Microbial Dynamics during Cheese Production and Ripening: Physicochemical and Biological Factors

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ABSTRACT

Microorganisms are an essential component of cheese, playing an important role during its manufacture and ripening. Cheese microbiota can be composed by starter cultures, group formed mainly by lactic acid bacteria (LAB) such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* involved in acid production and flavor development during ripening; adjunct microbial cultures and secondary microorganisms, which play an important role during ripening. Cheese is a typical example of a mixed fermentation process in which desirable and undesirable bacteria, yeasts and molds interact and compete during initial steps and ripening process, leading to a complex product whose characteristics depends on the dynamics of all these microbial populations, which is influenced by intrinsic and extrinsic factors, such as temperature, pH, moisture, manufacture conditions, biological factors, among others. The main biological factors that influence the increase of one population in detriment of other microbial populations are: direct competition for substrate, production of inhibitory metabolites (such as acetic acid, acetaldehyde, ethanol, diacetyl), and the secretion of bacteriocins. The inhibitory compounds are products of bacterial metabolism, whereas bacteriocins are antimicrobial peptides or proteins produced by different groups of bacteria, including LABs. Several examples show that the direct addition of bacteriocins or bacteriocin producing starter cultures interfere in the proliferation of undesirable NSLAB and harmful bacterial populations during cheese processing and ripening. Thus, the characterization of new bacteriocins and the selection of starter cultures, as well as their combination with non-thermal physical treatments offer excellent opportunities for the control of undesirable microbial communities in fermented dairy products.

CONTENTS

INTRODUCTION

Cheese manufacture is one of the classical examples of food preservation, as milk is so perishable, dating from 6000-7000 BC (Fox 2001). Actually, around a third of the world's milk production is used in cheese making (Farkye 2004).

Cheese manufacture essentially involves gelatinization of casein via isoeletric (acid) or enzymatic (rennet) coagulation (Fox 2001). The method used to clot milk for cheese-making influences the overall structure, characteristics and firmness of the cheese (Farkye 2004). Although the basic process of cheese fabrication is common to all kinds of cheeses, changes in milk origin, processing techniques and ripening time generate a huge range of the knowing varieties – around 1.000 kinds, from which 400 are produced in France (Perry 2004).

Cheese classification is based on the characteristics of milk, coagulation process, curdled consistence, amount of fat, rind formation, and conditions during ripening. In cheese, proteins are the primary structural material with fat entrapped in the protein matrix. Fat indirectly affects the water protein ratio and regulates firmness and elasticity of cheese by increasing the moisture retaining property of curd (Olson 1984). Texture development in cheese occurs due to the breakdown of α_{S1}-casein during ripening (Lawrence *et al.* 1987). Furthermore, milk fat normally provides a typical smoothness to a full fat cheese by being evenly distributed within the casein matrix of cheese. When fat is removed, such as in low-fat cheeses, casein plays a greater role in texture development. In low-fat variants there is an inadequate

Invited Review

breakdown of casein and, therefore, the cheese appears to have a relatively firm texture (Mistry 2001).

The milk used in cheese making may be pasteurized or not pasteurized, but fresh cheese (i.e. cottage, quark) are obligatory pasteurized. Cheeses as Prato, Danbo, Gouda, Edam, Mozzarella, among others, are made with heat-treated milk, while Camembert, Roquefort, Gorgonzolla, and several other varieties, are made with raw milk. Some cheeses as Gruyère, Emmental and Provolone can be made with pasteurized milk or not (Perry 2004).

Cheese ripening is a complex process and involves microbiological and biochemical changes to the curd resulting in flavor and texture characteristics of the particular variety (McSweeney 2004). Microorganisms are an essential component of all natural cheese varieties and play important roles during both cheese manufacture and ripening. During these processes, complex interactions occur between microbial populations, and between them and cheese environment (Beresford *et al.* 2001).

Cheese microbiota is formed by desirable microorganisms (bacteria, yeasts and molds), and undesirable populations of potential pathogens and/or deteriorating agents (Perry 2004).

Some microorganisms, mainly lactic acid bacteria, are desirable because they contribute to the coagulation and/or ripening processes, being involved in milk acidification and/or the development of flavor. They also compete with undesirable microorganisms by direct competition (substrate availability, pH reduction, changes in redox potential) and/or producing natural inhibitory compounds including bacteriocins. Lactic acid bacteria (LAB) can be added to cheese process (starter and adjunct culture) or spontaneously proliferate during cheese processing and ripening (non-starters).

