolates do not grow well in milk (Cogan *et al.*, 1997) and thus do not contribute to acid production during the manufacturing process. They can, however, grow in cheese during ripening and form a significant portion of the microbial flora of most cheese varieties. 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 and Cogan, 1993; Coppola *et al.*, 1997; Fitzsimons *et al.*, 1999). Members of group III are occasionally encountered, in particular *Lb. brevis* and *Lb. fermentum*. Group I contains the lactobacilli that are normally considered part of the starter flora.

Pediococci are Gram-positive, catalase-negative, spherical cells found in pairs or tetrads. They are unusual among lactic acid bacteria as they can divide in two perpendicular directions to form tetrads, although this formation may not always be present. Single cells are rarely found and pediococci do not form chains. They are non-motile and generally facultatively anaerobic (Schleifer, 1986). They can grow in the temperature range 25–50ºC. They ferment glucose to produce lactic acid but gas is not formed, lactose is not readily fermented and this limits their growth in milk. Species of pediococci vary in their tolerance to salt, with some able to grow in media containing 6.5% NaCl. These bacteria grow at pH 4.5 to 8.2. Pediococci are commonly found in fermenting vegetables, hay, silage, alcoholic beverages and soft drinks (Schleifer, 1986; Simpson and Taguchi, 1995). *Pediococcus pentosaceus* and *P. acidilactici* are the dominant species isolated from dairy products (Garvie, 1984).

While many enterococci will grow in milk, most strains isolated from cheese do not produce sufficient lactic acid to reduce milk to pH 5.3 in 6 hours at 30ºC (Cogan *et al*., 1997). Thus, they conform more to the definition of NSLAB rather than that of a starter and will be considered part of the NSLAB flora in this review. The genus *Enterococcus* consists of Gram-positive, catalase-negative, spherical or ovoid cells, which are typically arranged in pairs or chains. Enterococci are facultative anaerobes and most species within the genus will grow in the temperature range 10–45ºC. Most are capable of growth in media containing 6.5% NaCl, at pH 9.6 and can hydrolyse aesculin in the presence of 40% bile salts (Schleifer, 1986). Traditionally two species, *Streptococcus faecalis* and *S. faecium*, were considered part of the genus *Streptococcus* and were termed faecal group D streptococci (Deibel *et al*., 1963; Facklam and Moody, 1970; Facklam, 1973). Subsequently, molecular characterisation indicated that these two species were distinct from the majority of the species in the genus *Streptococcus* (Collins *et al*., 1984). Thus, Schleifer and Kilpper-Bälz (1984) proposed their transfer to the genus *Enterococcus*. In the intervening years a further 17 species have been added to the genus on the basis of phylogenetic evidence provided by 16S rRNA sequencing studies. Enterococci have been isolated from a variety of dairy products where they often co-exist with lactococci. Lactococci and enterococci share many phenotypic characteristics; however, traditionally they have been separated on the basis that all species of enterococci were capable of growth at 45ºC and in 6.5% NaCl, while lactococci were not. However, isolates have been identified which do not conform to these criteria; these include salt and/or temperature tolerant lactococci (Facklam and Collins, 1989; Teixeira *et al*., 1996) along with salt and/or temperature sensitive enterococci (Collins *et al*., 1984; Devriese *et al*., 1990). Genotypic methods are likely to provide more reliable identification and a genus-specific RCR based method was recently developed which can reliably differentiate lactococci from enterococci (Deasy *et al.*, 2000). The dominant species isolated from cheese include *E. faecium*, *E. faecalis* and *E. durans*.

While *Leuconostoc* form a component of mixed starter cultures, as they grow and produce acid very slowly in milk they do not conform to the definition of 'starter culture' given above and thus should be considered part of the NSLAB flora. The genus *Leuconostoc* consists of Gram-positive, catalase-negative cells with irregular coccoid morphology. Their optimum growth temperature is in the range 20–30ºC. Their distinction from gas-forming heterofermentative lactobacilli has long been controversial and the two genera are now considered to be phylogenetically intermixed (Stackebrandt and Teuber, 1988). They are also often confused with lactococci, but they can be distinguished on the basis of three fundamental characteristics:

1. They ferment sugars heterofermentatively rather than homofermentatively.
2. They produce the D rather than the L isomer of lactate.
3. With the exception of *Leuconostoc lactis*, they show no visual evidence of growth in litmus milk unless yeast extract (0.3 g per 100 ml) is added.

The taxonomy of dairy leuconostocs was recently reviewed in detail (Thunell, 1995). The leuconostocs are traditionally associated with plant material, fermented dairy products and wines. While they are found in mixed-strain starter cultures used in cheese manufacture, the exact species have not been clearly identified; however, both *L. mesenteroides* ssp. *cremoris* and *L. lactis* are involved.

20.3 NSLAB in different cheese varieties

While, with the exception of leuconostocs, NSLAB are not traditionally deliberately added to cheese, most cheeses studied to date contain bacteria from at least one group within the NSLAB complex. The mesophilic lactobacilli flora of Cheddar cheese has been extensively investigated in recent years using both phenotypic and molecular methods of characterisation. A study of 8-week-old commercial Irish Cheddar revealed that the flora consisted of 55% *Lb. paracasei*, 28% *Lb. plantarum* and 14% *Lb. curvatus* (Jordan and Cogan, 1993). A subsequent study on mature Irish Cheddar by Fitzsimons *et al.* (1999) which involved 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. A study of UK Cheddar ripened 6–9 months indicated that *Lb. paracasei* 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 and Banks, 1997). In New Zealand Cheddar, manufactured in six factories, *Lb. paracasei* and *Lb. rhamnosus* were the dominant species isolated (Crow *et al.*, 2001).

The mesophilic lactobacilli flora from a range of European traditional cheeses was recently reviewed (Beresford *et al.*, 2001). While there appears to be some variation in the *Lactobacillus* populations depending on the cheese variety and the duration of ripening, the dominant species identified throughout the range of cheeses reported include *Lb. paracasei, Lb. rhamnosus* and *Lb. plantarum. Lactobacillus curvatus* was identified in some of the cheeses reviewed. Studies on Fossa (pit) cheese, which is ripened in flask-shaped pits dug in the tufa ground in the Emilia-Romagna region of Italy, indicated that *Lb. plantarum, Lb. curvatus* and *Lb. paracasei* dominated the mesophilic lactobacillus flora (Gobbetti *et al.*, 1999). Characterisation of mesophilic lactobacilli from Fiore Sardo cheese demonstrated that *Lb. plantarum* and *Lb. paracasei* were the dominant species and that the population attained maximum numbers of $\sim 10^8$ cfu g^{-1} at 15 days of ripening and then slowly decreased to $\sim 10^4$ cfu g^{-1} after 7 months' ripening (Mannu *et al.*, 2000). In a study of 12 Italian ewes' milk cheeses (de Angelis *et al.*, 2001) 32% of the isolated mesophilic lactobacilli were phenotypically identified as *Lb. plantarum*, 15% *Lb. brevis*, 12% *Lb. paracasei*, 9% *Lb. curvatus*, 6% *Lb. fermentum*, 6% *Lb.*

casei, 5% *Lb. pentosus*, 3% *Lb. casei* ssp. *pseudoplantarum*, and 1% *Lb. rhamnosus*. *Lactobacillus paracasei* was identified as the predominant species of mesophilic lactobacilli in Caciocavallo Pugliese, a pasta-filata type cheese that is generally aged prior to consumption (Gobbetti *et al.*, 2002).

Pediococci were first reported in experimental New Zealand Cheddar cheese by Dacre (1958a, 1958b). A number of subsequent studies on Cheddar from the UK also reported the presence of pediococci in the NSLAB flora (Franklin and Sharpe, 1963; Fryer and Sharpe, 1966; Law *et al.*, 1976). In young Canadian Cheddar pediococci were observed to constitute approximately 1% of the NSLAB flora (Elliott and Mulligan, 1968). They have also been isolated from Cheddar cheese manufactured in the USA (Litopoulou-Tzanetaki *et al*., 1989). In varieties other than Cheddar strains of *P. pentosaceus* have been isolated from Manchego cheese (Nunez, 1976) and from a raw goats' milk cheese, Feta and Kaseri (Tzanetakis and Litopoulou-Tzanetaki, 1989), while *P. acidilactici* were reported in Parmigiano Reggiano cheese (Coppola *et al.*, 1997).