Dairy products have long been recognized as being susceptible to contamination by harmful microorganisms. The predominant contaminants of processed dairy products have origins in the raw milk supply. If one examines a list of bacterial pathogens commonly associated with raw milk, *Salmonella typhimurium*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Yersinia enterocolitica* are just a few of many contaminants (Bryan 1983; Potter *et al.* 1984).

Cheese contamination can be derived from milk, manipulators and manufacturing process. In raw milk and their cheeses, the main contamination source is bovine mastitis, which most important etiologic agent is *Staphylococcus au-*reus. Some researches showed that cross-contamination is the main source of undesirable microorganisms in cheeses made with pasteurized milk (Borges 2006).

Cheese is considered an usual vehicle of food-borne pathogens, especially artisanal fresh cheese like curd-cheese. In these cases, besides milk-borne contaminants, unsatisfactory conditions of hygiene during processing increase the chance of have a harmful product. Microbiological contamination represents important economic losses for producers, and health risk for consumers (Borges 2006).

CHEESE MICROORGANISMS

Cheese microbiota is divided into starter LAB, adjunct microbial cultures, and secondary microorganisms. The first group is involved in acid production during manufacture and also contributes to the ripening process, while the last two groups play an important role during ripening. The secondary microbiota is formed by spontaneous occurring non-starter lactic acid bacteria (NSLAB), other bacteria, yeasts and moulds which grow internally or externally in most cheese varieties (Beresford *et al.* 2001).

LAB are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal microbiota (Coeuret *et al.* 2003). *Lactococcus*, *Leuconostoc* and *Lactobacillus* (homofermentative and heterofermentative species) are the most prevalent genera of LAB in cheeses (Pérez 2000). They are potentially important in autolysis, and associated cheese-ripening events (Crow *et al.* 1995).

The term "starter culture" refers to one or more selected strains of food grade microorganisms used to produce fermented foods with some desirable characteristics in appearance, body, texture and flavor (Ray 2001). The most often starter bacteria present in cheeses are from the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* (Beresford *et al.* 2001). The most common starter species are listed in **Table 1**.

Secondary cheese microbiota probably has its origins in raw material, post-pasteurization airborne contamination, cheese-making equipment or ingredients, or pasteurization survival. In Irish cheddar cheese the main NSLAB isolated were *Lb. paracasei*, *Lb. plantaram*, *Lb. curvatus* and *Lb. brevis* (Fitzsimons *et al.* 2001). Beside these bacterial species, *Lb. pentosus*, *Lb. fermentum* and *Lb. casei*, were iso-

Table 1 LAB species commonly found in cheeses.

LAB species	Major known function	Examples
STARTERS		
Enterococcus faecalis	Acid and flavor	Soft Italian, Cheddar, Swiss cheese, Domiati (Egyptian cheese)
Lactobacillus delbrueckii subsp. *bulgaricus*	Acid and flavor	Swiss, Emmental, Italian cheese
Lactobacillus delbrueckii subsp. *lactis*		
Lactobacillus helveticus		
Lactococcus lactis subsp. *cremoris*	Acid	Sour cream, Feta, Camembert, Brie, Gouda, Blue
Lactococcus lactis subsp. *lactis*		
Lactococcus lactis subsp. *lactis* (biovar. *diacetylactis*)	Acid and flavor	Kareish cheese, Cottage cheese, Continental cheese, Cheddar, Camembert, Brie
Leuconostoc lactis	Flavor	Cottage cheese, Emmental, Tenerife cheese, Camembert, Brie
Leuconostoc mesenteroides subsp. *dextranicum*		
Leuconostoc paramesenteroides		
Pediococcus pentosaceus	Flavor	Cheddar, Gouda
Propionibacterium freudenrechii subsp. *shermanii*	Flavor and eye formation	Emmental and Swiss cheese
Streptococcus thermophilus	Acid	Emmental, Cheddar and Italian cheese
NON-STARTERS		
Lactococcus lactis	Flavor and/or acid	Most cheeses, especially artisanal cheeses
Lactobacillus plantarum		
Lactobacillus casei		
Lactobacilus paracasei		
Lactobacillus curvatus		
Lactobacillus brevis		
Leuconostoc mesenteroides		
Enterococcus faecalis		

lated from Italian ewe cheeses by De Angelis *et al.* (2001).

Milk pasteurization reduces or eliminates undesirable, as well as desirable microorganisms which contribute to the sensorial characteristics of dairy products. In these situations, the use of adjunct cultures in addition to the commercial starters may improve cheese flavor, achieving sensory scores similar to those made from raw milk (Broome 2007). The adjunct culture increases flavor intensity and acceptability during ripening (Lynch *et al.* 1999). This group of LAB, termed starter adjuncts, refers to non-starter strains intentionally added with starter cultures. LAB commonly used as starter adjuncts are leuconostocs, lactobacilli and *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* (Crow *et al.* 1995).

Propionic acid bacteria (PAB) grow in many cheese varieties during ripening, and are the characteristic microbiota associated with Swiss-type cheeses such as Emmental, Gruyère, Appenzell and Comté. PAB are Gram-positive short rod-shaped bacteria which can metabolize lactate (Steffen 1973). Traditionally, PAB are known for their ability to convert lactate to propionate, acetate and carbonic dioxide (Hettinga and Reinbold 1972) and for their high lipolytic activity – 10 to 100 times higher than LAB (Knaut and Mazurek 1974; Dupuis 1994).

Another important group of microorganisms in ripened cheeses are moulds. Mould-ripened cheeses are divided into two groups: those which are ripened due to the presence of *Penicillium roqueforti* which grows and form blue veins within the cheese, such as Roquefort, Gorgonzola, Stilton and Danish blue, and those which are ripened with *P. camemberti* which grows on the surface of the cheese, such as Camembert and Brie. Interior or surface mould ripened cheeses have different appearances and the high biochemical activities of these moulds produce the typical aroma and taste (Beresford *et al.* 2001).

The presence of yeasts in cheese is largely known, and these microorganisms may be originated from unpasteurized milk or from the environment where the cheese is manufactured, mainly the equipment and brines (Landell *et al.* 2006). Some yeasts species, as *Yarrowia lipolytica* and *Geotrichum candidum*, produce extra cellular enzymes in enough amounts to modify the organoleptic characteristics of cheese and contribute directly to its ripening (Wyder and Puhan 1999; Cosentino *et al.* 2001). Other species produce growth factors, such as amino acids and vitamins, supporting the growth of others microorganisms which in turn contribute to cheese maturation (Fleet 1990).

Beyond the desirable microbiota cheese may also present spoiling and/or pathogenic microorganisms, like *Escherichia coli*, *Salmonella*, *S. aureus*, *L. monocytogenes*, among others.

The fecal coliform group, defined as Gram-negative rods that produce CO_2 from lactose and grow at 45°C, is formed by *E. coli*, and in a less stand by other members of the *Enterobacteriaceae* family, *Klebsiella*, and *Citrobacter* (Garrity *et al.* 2004). *E. coli* is a predominant species among many kinds of Gram-negative bacteria, anaerobic facultative, and part of the microbiota of humans and hot-blooded animals guts. This bacterium is also found in water, soil, vegetables and foods, mainly those of animal origin as milk and dairy products. Its presence in food is evaluated under two aspects: (1) its presence are indicative of a fecal origin contamination, generally associated with unsatisfactory hygiene conditions; and (2) a potential risk for consumers, as many *E. coli* are etiologic agents of gastrointestinal disorders (Feng and Weagant 2002).

The presence of *L. monocytogenes* in milk and dairy products has been largely reported, especially, in high and medium moisture cheeses (Silva *et al.* 1998; Catão and Ceballos 2001; Kabuki *et al.* 2004). Cheese contamination by *L. monocytogenes* is related to raw milk contamination, what can occur during the milking process, storage, transportation, and cheese processing (Tompkin 2002; Waak *et al.* 2002; Kabuki *et al.* 2004).

Salmonella spp. frequency in food is worldwide, being main microbial agents involved in outbreaks of food-borne diseases, most of which are associated with the ingestion of animal origin foods, like poultry, eggs, meat, milk and lactic products (CDC 2004; Dalton *et al.* 2004; Colak *et al.* 2006). *Salmonella* has been reported in curd cheese and butter cheese (Pinto *et al.* 1996; Florentino and Martins 1999; Nassu *et al.* 2000), as well as in long ripening and storage cheeses (Borges *et al.* 1990; Modi *et al.* 2001).

An acid adaptation was found in *Salmonella* sp., including *S. enteritidis*, *S. choleraesuis* ssp. *choleraesuis* serotype *heidelberg*, and *S. choleraesuis* ssp. *choleraesuis* serotype *javiana*, associated with food poisoning. Acid-adapted cells also showed enhanced survival over a period of two months in cheddar, Swiss, and mozzarella cheeses kept at 5°C. These observations support the theory that acid adaptation is an important survival mechanism enabling *Salmonella* sp. to persist in fermented dairy products and possibly other acidic food products (Leyer and Johnson 1992).