Enterococci occur in a variety of artisanal cheeses made from raw or pasteurised goat, ewe, water buffalo or bovine milk in southern Europe (Cogan *et al*., 1997). Enterococci were identified in 96% of samples in a survey of 48 Italian fresh, soft and ripened semihard cheeses by Giraffa *et al*. (1997). Enterococci have been detected as a significant portion of the bacterial flora of a range of cheeses such as Manchego (Ordoñez *et al*., 1978), Le Serena (Del Pozo *et al.*, 1988), Mozzarella (Coppola *et al.*, 1988), Kefalotyri (Litopoulou-Tzanetaki, 1990), Feta and Teleme (Tzanetaki and Litopoulou-Tzanetaki, 1992), Picante de Beira Baixa (Freitas *et al.*, 1995), Serra (Macedo *et al.*, 1995), Cebreiro (Centeno *et al.*, 1996), Comté (Bouton *et al.*, 1998) and in a farmhouse Cheddar type cheese (Gelsomino *et al.*, 2001). Enterococcal numbers vary with cheese type and production season and ranged from 10^4 to 10^6 cfu/g for Emmental cheese during a 15-year survey to 10^4 to 10^7 cfu g^{-1} for Appenzeller cheese (see Franz *et al.*, 1999). The dominant species isolated were *E. faecium*, *E. faecalis* and *E. durans*.

Cultures containing leuconostocs are used in the manufacture of Dutch varieties such as Gouda and Edam, Danish varieties such as Danbo, Havarti, Maribo and Danish Blue, Swedish cheeses such as Herrgård, Drabantost, Wästerbottensost, Prästost and Svecia, and Finnish cheeses such as Turunmaa and Karelia. *Leuconostoc mesenteroides* and *Leuc. citreum* have also been isolated from cheeses made using traditional technology in France (Cibik *et al.*, 2000). They have been isolated from Casar de Cáceres (Poullet *et al.*, 1993), Manchego (Garcia *et al.*, 1995), Cebreiro (Centeno *et al.*, 1996), Roncal and Idiazabal (Arizcun *et al.*, 1997), San Simon (Fontan *et al.*, 2001), Tetilla (Menendez *et al.*, 2001) and Tenerife cheese (Pérez *et al.*, 2002) from Spain, and Serra de Estrela cheese from Portugal (Dahl *et al.*, 2000). *Leuconostoc lactis* was reported in the Greek cheese Kefalotyri (Litopoulou-Tzanetaki, 1990) while *Leuc. lactis* and *Leuc. mesenteroides* were isolated from Teleme cheese (Tzanetakis and Litopoulou-Tzanetaki, 1992). Mozzarella cheese made from water-buffalo milk in Italy is traditionally produced using a natural whey starter

culture. The composition of these cultures is complex and while lactococci and thermophilic lactobacilli were dominant, relatively high numbers of *Leuconostoc* were also isolated (Coppola *et al.*, 1988). A more recent study (Morea *et al.*, 1999) demonstrated that *Leuc. lactis* and *Leuc. mesenteroides* were present in raw cows' milk Mozzarella post-manufacture.

20.4 The source of NSLAB in cheese

With the exception of leuconostocs which in some situations are added as part of the mixed-strain starter culture, NSLAB are adventitious bacteria which gain access to the cheese either from the ingredients used in its manufacture or from the environment. Mesophilic lactobacilli are found in all natural cheeses investigated to date. For cheeses made from raw milk the likely source of these bacteria is the cheese milk. A study using molecular techniques to type strains of mesophilic lactobacilli in Comté cheese made from raw milk indicated that a large number of the strains investigated could have originated from the milk (Berthier *et al.*, 2001). A number of studies have reported on the heat sensitivity of mesophilic lactobacilli but the findings have been equivocal. In one study, which involved 21 cultures and included typical Cheddar cheese isolates, the most heat-resistant strain *Lb. casei* NCDO161 suffered a 3.5 log reduction when heated to 72ºC for 15 s, while the majority of the other cultures were reduced by 6 log cycles, suggesting that mesophilic lactobacilli would be inactivated by pasteurisation (Turner *et al.*, 1986). However, a subsequent study indicated that small numbers of mesophilic lactobacilli may survive pasteurisation in an injured state, revive during cheese ripening and subsequently grow in the cheese (Jordan and Cogan, 1999). This hypothesis was supported by the data of McSweeney *et al.* (1994) who detected no mesophilic lactobacilli in pasteurised cheese milk, but noted that the resulting cheese manufactured under aseptic conditions supported a low population of mesophilic lactobacilli from the beginning of ripening.

Mesophilic lactobacilli that survive pasteurisation or gain access to the cheese plant from other cheese-making ingredients or the environment may survive within the plant in the form of biofilms. This was demonstrated in pilot plant studies where Cheddar cheese manufactured in the presence of biofilms of *Lb. curvatus* and *Lb. fermentum* was found to be contaminated with the *Lb. curvatus* strain used to make the biofilm (Somers *et al.*, 2001). The biofilm made with the *Lb. curvatus* was also able to survive the cleaning process used. In Cheddar cheese manufactured under commercial conditions the mesophilic *Lactobacillus* flora varied between factories in studies conducted in Ireland and New Zealand (Fitzsimons *et al.*, 1999; Crow *et al.*, 2001). Similar observations were reported for Herrgård cheese (Antonsson *et al.*, 2001) and a range of Italian ewe cheeses (de Angelis *et al.*, 2001). In three Irish Cheddar cheeses manufactured over a three-week period it was observed that the majority of the most prevalent strains were common (Fitzsimons *et al.*, 2001); however,

seasonal variation was observed in the mesophilic lactobacillus flora of New Zealand Cheddar (Crow *et al.*, 2001). These data imply that a unique and persistent flora is not associated with particular factories, though this topic demands further investigation. Pediococci have been reported in only a limited number of cheeses as outlined above and no reports on the manner in which they gain access to the cheese have been published.

The source of enterococci in cheese is not clearly defined; however, it is generally assumed that their presence in milk is the primary source. It has been considered that they contaminate milk during production and processing since they are present in bovine faeces (Devriese *et al.*, 1992), animal hides and dairy equipment. In a set of recent studies (Gelsomino *et al.*, 2001, 2002) enterococci were isolated from a Cheddar type cheese during manufacture and ripening, the milk used in its manufacture, the faeces of the personnel involved in cheese making, and the faeces of the dairy cows present on the farm. In addition, strains were isolated from the environment, the tap water, the milking machine and the cows' teats. Isolates were typed using pulse field gel electrophoresis. The key finding was that the same three clones, one of *E. faecalis* and two of *E. casseliflavus*, dominated almost all of the milk, cheese and human faecal samples. The two *E. casseliflavus* clones were also found in the bulk tank and the milking machine even after chlorination, suggesting that contamination of milk with enterococci is a result of contaminated milking equipment. Cows' faeces were not considered the source of enterococci in the cheese, as *E. faecium* and *Streptococcus bovis*, which largely dominated the cows' intestinal tracts, were not found in either the milk or the cheese.

20.5 The growth of NSLAB in cheese

Environmental parameters within cheese which influence bacterial growth and survival such as pH, level of salt, water activity and temperature were reviewed recently (Beresford *et al.*, 2001). These parameters combine to inhibit the growth of most microorganisms, including starter bacteria. However, many NSLAB will survive in cheese and in some cases grow during ripening. Cheese is a nutritious food and contains high levels of protein and fat but would appear to be lacking in carbohydrates.

Mesophilic lactobacilli grow in all cheese types studied to date and in Cheddar cheese ripened at 6ºC they have a generation time of 8.5 days (Jordan and Cogan, 1993). In Cheddar cheese manufactured from pasteurised milk the initial numbers of mesophilic lactobacilli in the cheese are low, sometimes below the detectable limit; however, they grow to levels of at least 10^7 cfu g^{-1} over the first 10–20 weeks of ripening, after which their population remains relatively constant for the duration of ripening (Fig. 20.1).