Enterotoxigenic *Staphylococcus* presence and its enterotoxins have been evidenced frequently in lactic products, mainly in raw milk and cheeses (Sena 2000; Rosec *et al.* 2002; Le Loir *et al* 2003; Lamaita *et al.* 2005). Raw milk from animals with staphylococcal infection in the mammal glands (mastitis) is the main source of enterotoxigenic *S. aureus* strains (Borges 2006). Outbreaks and sporadic cases associated to staphylococcal intoxication due to the consumption of lactic products, mainly cheeses, have been evidenced in many countries (Sabioni *et al.* 1994; Altekruse *et al.* 1998; Meyrand and Vernozy-Rozand 1999; De Buyser *et al.* 2001; Carmo *et al.* 2002; Leclerc *et al.* 2002).

DYNAMICS OF MICROBIAL POPULATIONS IN CHEESE

Cheese is a typical example of mixed fermentation process in which desirable and undesirable bacteria, yeasts and molds interact and compete during initial steps and ripening process, leading to a complex product which characteristics depends on the dynamic of all these microbial populations. The dynamics of these microbial populations is influenced by intrinsic and extrinsic factors, such as temperature, potential of hydrogen (pH), moisture, manufacture conditions, substrate, natural compounds, among others.

In pasteurized milk cheese, the microbiota of milk immediately after the heat treatment consists primarily of thermoduric bacteria and spores. The numbers and types of thermoduric bacteria are dependent on the microbial population of raw milk. The genera *Lactobacillus*, *Streptococcus* and *Enterococcus* are among the more common thermoduric lactic acid bacteria (Richter and Vedamuthu 2001).

In the conventional fermentation process, a bulk culture containing about 10^8 to 10^9 cell per milliliter is inoculated at about 1% level to the raw material (Ray 2001). For many kinds of cheese, these starters are the only microorganisms deliberately added to the cheese milk, and usually in the next day after manufacture the viable starter densities in cheese are at least 4.5 log cycles higher than other lactic acid bacteria (Crow *et al.* 1995).

The growth and activity of the starter culture, during the first stages of cheese manufacture, is supported by water activity (a*w*), which is around 0.99. However, after whey drainage, salting and during ripening the prevailing a*w* levels are significantly lower then the optimal requirements of starter bacteria (Beresford *et al.* 2001). It is thus likely that a*w* contributes to the control of their metabolic activity and multiplication (Brown 1976). Starters are also presents during the ripening process, where their enzymes are involved in proteolysis and conversion of amino acids into flavor compounds (Fox and Wallace 1997).

The dynamic of starter LAB during Cheddar ripening showed that lactococci, represented by *Lc. lactis*, *Lc. cremoris* and *Lc. diacetylactis*, have a gradual decline, resulting in 2 log reduction after 6 month. *Lb. casei* increased significantly during the first month and showed a slight decrease after 6 month. Conversely, *Lb. bulgaricus* population showed a rapid increase after inoculation, followed by a

drastic reduction, indicative of autolytic activity, during the first month (Sallami *et al.* 2004). Similar results were obtained by other authors (Trépanier *et al.* 1992; Wilkinson *et al.* 1994; Benech *et al.* 2003).

NSLAB do not grow well in milk (Cogan *et al.* 1997), and are usually present at low cell densities at the beginning of cheese production, approximately 10^2 CFU/g (colony forming unit/gram) (Crow *et al.* 1995). Thus, NSLAB do not contribute to acid production during manufacture (Beresford *et al.* 2001), but they rapidly proliferate during ripening, reaching and maintaining densities of about 10^5 to 10^8 CFU/g (Crow *et al.* 1995; De Angelis *et al.* 2001). NSLAB are responsible for important modifications in cheese texture, acidity, and flavor by fermenting lactose, converting milk proteins (primarily caseins) into peptides and free amino acids, and breaking down citrate, lipids, esters, and amino acids into volatile aroma compounds (Broadbent and Steele 2005).

Starter adjuncts do not play a significantly role in acid production during curd manufacture and in one day curd their densities are usually less than 10^6 CFU/g cheese (Crow *et al.* 1995).

In pasteurized milk cheese, PAB may be added at the beginning to reach approximately 10^3 CFU/g after manufacture (Vorobjeva 1999). In Morbier, a raw milk cheese usually made without inoculum, the two PAB strains used as starters reached 10^8 CFU/g at the end of ripening in the inoculated cheeses, whereas indigenous PAB reached just 5×10^6 CFU/g in non-inoculated products (Thierry and Maillard 2002).

Regardless of the general behavior explained above, each cheese has its own characteristic and microbial dynamic. Some examples of different microbial relations are given below.

Studies on Serra cheese indicated that the lactic acid fermentation results mainly from the action of lactic acid-type *Streptococcus*, *Leuconostoc*, and *Lactobacillus* species (Macedo *et al.* 1993). Tavaria and Malcata (2000), studying bacterial populations in 60-days old Serra cheese, found LAB (lactobacilli, lactococci and enterococci), as well as Enterobacteriaceae, staphylococci and yeasts. Significant differences in the number of lactococci, lactobacilli, enterococci, Enterobacteriaceae and staphylococci were among geographical origins, whereas the year of manufacture played a significant role only upon the number of yeasts and staphylococci. These data back up the claim that those microbial families usually implicated with health hazards are prone to a wide variability, which reflects the intrinsic heterogeneity associated with production and handling of raw milk in traditional cheesemaking.

Fenelon *et al.* (2000) investigated the effect of incremental reduction in fat content, in the range of 33 to 6% (w/w), on microbial populations and proteolysis in Cheddar cheese. The populations of nonstarter lactic acid bacteria decreased with fat content, and counts in the low fat cheese (6% w/w) were significantly lower than those in the full fat cheese (33% w/w) at ripening times >1 and <180 days.

Albenzio *et al.* (2001) in their research on the effect of raw ewes' milk, pasteurized ewes' milk and curd/whey heating, on Canestrato Pugliese cheese production, found that cheeses made with raw milk showed higher concentration (one or two orders of magnitude) of cheese-related bacteria, such as adventitious mesophilic lactobacilli, enterococci and staphylococci. At the end of ripening, all cheeses contained less than 1.0 log CFU/g of total and fecal coliforms, and *E. coli* and *S. aureus* were not detected. Using phenotypic identification and RAPD-PCR (randomly amplified polymorphic DNA – polymerase chain reaction), they found that raw milk cheeses contained the largest number of mesophilic lactobacilli species and the greatest diversity of strains within the *Lb. plantarum* species.

Randazzo *et al.* (2006) studying traditional artisanal and experimental Pecorino Siciliano cheeses manufactured by using either raw or pasteurised ewes' milk with the addition of starter cultures found that both, artisanal and experimental cheese types showed similar microbial counts, and species, belonging to *Lc. lactis*, *Stp. thermophilus*, *E. faecalis* and *Ln. mesenteroides*. They also found a dominance of *Stp. bovis* and *Lc. lactis* species in the traditional artisanal Pecorino Siciliano cheese.

High prevalence of contaminant bacteria was detected by Tandir (2005) analyzing 212 samples of Travnik cheese: *E. coli* (84.43%), *Proteus* spp. (37.26%), *Staphylococcus* positive coagulase (41.98%) and sulfo-reducing *Clostridium* (32.54%). It is estimated that the health inaccuracy of Travnik cheese results from unsanitary conditions of sheep milk production, as well as the final product of Travnik cheese.

The contamination with microorganisms indicative of hygieinic status (enterococci and coliforms) was studied in 98 samples of white brine cheese by Aleksieva (1980), including 33 samples of cow's milk cheese and 65 of ewe's milk cheese. It was found that 22.4% of the cheese samples do not contain enterococi in 0.01 g product although in 15.3% enterococci amount exceeds 10^5 CFU/g. No relationship was found between enterococci finding and cheese content of salt, titratable and active acidity. Cheese contamination with coliforms bacteria is limited-merely 3.1% of the samples contain coliforms in quantity of 10^1 to 10^2 MPN/g (most probable number/gram). No difference was found in the contamination of cow's and ewe's milk white brine cheese with enterococci and coliforms. The enterococci microflora in white brine cheese and in the brine is represented predominantly by *Stp. faecium* and *Stp. durans* (91.3% of the isolated strains). The coliforms are represented by *E. coli*, *Enterobacter aerogenes* and *Citrobacter intermedium*.

The microbiological analysis of Cameros cheese produced with raw milk, and raw milk and pasteurized milk with a starter culture (Olarte *et al.* 1999), showed that although lactic acid bacteria in raw milk cheese were lower than the inoculated ones at the beginning of the ripening period, non significant differences were found after 5 days. *Lactococcus*, particularly *Lc. Lactis* ssp. *lactis*, were the main lactic acid bacteria isolated at the beginning of the process. However, after 15 days, the *Lactococcus* counts decreased due to their inability to grow under the conditions found in cheeses at the end of the ripening period. At this time, *Lactobacillus* was the predominant genus in raw milk derived cheeses, with *Lb. plantarum* predominance. The addition of a starter improved the microbiological quality of the cheeses, leading to a significant reduction on *Enterobacteriaceae* and faecal coliforms populations. Although high counts of *S. aureus* were obtained in raw milk cheese (6 log CFU/g), no enterotoxins were detected.