The energy source used by mesophilic lactobacilli for growth in cheese has not been clearly defined but a range of suggested sources have been identified (for review see Beresford *et al.*, 2001). It is likely that mesophilic lactobacilli do

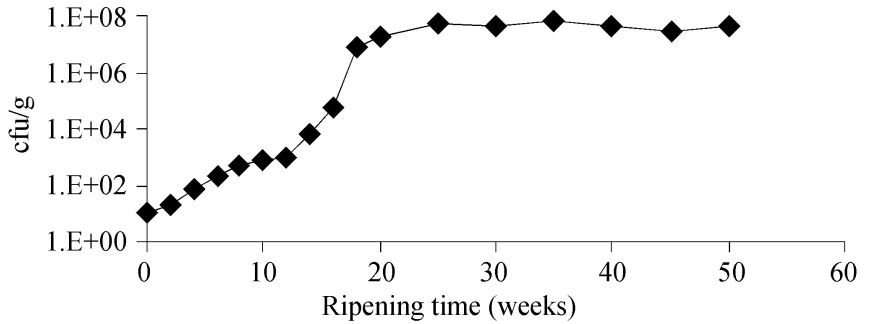

Fig. 20.1 Development of mesophilic lactobacilli in Cheddar cheese during ripening.

not depend on a single energy source to support their growth in cheese, and a recent study indicated that individual species and strains were apparently non-demanding and able to metabolise several different substrates potentially available in cheese (Williams *et al.*, 2000). This metabolic characteristic would assist their establishment and survival in cheese.

Substrate availability in cheese will vary throughout ripening; this will affect the heterogeneity of the mesophilic lactobacillus population and will thus influence population dynamics during ripening. In a study of Irish Cheddar over 39 weeks of ripening a mixture of *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* and unidentified isolates was found up to 6 weeks' maturation; thereafter only *Lb. paracasei* was isolated (Fitzsimons *et al.*, 2001). Similar species dynamics were reported in New Zealand Cheddar except that the dominant species were *Lb. paracasei* and *Lb. rhamnosus* (Crow *et al.*, 2001). Evidence that strain dynamics occurs in Cheddar cheese was also reported (Fitzsimons *et al.*, 2001; Crow *et al.*, 2001). Species dynamics during ripening have also been reported for cheeses such as Fiore Sardo (Mannu *et al.*, 2000), Tenerife goats' cheese (Zarate *et al.*, 1997) and a Swiss type cheese (Demarigny *et al.*, 1996).

Few studies have reported on the growth of pediococci in cheese. Dacre (1958b) indicated that pediococci were not detected in Cheddar cheese in the first days post-manufacture but appeared within 18 days. They subsequently grew and comprised about one-fourth of the NSLAB population in 6-month-old cheese. The growth substrates for pediococci in cheese have not been studied; however, one report indicated that in buffer systems they could grow on the products released due to autolysis of starter cultures (Thomas, 1987).

As indicated above, enterococci occur in both raw and pasteurised milk cheeses. In Cheddar cheese produced in modern processing plants from pasteurised milk the numbers of enterococci in the cheese are very low or non-detectable. The sanitary methods of milk handling and refrigerated storage prior to processing contribute to such low levels. In Cheddar cheese manufactured with raw milk, enterococci are detected at levels in the range 10^2–10^3 cfu g^{-1} at day 1 of ripening and they maintain these levels during ripening (Gelsomino *et*

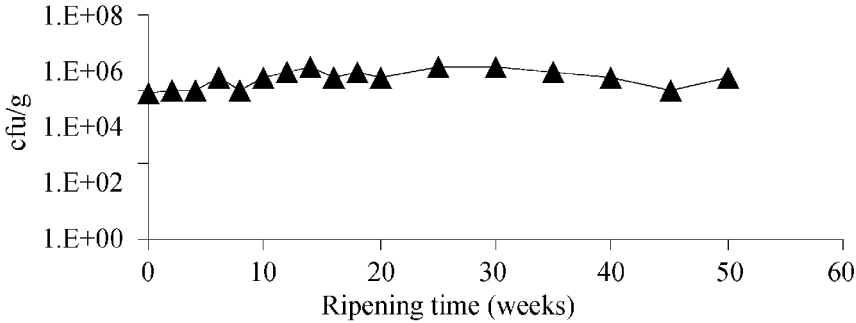

Fig. 20.2 Development of enterococci in a raw milk Cheddar cheese during ripening.

al., 2001). Typical growth of enterococci in raw milk Cheddar is illustrated in Fig. 20.2. Similar growth patterns have been reported for other cheese varieties (Del Pozo *et al.*, 1988; Litopoulou-Tzanetaki, 1990; Bouton *et al.*, 1998).

While a number of studies have indicated that leuconostocs are present in cheese, few reports on their growth dynamics during ripening have been published. Turner (1988) investigated the behaviour of two strains in experimental Gouda. One of the strains increased from ~10^5 to 5×10^5 cfu g^{-1} during the period from moulding of the curd to day 1 of ripening but thereafter decreased to ~10^4 cfu g^{-1} at 35 days of ripening. The other strain grew during the moulding period from ~5×10^5 cfu g^{-1} to 10^7 cfu g^{-1} and maintained this level in the cheese throughout the 35 days of ripening. These data suggest that growth and survival are strain dependent. In Serra da Estrela cheese, manufactured using traditional technology, the *Leuconostoc* population was ~10^7 cfu g^{-1} throughout the ripening period of 180 days (Dahl *et al.*, 2000).

20.6 The influence of NSLAB on cheese quality

Most research to date on the influence of various NSLAB on cheese quality and flavour has used Cheddar as a model. This is due in part to the 'relative' lack of complexity of the microflora associated with this cheese variety and the ability of the researcher to manipulate the flora to at least a limited extent. It should be noted, however, that in most experiments reported there are few, if any, examples where total control of the flora was achieved. This makes interpretation of the data difficult and definitive statements regarding the role of NSLAB problematic.

As demonstrated in Fig. 20.1, mesophilic lactobacilli are present in Cheddar at levels of >10^7 cfu g^{-1} from early in ripening. This represents a significant biocatalytic potential that would be expected to exert some impact on cheese quality. Traditionally Cheddar was manufactured from raw milk using undefined strain starter cultures. Since the introduction of pasteurisation, more hygienic milk handling practices and the defined strain starter system a perception has

developed among many commentators that Cheddar cheese does not develop as mature a flavour as when it was manufactured using traditional methods.

Mesophilic lactobacilli as discussed above are adventitious microorganisms, which are ubiquitous in Cheddar. Attempts to investigate their influence on cheese quality have centred on approaches involving one or more of the following:

- Manufacture of cheese under conditions which hinder their access to the cheese
- Methods which inhibit their growth in the cheese
- Addition of strains to the cheese milk in an effort to ensure that the specific strains dominate the NSLAB flora during ripening.

The technology for the manufacture of cheese under controlled microbiological conditions was developed by Mabbitt *et al.* (1959) who used a combination of pasteurisation (78ºC for 17 s) and hygienic cheese manufacture in enclosed vats. This technology was later modified and the conclusion was that mesophilic lactobacilli did not have a significant effect on flavour development (Chapman *et al.*, 1966). McSweeney *et al.* (1994) and Lynch *et al.* (1996) subsequently used this approach to study the effect of adjunct lactobacilli, which they reported to have a positive effect on cheese flavour and quality. Microfiltration is a technology that facilitates the removal of indigenous microorganisms from milk without the concomitant heat-induced changes resulting from pasteurisation. A study comparing Cheddar manufactured from either raw, pasteurised or microfiltered milks indicated that the flavour of the raw milk cheese was substantially more intense than that of the cheeses manufactured from either pasteurised or microfiltered milk, suggesting that the indigenous lactobacilli play a role in flavour development (McSweeney *et al.*, 1993). It should be noted, however, that growth of lactobacilli in the microfiltered milk cheese was similar to growth in the pasteurised milk cheese, indicating that microfiltration in that series of experiments was no more efficient than pasteurisation at removal of lactobacilli.

The metabolic activity of the starter bacteria is essentially finished at the milling stage in Cheddar cheese. Thus it was proposed that the mesophilic lactobacillus population which develop during ripening could be controlled through the addition of antibiotics to the curd at salting without any deleterious impact on starter activity. A suitable combination of antibiotics was developed and applied during Cheddar manufacture (Walsh *et al.*, 1996; Shakeel-Ur-Rehman *et al.*, 1999). In both studies growth of mesophilic lactobacilli was greatly reduced, though not totally inhibited. While the overall pattern of proteolysis in the cheese was not impacted upon by the antibiotic treatment, higher levels of amino acids were reported in cheese containing antibiotic. It was not clear why this should be the case but it was speculated that the antibiotics might have induced autolysis of the starter culture.

Bacteriocins, which may be produced by the starter culture within the cheese, are an alternative to antibiotics to control growth of secondary flora in cheese.

Ryan *et al.* (1996) reported on the use of starter cultures capable of producing the broad-spectrum bacteriocin, lacticin 3147, to inhibit growth of mesophilic lactobacilli. In trials using a combination of three strains which naturally produced lacticin 3147, no mesophilic lactobacilli were detected in the cheese up to 6 months of ripening. In trials using a single transconjugant strain capable of producing lacticin 3147, mesophilic lactobacilli did grow in the cheese but at significantly lower levels (~100-fold) than the mesophilic population in the control cheese. Application of this technology to reduced-fat Cheddar also resulted in growth inhibition of mesophilic lactobacilli, and it was concluded that the differences in the rate of growth and the final population of mesophilic lactobacilli had no significant effect on proteolysis, or flavour and aroma, or body and texture of the resulting cheese (Fenelon *et al.*, 1999). Manipulation of temperature during ripening has been used extensively to control the rate of cheese ripening and the growth of mesophilic lactobacilli and has been reviewed recently by Shakeel-Ur-Rehman *et al.* (2000c). In all such studies to date, while reducing the ripening temperature inhibits growth of mesophilic lactobacilli, no cheese was maintained free of lactobacilli using this technology, even when the temperature was reduced to 1ºC (Shakeel-Ur-Rehman *et al.*, 2000b).

The effect of adding adjunct cultures of mesophilic lactobacilli, isolated from cheese, to milk for cheesemaking has been researched for several decades. The results of these studies are equivocal, with some studies showing positive effects while others report negative effects on flavour formation (Peterson and Marshall, 1990; Fox *et al.*, 1998). The reason for the equivocal nature of the findings probably results from the flavouring potential of the isolates selected, combined with growth of adventitious strains during ripening. Most of the more recent studies on this topic have indicated that mesophilic adjuncts exert a positive effect on flavour (Puchades *et al.*, 1989; Broome *et al.*, 1990; Trépanier *et al.*, 1991, 1992; McSweeney *et al.*, 1993; Lynch *et al.*, 1996; Swearingen *et al.*, 2001). Gas production, which may result from growth of heterofermentative lactobacilli during ripening, is undesirable in Cheddar cheese. This defect could be controlled by addition of homofermentative lactobacilli to Cheddar during production (Laleye *et al.*, 1990). Direct comparison of the results from the various studies on the influence of mesophilic lactobacilli on cheese quality is difficult, as many differences exist in the experimental design and the level at which the adjunct lactobacilli were added.

Puchades *et al.* (1989) demonstrated that *Lb. casei* L2A when used as an adjunct resulted in a strong Cheddar flavour after 7 months of ripening at 6ºC; however, growth of mesophilic lactobacilli was not monitored during this study. In a subsequent study the effect on flavour was confirmed (Trépanier *et al.*, 1992) and maximum counts of lactobacilli were obtained at two weeks. Interpretation is further complicated by the fact that adventitious lactobacilli almost invariably gain access to the 'control' cheese and eventually reach similar, and in some cases higher (e.g. Trépanier *et al.*, 1991), numbers in comparison to those in the experimental cheeses. In studies by Broome *et al.* (1990) two strains of *Lb. casei* were investigated as potential adjunct strains.

Neither had an effect on flavour after 6 months' ripening at 8ºC, but cheese containing the adjunct had more pronounced Cheddar flavours after 9 and 12 months' ripening. Initial numbers of mesophilic lactobacilli were ~10^2 cfu g^{-1} and 10^8 cfu g^{-1} in the control and experimental cheeses respectively; however, the counts in the control cheese were ~10^8 cfu g^{-1} by 3 months of ripening. Improved flavour and reduced bitterness were obtained in American Cheddar using two strains of *Lb. paracasei* (Swearingen *et al.*, 2001); in these cheeses the counts for mesophilic lactobacilli were similar to those reported by Broome *et al.* (1990).

In another series of experiments (McSweeney *et al.*, 1993; Lynch *et al.*, 1996) adjuncts of *Lb. casei* ssp. *casei*, *Lb. casei* ssp. *pseudoplantarum*, *Lb. curvatus* and *Lb. plantarum* were added during the manufacture of Cheddar under controlled microbiological conditions. Mesophilic lactobacilli were detected in the control vats at initial levels of ~10^2 cfu g^{-1} and their population increased to ~10^6 cfu g^{-1} after 5 months' ripening at 8ºC. In contrast, mesophilic lactobacilli in the experimental cheeses were ~10^8 cfu g^{-1} from the beginning of ripening. The cheeses containing the mesophilic lactobacillus adjuncts were generally reported to have better flavour intensities and flavour acceptabilities than the control cheeses. Thus, mounting evidence appears to support the hypothesis that mesophilic lactobacilli do exert an influence on cheese quality.

Although pediococci are recognised as part of the non-starter flora of cheese, their impact on cheese quality has not been well studied, due primarily to the infrequency with which they have been encountered (for review, see Bhowmik and Marth, 1990). A number of enzymes with potential to promote cheese ripening have been identified in various strains and include protease, peptidase and lipase activities. Acetate and diacetyl production by pediococci may contribute to flavour development. Further investigation regarding the prevalence of pediococci and their growth and survival during cheese ripening is required prior to defining their role in flavour development.

While enterococci occur in a variety of dairy products made from raw or pasteurised milk (Cogan *et al.*, 1997) their impact on cheese quality is unclear. Indeed their presence in cheese may be considered an indicator of insufficient sanitary conditions during the production and processing of milk. A study of 129 strains of food, human and veterinary origin indicated that the majority of the isolates exhibited low milk acidifying ability and extracellular proteolytic activity (Sarantinopoulos *et al.*, 2001). However, relatively high lipolytic activities were reported and many of the strains utilized citrate and pyruvate. Some studies have concluded that high levels of enterococci result in deterioration in the sensory properties of cheese (Thompson and Marth, 1986; López-Diaz *et al.*, 1995). However, others have reported improved characteristics (Jensen *et al.*, 1975a, 1975b; Ordoñez *et al.*, 1978; Trovatelli and Schiesser, 1987; Centeno *et al.*, 1999). The perceived beneficial impact of enterococci on cheese quality has resulted in their proposed inclusion as adjuncts for the production of a range of cheeses including Mozzarella (Coppola *et al.*, 1988; Parente *et al.*, 1989), Feta (Litopoulou-Tzanetaki *et al.*, 1993), Cebreiro

(Centeno *et al.*, 1996) and Venaco (Casalta and Zennaro, 1997). The British Advisory Committee on Novel Foods and Processes (ACNFP) recently approved the use of *E. faecium* K77D (Coppola *et al.*, 1988; Parente *et al.*, 1989) for use in the manufacture of Mozzarella cheese (ACNFP, 1996).

As part of the non-starter flora *Leuconostoc* differ from the other members of the group in that their primary role in the production of acetate, CO_2, diacetyl, acetoin and 2,3-butanediol resulting from citrate metabolism is well documented. The CO_2 produced is responsible for small eye formation in Dutch cheeses such as Edam and Gouda, while diacetyl and acetate contribute to the flavour of products such as Quark, Fromage Frais and Cottage Cheese. *Leuconostoc* are also rich in intercellular proteolytic enzymes (El-Shafei *et al.*, 1990) and esterase activities were reported from wild strains of leuconostocs from Greek cheese (Vafopoulou-Mastrojiannaki *et al.*, 1996). Thus, they may have a secondary impact on cheese quality through proteolysis and lipolysis but this requires clarification.