Sixty-two samples of a few days ripened Pichtogalo Chanion cheese traditionally produced in Crete, were analyzed by Papageorgiou *et al.* (1998). High counts of lactic acid streptococci, lactococci and lactobacilli, as well as enterococci, yeasts, molds, and psychrotrophic bacteria, were evidenced in all samples. Among contaminant bacteria, high populations of coliforms were detected in all the samples, and *Staphylococcus, Bacillus cereus* and sulfite-reducing *Clostridium* were isolated in 6.45, 14.51 and 40.32% of the samples, respectively. Moreover, *Salmonella* spp. and *L. monocytogenes* were not detected.

FACTORS THAT AFFECT CHEESE MICROBIOTA

Physico-chemical factors

Pasteurization is the most important physical system used in the control of microbial populations in cheese processing. Heat treatments drastically reduce the number of microorganisms, specially pathogenic and/or spoiling bacteria, but also NSLAB that are important during ripening been responsible for the development of aroma and flavor. Although there are no evidence that the nutritional value of raw milk cheeses is different than that of cheese made of pasteurized milk, the flavor and aroma profile of raw milk cheeses dif-

fer, to a certain extent, from that of pasteurized milk cheeses. Moreover, since secondary cheese microbiota may have its origins in post-pasteurization airborne contamination, cheese-making equipment or ingredients, or pasteurization survival, heat treatment alone is not enough to ensure the microbiological quality of cheese.

In both pasteurized milk and raw milk cheeses the microbial grow during cheese processing and ripening is influenced by a number of physical/chemical parameters, including water activity, salt concentration and pH, which depend on the cheese making process (Beresford *et al.* 2001).

All microorganisms require water to grow, and one of the most effective ways to control their growth is to reduce the available water either through dehydration or addition of some water soluble component such as sugar or salt. 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 and Marcos 1989). In fact, an increase in the moisture content of cheese leads to increased susceptibility to spoilage (Beresford *et al.* 2001).

Feeney *et al.* (2002) studied the effect of pH and calcium concentration on Mozzarella cheese proteolysis with low rate of moisture. The authors verified that the pH and calcium significantly affected the moisture rate, besides the rate of proteolysis during cheese storage at 4°C. pH is a very important parameter affecting cheese identity and quality, because it affects cheese structure, flavor and rheologic properties, interfering in the chemical interactions between the structural compounds (proteins, water and minerals), as well as the proteolytic activity and the structure of microbial populations (Lawrence *et al.* 1983, 1984, 1987). In this sense, Monteiro (2004) found that pH increase is directly correlated with the melting capacity and inversely correlated the firmness of Brazilian cream cheese.

The microorganisms involved in cheese manufacture and ripening are either mesophilic or thermophilic having optimal growing temperature at about 30 and 42°C, respectively. The optimal temperature for cheese ripening is a relation between the need to promote ripening reactions ensure the development of a desirable secondary flora, and the need to prevent the propagation of potential spoilage and pathogenic bacteria (Beresford *et al.* 2001).

The redox potential of cheese is one of the major factors in determining the types of microorganisms which will grow in these dairy products (Beresford *et al.* 2001). In general, cheese offers a reduce environment developed during the fermentation process by the starter cultures or NSLAB responsible for the initial fermentation, and the reduction of small amounts of O_2 in the milk to water (Crow *et al.* 1995). As a consequence of these reactions, the cheese interior is essentially an anaerobic system, which can only support the growth of obligatory or facultative anaerobic microbe. Therefore, obligate aerobes, such as *Pseudomonas*, *Brevibacterium*, *Bacillus* and *Micrococcus* spp. are excluded from growth in the interior of the cheese. Bacteria that develop on the cheese surface are predominantly obligate aerobes (Beresford *et al.* 2001).

Biological factors

During cheese processing and ripening, the factors that influence the increase of one population in detriment of other microbial populations are mainly: direct competition for substrate, production of natural inhibitory metabolic compounds, and bacteriocins. In direct competition, LAB dispute nutrients among themselves and with contaminant microorganisms. The inhibitory compounds are products of bacteria metabolism, and bacteriocins are antimicrobial protein produced by LAB.