20.7 Selection of NSLAB adjuncts for quality improvement of cheese

Identification of suitable strains of NSLAB for use as starter adjunct for quality improvement of cheese offers considerable financial benefit to cheese manufacturers. Suitable strains would improve cheese flavour through production of key flavour compounds in the cheese and by inhibiting growth of deleterious adventitious strains. An important characteristic of NSLAB and mesophilic lactobacilli in particular is their ability to grow in the cheese during ripening, thus enabling the cheesemaker to add them at very low levels to the cheese milk at the beginning of manufacture and still obtain a positive impact in the cheese. However, correct selection of the adjunct strain is crucial, as it was demonstrated that some 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 and Gilles, 1987).

As the perceived deterioration in Cheddar flavour has been associated with the introduction of pasteurisation by some commentators, selection of non-starter bacteria from raw milk for use as adjuncts during commercial manufacture of Cheddar was proposed (Reiter *et al.*, 1967). This approach involved the development of a ‘reference flora’ whereby bacteria isolated from raw milk or fresh cheese curd by selective plating techniques were used as an adjunct blend during Cheddar manufacture. These experiments indicated that such an approach could result in cheese of improved flavour, but that off-flavours may also occur. This approach assumes that all the relevant microflora in the raw milk can be recovered by the selective plating techniques used and that the resulting reference flora is representative of the raw milk. This assumption may not be correct as it is recognised that many strains of particular species may be unable to grow on selective media designed for that particular species.

A similar approach was adopted by Beuvier *et al.* (1997) who added microfiltration retentate from raw milk to pasteurised milk to study the influence of the raw milk microflora on the quality of Swiss cheese. The cheese made using the retentate was reported to have a better flavour than the control cheeses. A further modification of this approach was used by Shakeel-Ur-Rehman *et al.* (2000a) who used blends of raw and pasteurised milk for Cheddar cheese manufacture. They reported that cheese made from a blend containing as little as 1% raw milk had a significantly higher population of mesophilic lactobacilli and graded better than cheeses made from pasteurised milk. The approaches outlined above use the milk as the source of mesophilic lactobacilli. Folkertsma (1999) suggested that if mesophilic lactobacilli have an impact on cheese quality, suitable strains should be present in mature high quality cheese. To test this hypothesis cheese milk was inoculated with slurries of mature Cheddar prior to cheese manufacture. Unfortunately the quality of the cheese made from the inoculated milk was not significantly better than that of the control cheese. Recent studies have indicated that the populations of mesophilic lactobacilli in Cheddar cheese are dynamic and that few cheeses are dominated by single strains or groups of strains during ripening (Crow *et al.*, 2001; Fitzsimons *et al.*, 2001). These findings may explain why the hypothesis proposed by Folkertsma (1999) was not verified.

The growing knowledge of mesophilic *Lactobacillus* populations in Cheddar cheese led Crow *et al.* (2001) to apply molecular and biochemical techniques to the selection of strains from cheese during ripening. Use of pulse field gel electrophoresis and fermentation patterns on 22 carbohydrates resulted in the identification of 140 strains. This bank of strains was further screened for their responses to salt, temperature and pH, their ability to metabolise citrate, produce biogenic amines and their proteolytic and lipolytic activities. This reduced the number of potential strains to 60, which were screened in a model cheese system for flavour development and biochemical changes (excess fermentation of glutamate, lactate racemisation). This resulted in 24 potential strains being selected and 20 adjunct combinations (made up of 2–4 strains each) were tested in Cheddar cheese trials. Twelve of the adjunct blends showed some flavour improvement over the control cheeses and seven of the blends resulted in significant improvement and accelerated flavour development. This demonstrated that a systematic approach to strain selection based on the available knowledge of cheese microbiology and biochemistry of flavour development can lead to the selection of strains of mesophilic lactobacilli with the potential to improve the quality of Cheddar cheese.

Studies on the selection of other NSLAB for use as starter adjuncts are limited but in general similar approaches to those for selection of adjuncts of mesophilic lactobacilli are used. Pediococci have been selected from raw milk by selective plating (Robertson and Perry, 1961) or from cheese (Law *et al.*, 1976), while enterococci have been isolated from natural whey cultures (Coppola *et al.*, 1988; Parente *et al.*, 1989) and cheese (Centeno *et al.*, 1999).

20.8 Conclusions

In summary, the NSLAB population of most cheese varieties is complex, composed of a number of species and strains, which are in a dynamic state during ripening. Significant progress has been made in understanding the behaviour of NSLAB in cheese and this has been greatly aided by the application of suitable molecular techniques. The potential of members of the NSLAB complex, in particular mesophilic lactobacilli and enterococci, to function as probiotics and the capacity of cheese to act as a suitable delivery system for such strains to the human gastrointestinal tract has been demonstrated (Gardiner *et al.*, 1998, 1999). Genomic sequencing of members of the NSLAB complex is either recently completed or underway (for review, see Klaenhammer *et al.*, 2002), and the information generated will provide opportunities for further exploitation. There is mounting evidence to support the hypothesis that NSLAB have the potential to influence cheese quality. Thus, selection of suitable strains for use as starter adjuncts is crucial if the economic potential of these bacteria is to be realised, and such selections will be aided by the expanding scientific understanding of NSLAB.

20.9 References

ACNFP (1996), 'Report on *Enterococcus faecium*, strain K77D', MAFF Advisory Committee on Novel Foods and Processes, Report, Ergon House, c/o Nobel House, 17 Smith Square, London SW1 3JR.

ANTONSSON, M., ARDO, Y. and MOLIN, G. (2001), 'A comparison between the microflora of Herrgård cheese from three different dairies', *Int. Dairy J.* 11(4–7), 285–291.

ARIZCUN, C., BARCINA, Y. and TORRE, P. (1997), 'Identification of lactic acid bacteria isolated from Roncal and Idiazabal cheeses', *Lait* 77(6), 729–736.

BERESFORD, T.P., FITZSIMONS, N.A., BRENNAN, N.L. and COGAN, T.M. (2001), 'Recent advances in cheese microbiology', *Int. Dairy J.* 11, 259–274.

BERTHIER, F., BEUVIER, E., DASEN, A. and GRAPPIN, R. (2001), 'Origin and diversity of mesophilic lactobacilli in Comté cheese, as revealed by PCR with repetitive and species-specific primers', *Int. Dairy J.* 11(4/7), 293–305.

BEUVIER, E., BERTHAUD, K., CEGARRA, S., DASEN, A., POCHET, S., BUCHIN, S. and DUBOZ, G. (1997), 'Ripening and quality of Swiss-type cheese made from raw, pasteurized or microfiltered milk', *Int. Dairy J.* 7, 311–323.

BHOWMIK, T. and MARTH, E.H. (1990), 'Role of *Micrococcus* and *Pediococcus* species in cheese ripening: a review', *J. Dairy Sci.* 73, 859–866.

BOUTON, Y., GUYOT, P. and GRAPPIN, P. (1998), 'Preliminary characterization of microflora of Comté cheese', *J. Appl. Microbiol.* 85, 123–131.

BROOME, M.C., KRAUSE, D.A. and HICKEY, M.W. (1990), 'The isolation and characterization of lactobacilli from Cheddar cheese', *Australian J. Dairy Technol.* 45, 60–66.

CASALTA, E. and ZENNARO, R. (1997), 'Effect of specific starters on microbiological, biochemical and sensory characteristics of Venaco, a Corsican soft cheese', *Sciences des Aliments* 17, 79–94.

CENTENO, J.A., MENÉNDEZ, S. and RODRIGUEZ-OTERO, J.L. (1996), 'Main microflora present

in natural starters in Cebreiro raw cow's milk cheese (Northwest Spain)', *Int. J. Food Microbiol.* 33, 307–313.

CENTENO, J.A., MENÉNDEZ, S., HERMIDA, M.A. and RODRIGUEZ-OTERO, J.L. (1999), 'Effects of addition of *Enterococcus faecalis* in Cebreiro cheese manufacture', *Int. J. Food Microbiol.* 48, 97–101.

CHAPMAN, H.R., MABITT, L.A. and SHARPE, M.E. (1966), 'Apparatus and techniques for the manufacture of Cheddar cheese under controlled bacteriological conditions', *Proceedings of the 17th International Dairy Congress*, Munich, D, 774–775.

CIBIK, R., LEPAGE, E. and TAILLIEZ, P. (2000), 'Molecular diversity of *Leuconostoc mesenteroides* and *Leuconostoc citreum* isolated from traditional French cheeses as revealed by RAPD fingerprinting, 16S rDNA sequencing and 16S rDNA fragment amplification', *Syst. Appl. Microbiol.* 23, 267–278.

COGAN, T.M. (2000), 'Microbiology of cheese ripening', in Fox, P.F., Guinee, T.P., Cogan, T.M. and McSweeney, P.L.H., *Fundamentals of Cheese Science*, Maryland, Aspen, 206–232.

COGAN, T.M., BARBOSA, M., BEUVIER, E., BIANCHI-SALVADORI, B., COCCONCELLI, P.S., FERNANDES, I., GOMEZ, J., GOMEZ, R., KALANTZOPOULOS, G., LEDDA, A., MEDINA, M., REA, M.C. and RODRIGUEZ, V. (1997), 'Characterisation of the lactic acid bacteria in artisanal dairy products', *J. Dairy Res.* 64, 409–421.

COLLINS, M.D., JONES, D., FARROW, J.A.E., KILPPER-BÄLZ, R. and SCHLEIFER, K.H. (1984), '*Enterococcus avium* nom. rev., comb. nov.; *E. casseliflavus* nom. rev., comb. nov.; *E. durans* nom. rev., comb. nov.; *E. gallinarum* comb nov.; and *E. malodoratus* sp. nov.', *Int. J. Syst. Bacteriol.* 34(2), 220–223.

COPPOLA, R., NANNI, M., IORIZZO, M., SORRENTINO, A., SORRENTINO, E. and GRAZIA, L. (1997), 'Survey of lactic acid bacteria isolated during the advanced stages of the ripening of Parmigiano Reggiano cheese', *J. Dairy Res.* 64, 305–310.

COPPOLA, T.M., PARENTE, J.E., DUMONTET, S. and LA PECCRELLA, A. (1988), 'The microflora of natural whey cultures utilized as starters in the manufacture of Mozzarella cheese from water buffalo milk', *Lait* 68, 295–310.

CROW, V., CURRY, B. and HAYES, M. (2001), 'The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar', *Int. Dairy J.* 11, 275–283.

DACRE, J.C. (1958a), 'Characteristics of a presumptive *Pediococcus* occurring in New Zealand Cheddar cheese', *J. Dairy Res.* 25, 409–413.

DACRE, J.C. (1958b), 'A note on pediococci in New Zealand Cheddar cheese', *J. Dairy Res*. 25, 414–417.

DAHL, S., TAVARIA, F.K. and MALCATA, F.X. (2000), 'Relationships between flavour and microbiological profiles in Serra da Estrela cheese throughout ripening', *Int. Dairy J*. 10(4), 255–262.

DE ANGELIS, M., CORSETTI, A., TOSTI, N., ROSSI, J., CORBO, M.R. and GOBBETTI, M. (2001), 'Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analyses', *Appl. Environ. Microbiol*. 67, 2011–2020.

DEASY, B.M., REA, M.C., FITZGERALD, G.F., COGAN, T.M. and BERESFORD, T. (2000), 'A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus', Syst. Appl. Microbiol.* 23, 510–522.

DEIBEL, R.H., LAKE, D.E. and NIVEN JR, C.F. (1963), 'Physiology of the enterococci as related to their taxonomy', *J. Bacteriol*, 86(6), 1275–1282.

DEL POZO, B.F., GAYA, P., MEDINA, M., RODRIGUEZ-MARIN, M.S. and NUNEZ, M. (1988),

'Changes in the microflora of la Serena ewes' milk cheese during ripening', *J. Dairy Res.* 55(3), 449–455.

DEMARIGNY, Y., BEUVIER, E., DASEN, A. and DUBOZ, G. (1996), 'Influence of raw milk microflora on the characteristics of Swiss-type cheeses. I. Evolution of microflora during ripening and characterisation of facultatively heterofermentative lactobacilli', *Lait* 76, 371–387.

DEVRIESE, L.A., CEYSSENS, K., RODRIGUES, U.M. and COLLINS, M.D. (1990), '*Enterococcus columbae*, a species from pigeon intestines', *FEMS Microbiol. Lett.* 71, 247–252.

DEVRIESE L.A., LAURIER, L., DE HERDT, P. and HAESEBROUCK, F. (1992), 'Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows', *J. Appl. Bacteriol*. 72, 29–31.

ELLIOTT, J.A. and MULLIGAN, H.T. (1968), 'Pediococci in Canadian Cheddar cheese', *Can. Inst. Food Technol. J*. 1, 61.

EL-SHAFEI, H., EL-SODA, M. and EZZAT, N. (1990), 'The peptidase hydrolyase system of the *Leuconostoc'*, *J. Food Prot*. 53, 165–169.

FACKLAM, R.R. (1973), 'Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci', *Appl. Microbiol.* 26(2), 138–145.

FACKLAM, R.R. and COLLINS, M.D. (1989), 'Identification of *Enterococcus* species isolated from human infections by a conventional test scheme', *J. Clin. Microbiol*. 27(4), 731–734

FACKLAM, R.R. and MOODY, M.D. (1970), 'Presumptive identification of group D streptococci: the bile aesculin test', *Appl. Microbiol*. 20(2), 245–250.

FENELON, M.A., RYAN, M.P., RAE, M.C., GUINEE, T.P., ROSS, R.P., HILL, C. and HARRINGTON, D. (1999), 'Elevated temperature ripening of reduced fat Cheddar cheese made with or without lacticin 3147-producing starter culture', *J. Dairy Sci*. 82, 10–22.

FITZSIMONS, N.A., COGAN, T.M., CONDON, S. and BERESFORD, T. (1999), 'Phenotypic and genotypic characterisation of non-starter lactic acid bacteria in mature Cheddar cheese', *Appl. Environ. Microbiol*. 65, 3418–3426.

FITZSIMONS, N.A., COGAN, T.M., CONDON, S. and BERESFORD, T. (2001), 'Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese', *J. Appl. Microbiol.* 90, 600–608.

FOLKERTSMA, B. (1999), 'Studies on ripening of Cheddar cheese', MSc dissertation, Department of Food Science and Technology, National University of Ireland, Cork.

FONTAN, M.C.G., FRANCO, I., PRIETO, B., TORNADIJO, M.E. and CARBALLO, J. (2001), 'Microbiological changes in ''San Simon'' cheese throughout ripening and its relationship with physico-chemical parameters', *Food Microbiol*. 18(1), 25–33.

FOX, P.F., MCSWEENEY, P.L.H. and LYNCH, C.M. (1998), 'Significance of non-starter lactic acid bacteria in Cheddar cheese', *Australian J. Dairy Technol*. 53, 83–89.

FRANKLIN, J.G. and SHARPE, M.F. (1963), 'The incidence of bacteria in cheese milk and Cheddar cheese and their association with flavour', *J. Dairy Res*. 30, 87–99.

FRANZ, C.M.A.P., HOLZAPFEL, W.H. and STILES, M.F. (1999), 'Enterococci at the crossroads of food safety?', *Int. J. Food Microbiol*. 47, 1–24.

FREITAS, A.C., PAIS, C., MALCATA, F.X. and HOGG, T.A. (1995), 'Microbiological characterization of Picante de Baixa cheese', *J. Food Prot.* 59, 155–160.

FRYER, T.F. and SHARPE, M.F. (1966), 'Pediococci in Cheddar cheese', *J. Dairy Res*. 33, 325–331.

GARCIA, A., CABEZAS, L. and PALOP, M.L. (1995), 'Identification of the lactic acid bacteria

from origin labelled ''Manchego'' cheeses', *Microbiologie Aliments Nutrition* 13(3), 275–280.

GARDINER, G., ROSS, R.P., COLLINS, J.K., FITZGERALD, G. and STANTON, C. (1998), 'Development of a probiotic Cheddar cheese containing human-derived *Lactobacillus paracasei* strains', *Appl. Environ. Microbiol*. 64(6), 2192–2199.