LABs, and particularly, starter culture rapidly ferments lactose into lactic acid/lactate, limiting the availability of this carbon source for harmful lactose fermenting microorganisms like coliforms or spoilage bacteria. Other substrates converted or sequestered by LAB during cheese making are citrate and protein, which mean that these compounds can not serve as substrate for pathogens. Moreover, the formation of lactic acid/lactate drastically reduces pH and increase acidity to levels that limit the growth of many harmful bacteria (Hui *et al.* 2004).

Residual lactose is metabolized quickly to L-lactate during the early stage of ripening at a rate largely determinate by the presence of starter cultures, temperature and salt-in-moisture levels of the curd (Turner *et al.* 1980; Parente and Cogan 2004). Lactose that is not metabolized by starter is probably fermented by NSLAB (McSweeney and Fox 2004).

Although, in ripened cheeses, some heterofermentative facultative lactobacilli can be develop well under high selective conditions of the media. Lactose and galactose are usually metabolized after primary fermentation by a starter culture. Others energy sources for NSLAB may be: citrate, lactate, milk components, microbial metabolites, and microbial cell lyse products (Peterson *et al.* 1990; Fox *et al.* 1998).

Under normal food fermentation conditions, LAB produce lactic acid, as the main product of the metabolism, but also other products, such as acetic acid, acetaldehyde, ethanol, and diacetyl, which contribute to cheese flavor and that are inhibitors of other microorganisms (Hugenholtz and Kleerebezem 1999).

During manufacture, starter LAB take part in the acidification of cheese milk to the desired pH (Farkye 2004). Starter cultures with coagulating enzymes such as rennet promote curd formation, lowering the redox potential, which can destroy or prevent the growth of pathogens and spoilage organisms (Richter and Vedamuthu 2001). Other function of starter LAB is the contribution to increase the biomass in young curd, representing a biocatalytic potential for cheese-ripening reactions. This potential can be measured by starter cell autolysis (Crow *et al.* 1995). The environment promoted by starter, with respect to redox potential, pH and moisture content, stimulate the growth of secondary flora, which in turn contribute for cheese ripening (Beresford *et al.* 2001).

During the last decade, fundamental studies have opened a new field of research dealing with bioactive or biogenic substances derived from dairy products. These antimicrobial pore forming peptides are generated by the proteolytic hydrolysis of milk proteins (Floris *et al.*, 2003). Some examples of these peptides are casecidins, a group of basic, glycosylated, and polypeptides, released form chymosin treated casein, and casocidin isolated form acidified milk and corresponding to the residues 150 to 188 of α-casein. Several antibacterial active peptides were identified in water-soluble extracts of nine Italian cheese varieties. These peptides characterized as casokinin, iracidin, kappacin, casoplatelin, and casomorphin showed a large spectrum of inhibition towards gram-positive, including some NSLAB, and Gram-negative bacteria (Rizzello *et al.* 2005).

The most interesting antimicrobial compounds in cheese are small peptides produced by LAB and known as bacteriocins. These peptides are effective against a large range of food-borne, spoiling and NSLAB bacteria, and can be directly applied as supplement or produced by bacteriocinogenic strains during cheese processing (Ray 2001; Bagenda and Yamazaki 2007).

LAB bacteriocins: classification, genetics and mode of action

The term bactericin was introduced in the 50th to design antibacterial proteins produced by enterobacteria. Today, the definition of bacteriocin was modified to include antibacterial peptides and proteins from both gram-negative and gram-positive bacteria. Bacteriocins can be active against other bacteria, either of the same species, or across genera (Cotter *et al.* 2006; Bernardeau *et al.* 2007).

Most of the bacteriocins from LAB are cationic, hydrophobic, or amphiphilic peptides composed of 20 to 60 amino

Table 2 Classification of bacteriocins.