GARDINER, G., ROSS, R.P., WALLACE, J.M., SCANLAN, F.P., JÄGERS, P.P.J.M., FITZGERALD, G.F., COLLINS, J.K. and STANTON, C. (1999), 'Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of Cheddar cheese', *J. Agric. Food Chem*. 47, 4907–4916.

GARVIE, E. (1984), 'Taxonomy and identification of bacteria important in cheese and fermented dairy products', in Davis, F.L. and Law, B.A., *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*, London and New York, Elsevier.

GELSOMINO, R., VANCANNEYT, M., CONDON, S., SWINGS, J. and COGAN, T.M. (2001), 'Enterococcal diversity in the environment of an Irish Cheddar-type cheesemaking factory', *Int. J. Food Microbiol*. 71, 177–188.

GELSOMINO, R., VANCANNEYT, M., COGAN, T.M., CONDON, S. and SWINGS, J. (2002), 'Source of enterococci in a farmhouse raw-milk cheese', *Appl. Environ. Microbiol*. 68(7), 3560–3565.

GIRAFFA, G., CARMINATI, D. and NEVIANI, F. (1997), 'Enterococci isolated from dairy products: a review of risks and potential technological use', *J. Food Prot*. 60(6), 732–738.

GOBBETTI, M., FOLKERTSMA, B., FOX, P.F., CORSETTI, A., SMACCHI, E., DE ANGELIS, M., ROSSI, J., KILCAWLEY, K. and CORTINI, M. (1999), 'Microbiology and biochemistry of Fossa (pit) cheese', *Int. Dairy J.* 9(11), 763–773.

GOBBETTI, M., MOREA, M., BARUZZI, F., CORBO, M.R., MATARANTE, A., CONSIDINE, T., DI CAGNO, R., GUINEE, T. and FOX, P.F. (2002), 'Microbiological, compositional, biochemical and textural characterisation of Caciocavallo Pugliese cheese during ripening', *Int. Dairy J*. 12(6), 511–523.

JENSEN, J.P., REINBOLD, G.W., WASHAM, J.C. and VEDAMUTHU, E.R. (1975a), 'Role of enterococci in Cheddar cheese: proteolytic activity and lactic acid development', *J. Milk Food Technol.* 38, 3–7.

JENSEN, J.P., REINBOLD, G.W., WASHAM, J.C. and VEDAMUTHU, E.R. (1975b), 'Role of enterococci in Cheddar cheese: free fatty acid appearance and citric acid utilization', *J. Milk Food Technol.* 38, 78–83.

JORDAN, K.N. and COGAN, T.M. (1993). Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. *Irish J. Agric. Food Res*. 32, 47–55.

JORDAN, K.N. and COGAN, T.M. (1999), 'Heat resistance of *Lactobacillus* spp. isolated from Cheddar cheese', *Lett. Appl. Microbiol*. 29, 136–140.

KANDLER, O. and WEISS, N. (1986), 'Regular, non-sporing, Gram positive rods', in Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., *Bergey's Manual of Systematic Bacteriology*, Baltimore, Williams and Wilkins, Vol. 2, 1208–1234.

KLAENHAMMER, T., ALTERMANN, E., ARIGONI, F., BOLOTIN, A., BREIDT, F., BROADBENT, J., CANO, R., CHAILLOU, S., DEUTSCHER, J., GASSON, M., VAN DE GUCHTE, M., GUZZO, J., HARTKE, A., HAWKINS, T., HOLS, P., HUTKINS, R., KLEEREBEZEM, M., KOK, J., KUIPERS, O., LUBBERS, M., MAGUIN, E., MCKAY, L., MILLS, D., NAUTA, A., OVERBEEK, R., PEL, H., PRIDMORE, D., SAIER, M., VAN SINDEREN, D., SOROKIN, A., STEELE, J., O'SULLIVAN, D., DE VOS, W., WEIMER, B., ZAGOREC, M. and SIEZEN, R. (2002), 'Discovering lactic acid bacteria by genomics', in Siezen, R.J., Kok, J., Abee, T. and Schaafsma, G.,

Proceedings of the Seventh Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications, The Netherlands, *Antonie van Leeuwenhoek*, Kluwer Academic Publishers, 29–58.

LALEYE, L.C., SIMARD, R.E., LEE, B.H. and HOLLEY, R.A. (1990), 'Quality attributes of Cheddar cheese containing added lactobacilli', *J. Food Sci*. 55, 114–118.

LAW, B.A., CASTANON, M. and SHARPE, M.E. (1976), 'The effect of nonstarter bacteria on the chemical composition and the flavour of Cheddar cheese', *J. Dairy Res*. 43, 117–125.

LAWRENCE, R.C. and GILLES, J. (1987), 'Cheddar cheese and related dry-salted varieties', in Fox, P.F., *Cheese: Chemistry, Physics and Microbiology*, London, Elsevier Applied *Science*, Vol. 2, 1–44.

LITOPOULOU-TZANETAKI, E. (1990), 'Changes in numbers and kinds of lactic acid bacteria during ripening of Kefalotyri cheese', *J. Food Sci*. 73, 111–113.

LITOPOULOU-TZANETAKI, E., GRAHAM, D.C. and BEYATLI, Y. (1989), 'Detection of pediococci and other non-starter organisms in American Cheddar cheese', *J. Dairy Sci*. 72(4), 854–858.

LITOPOULOU-TZANETAKI, E., TZANETAKIS, N. and VAFOPOULOU-MASTROJIANNAKI, A. (1993), 'Effect of type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese', *Food Microbiol*. 10, 31–41.

LÓPEZ-DIAZ, T.M., SANTOS, J.A., GONZALEZ, C.J., MORENO, B. and GARCIA, M.L. (1995), 'Bacteriological quality of a traditional Spanish blue cheese', *Milchwissenschaft* 50(9), 503–505.

LYNCH, C.M., MCSWEENEY, P.L.H., FOX, P.F., COGAN, T.M. and DRINAN, F.D. (1996), 'Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions', *Int. Dairy J*. 6, 851–867.

MABBITT, L.A., CHAPMAN, H.R. and SHARPE, M.E. (1959), 'Making Cheddar cheese under controlled bacteriological conditions', *J. Dairy Res*. 26, 105–112.

MACEDO, A.C., MALCATA, F.X. and HOGG, T.A. (1995), 'Microbiological profile in Serra ewe's cheese during ripening', *J. Appl. Bacteriol*. 79, 1–11.

MANNU, L., COMUNIAN, R. and SCINTU, M.F. (2000), 'Mesophilic lactobacilli in Fiore Sardo cheese: PCR-identification and evolution during cheese ripening', *Int. Dairy J*. (5-6), 383–389.

MCSWEENEY, P.L.H., FOX, P.F., LUCEY, J.A., JORDAN, K.N. and COGAN, T.M. (1993), 'Contribution of indigenous microflora to the maturation of Cheddar cheese', *Int. Dairy J*. 3, 613–614.

MCSWEENEY, P.L.H., WALSH, E.M., FOX, P.F., COGAN, T.M., DRINAN, F.D. and CASTELO-GONZALEZ, M. (1994), 'A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality', *Irish J. Agric. Food Res*. 33, 183–192.

MENENDEZ, S., GODINEZ, R., CENTENO, J.A. and RODRIGUEZ-OTERO, J.L. (2001), 'Identification and characterization of lactic acid bacteria isolated from Tetilla cows' milk cheese', *Alimentaria* 320, 77–83.

MOREA, M., BARUZZI, F. and COCCONCELLI, P.S. (1999), 'Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing', *J. Appl. Microbiol*. 87, 574-582.

NUNEZ, M. (1976), 'Microflora of Manchego cheese, VI. Pediococci', *An. Inst. Nac. Invest. Agrarias, General*, No 4: 75 (in *Dairy Sci. Abstr*. 1978: 458).

ORDOÑEZ, J.A., BARNETO, R. and RAMOS, M. (1978), 'Studies on Manchego cheese ripened in olive oil', *Milchwissenschaft* 33(10), 609–613.

PARENTE, V., VILLANI, F., COPPOLA, R. and COPPOLA, S. (1989), 'A multiple strain starter for

water-buffalo mozzarella cheese manufacture', *Lait* 69, 271–279.

PÉREZ, G., CARDELL, E. and ZÁRATE, V. (2002), 'Random amplified polymorphic DNA analysis for differentiation of *Leuconostoc mesenteroides* subspecies isolated from Tenerife cheese', *Lett. Appl. Microbiol*. 34, 82–85.

PETERSON, S.D. and MARSHALL, R.T. (1990), 'Non starter lactobacilli in Cheddar cheese: a review', *J. Dairy Sci*. 73, 1395–1410.

POULLET, B., HUERTAS, M., SÁNCHEZ, A., CÁCERES, P. and LARRIBA, G. (1993), 'Main lactic acid bacteria isolated during ripening of Casar de Cáceres cheese', *J. Dairy Res*. 60, 123–127.

PUCHADES, R., LEMIEUX, L. and SIMARD, R.E. (1989), 'Evolution of free amino acids during the ripening of Cheddar cheese containing added lactobacilli strains', *J. Food Sci*. 54, 885–888.

REITER, B., FRYER, T.F., PICKERING, A., CHAPMAN, H.R., LAWRENCE, R.B. and SHARPE, M.E. (1967), 'The effect of microbial flora on the flavour and free fatty acid composition of Cheddar cheese', *J. Dairy Res*. 26, 105–112.

ROBERTSON, P.S. and PERRY, K.D. (1961), 'Enhancement of flavour of Cheddar cheese by adding a strain of *Micrococcus* to the milk', *J. Dairy Res*. 28, 245–253.

RYAN, M.P., RAE, M.C., HILL, C. and ROSS, R.P. (1996), 'An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147', *Appl. Environ. Microbiol*. 62, 612–619.

SARANTINOPOULOS, P., ANDRIGHETTO, C., GEORGALAKI, M.D., REA, M.C., LOMBARDI, A., COGAN, T.M., KALANTZOPOULOS, G. and TSAKALIDOU, E. (2001), 'Biochemical properties of enterococci relevant to their technological performance', *Int. Dairy J*. 11(8), 621–647.

SCHLEIFER, K.H. (1986). 'Gram-positive cocci', in Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., *Bergey's Manual of Systematic Bacteriology*, Baltimore, Williams and Wilkins, 999–1103.

SCHLEIFER, K.H. and KILPPER-BÄLZ, R. (1984), 'Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov.', *Int. J. Syst. Bacteriol*. 34(1), 31–34.

SHAKEEL-UR-REHMAN, MCSWEENEY, P.H.L. and FOX, P.F. (1999), 'A study on the role of indigenous microflora of raw milk on the ripening of Cheddar cheese', *Milchwissenschaft* 54(7), 388–392.

SHAKEEL-UR-REHMAN, BANKS, J.M., BRECHANY, E.Y., MUIR, D.D., MCSWEENEY, P.H.L. and FOX, P.F. (2000a), 'Studies on the ripening of Cheddar cheese made from blends of raw and pasteurized milk', *Int. Dairy J*. 10, 33–44.

SHAKEEL-UR-REHMAN, BANKS, J.M., MCSWEENEY, P.H.L. and FOX, P.F. (2000b), 'Effect of ripening temperature on the growth and significance of non-starter lactic acid bacteria in Cheddar cheese made from raw or pasteurized milk', *Int. Dairy J*. 10, 45–53.

SHAKEEL-UR-REHMAN, FOX, P.F. and MCSWEENEY, P.H.L. (2000c), 'Methods used to study non-starter micro-organisms in cheese: a review', *Int. J. Dairy Technol*. 53, 113–119.

SIMPSON, W.J. and TAGUCHI, H. (1995), 'The genus *Pediococcus* with notes on the genera *Tetragenococcus* and *Aerococcus*', in Wood, B.J.B. and Holzapfel, W.H., *The Genera of Lactic Acid Bacteria*, Blackie Academic & Professional, 125–172.

SOMERS, E.B., JOHNSON, M.E. and WONG, A.C.L. (2001), 'Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment', *J. Dairy Sci.* 84, 1926–1936.

STACKEBRANDT, E. and TEUBER, M. (1988), 'Molecular taxonomy and phylogenetic

position of lactic acid bacteria', *Biochimie* 70(3), 317–324.

SWEARINGEN, P.A., O'SULLIVAN, D.J. and WORTHESON, J.J. (2001), 'Isolation, characterization and influence of native non-starter lactic acid bacteria on Cheddar cheese quality', *J. Dairy Sci*. 84, 50–59.

TEIXEIRA, L.M., MERQUIOR, V.L.C., DA CONCEIÇÃO, M., VIANNI, M. DA C.E., CARVALHO, M. DA G.S., FRACALANZZA, S.E.L., STEIGERWALT, A.G., BRENNER, D.J. and FACKLAM, R.R. (1996), 'Phenotypic and genotypic characterisation of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida*', *Int. J. Syst. Bacteriol.* 46(3), 664–668.

THOMAS, T.D. (1987), 'Cannibalism among bacteria found in cheese', *NZ J. Dairy Sci. Technol.* 22, 215–219.

THOMPSON, T.L. and MARTH, E.H. (1986), 'Changes in Parmesan cheese during ripening: icroflora – coliforms, enterococci, anaerobes, propionibacteria and staphylococci', *Milchwissenschaft* 41(4), 201–205.

THUNELL, R.K. (1995), 'Taxonomy of leuconostocs', *J. Dairy Sci*. 78, 2514–2522.

TRÉPANIER, G., SIMARD, R.E. and LEE, B.H. (1991), 'Effect of added lactobacilli on composition and texture of Cheddar cheese during accelerated maturation', *J. Food Sci*. 56, 696–700.

TRÉPANIER, G., ABBOUDI, M.E., LEE, B.H. and SIMARD, R.E. (1992), 'Accelerated maturation of Cheddar cheese: microbiology of cheese supplemented with *Lactobacillus casei* subsp. *casei* L2A', *J. Food Sci*. 57, 345–349.

TROVATELLI, L.D. and SCHIESSER, A. (1987), 'Identification and significance of enterococci in hard cheese made from raw cow and sheep milk', *Milchwissenschaft* 42, 717–719.

TURNER, K.W. (1988), 'Some aspects of the microbiology of cheese ripening investigated using aseptic manufacturing techniques', PhD thesis, Massey University, Palmerston North, New Zealand.

TURNER, K.W., LAWRENCE, R.C. and LE LIEVRE, J. (1986), 'A microbiological specification for milk for aseptic cheesemaking', *NZ J. Dairy Sci. Technol*. 21, 249–254.

TZANETAKIS, N. and LITOPOULOU-TZANETAKI, E. (1989), 'Biochemical activities of *Pediococcus pentosaceus* isolates of dairy origin', *J. Dairy Sci.* 72, 859–863.

TZANETAKI, N. and LITOPOULOU-TZANETAKI, E. (1992), 'Changes in the numbers and kinds of lactic acid bacteria in Feta and Teleme, two Greek cheeses from ewe's milk', *J. Dairy Sci.* 75, 1389–1393.

VAFOPOULOU-MASTROJIANNAKI, A., LITOPOULOU-TZANETAKI, E. and TZANETAKIS, N. (1996), 'Esterase activities of cell-free extracts from ''wild'' strains of leuconostocs and heterofermentative lactobacilli isolates from traditional Greek cheese', *Lett. Appl. Microbiol*. 23(6), 367–370.

WALSH, E.M., MCSWEENEY, P.H.L. and FOX, P.F. (1996), 'Use of antibiotics to inhibit non-starter lactic acid bacteria in Cheddar cheese', *Int. Dairy J.* 6, 425–431.

WILLIAMS, A.G. and BANKS, J.M. (1997), 'Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom', *Int. Dairy J.* 7, 763–774.

WILLIAMS, A.G., WITHERS, S.E. and BANKS, J.M. (2000), 'Energy sources of non-starter lactic acid bacteria isolated from Cheddar cheese', *Int. Dairy J.* 10, 17–23.

ZARATE, V., BELDA, F., PEREZ, C. and CARDELL, E. (1997), 'Changes in the microbial flora of Tenerife goat's milk cheese during ripening', *Int. Dairy J.* 7, 635–641.