Class		Characteristics	Bacteriocins	Producers	Active against
I		Lantibiotics, small peptides (<5KDa) containing lantionine and β- metal lantionine	Nisin, lacticin 481, carnocin UI49, lactocine S, epidermin, gallidermin, lacticin 481, mersacidin, ancovenin, duramycin, actagardin.	*Lc. lactis, Lb. sake, Staphylococcus epidermidis, Staphylococcus gallinarum, Lc. lactis, Bacillus subtilis, Streptomyces cinnamoneus, Streptomyces* sp., *S. cinnamoneus, Actinoplanes* sp.	*Lactobacillus, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Listeria, Staphylococcus* sp., *Bacillus* sp., *Clostridium* sp.
II	II$_a$	Small peptides, thermo stables and synthesized in a precursor form (ended processed after two gly residues adhesion). Show a YGNGV-C common sequence in the molecule N-terminal part.	Pediocin PA-1, sakacins A & P, curvacin A, leucocin A-UAL 187, mesentericin Y 105, enterocin A, divercin V41, lactococcin MMFII.	*Pediococcus acidilactici, Lb. sake, Leuconostoc gelidum, Leuconostoc mesenteroides, Enterococcus faecium, Carnobacterium divergens, Lc. Lactis*	*Lactobacillus, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Listeria* sp., *Carnobacterium*
	II$_b$	A two compounds system, two different peptides needed for complex formation.	Lactococcins G & M, lactacin F, plantaricin A, S, EF & JK.	*Lc. lactis, Lb. johnsonii, Lb. plantarum*	-
	II$_c$	Peptides that required cys residues presence in the reduced form for molecule biological activity.	Acidocin B, enterocin P & B carnobacteriocin A, divergicin A.	*Lb. acidophilus, Carnobacterium piscicola, Carnobacterium divergens, E. faecium*	-
III		Big molecules heat sensitive.	Helveticin J & V-1829, acidophilucin A, lactacins A & B	*Lb. helveticus, Lb. Acidophilus*	-
IV		Complex molecules formed by proteins and one or more molecules like lipids or carbohydrates.	Plantaricine S, leuconocine S, lactocine 27, pediocine SJ 1	*Lb. plantarum, Leuconostoc paramesenteroides, Pediococcus pentosaceus*	-

Lc: Lactococcus
Lb: Lactobacillus

acids with 3 to 6 kDa (Nes *et al.* 1996), although there are exceptions (Joerger and Klaenhammer 1990). The bacteriocins produced by gram-positive bacteria are classified into four groups (Jimenez-Diaz *et al.* 1993). Examples of the bacteriocins of these classes are summarized in **Table 2**.

Lantibiotics (Class I) are small peptides (<5 kDa) containing lantionine, α-methyllanthionine, dehydroalanine, and/or dehydrobutyrine (Twomey *et al.* 2002). According with their chemical structure and antimicrobial activities, lantibiotics are further in subclasses A and B. A lantibiotics are elongated and positive charged peptides that induce pore formation in sensitive bacteria, whereas B lantibiotics are smaller globular negative charged peptides which antimicrobial activity is related to the inhibition of specific enzymes.

Class II bacteriocins are heat-stable small peptides (<10 kDa). These bacteriocins are subdivided in the subgroups. Among these, class IIa or pediocin-like peptides, characterized by the presence of a consensus N-terminal sequence (Tyr-Gly-Asn-Gly-Val-Unspecific-Cys) are particularly important due to their anti-*Listeria* activity. The class III bacteriocins are heat-labile proteins with >30 kDa, and class IV is formed by complex glyco or lipoproteins.

The genes encoding bacteriocin production and immunity are usually organized in polycistronic operons, that can be located on the chromosome (e.g. mersacidin), on plasmids (e.g. divergicin A and sakacin A), or in transposable elements (e.g. nisin and lacticin 481) (Nes *et al.* 1996; McAuliffe *et al.* 2001).

Lantibiotic coding operons generally contain a pre-lantibiotic gene, a modification enzyme gene, a processing serine-protease gene (responsible for the removal of the leader peptide), a group of genes that codify a specific ABC transporter, a transport protein gene involved in peptide translocation, regulatory genes, and self immunity genes. Conversely, class II bacteriocin operons are formed by a structural gene coding for precursor peptide, followed immediately by a immunity gene and genes for an ABC-transporter and an accessory protein essential for the export of the bacteriocin (Nes *et al.* 1996; Sablon *et al.* 2000; McAuliffe *et al.* 2001; Rosa and Franco 2002).

Most bacteriocins are firstly synthesized as biologically inactive pre-peptides or pre-bacteriocins carrying an N-terminal leader peptide that is attached to the C-terminal propeptide. Those pre-peptides have 18 to 27 amino acids, with two glycines in the N-terminal region (Nes *et al.* 1996). Ac-

cording to Moll *et al.* (1999), the two glycines present in the pre-peptide sequence are responsible for pre-bacteriocin recognition by the transport system.

The lantibiotics biosynthesis follows a general pathway that involves the synthesis of a pre-peptide, several modification reactions (mainly deshydration of some hydroxyl amino acids), the proteolytic cleavage of the leader peptide, and the translocation of the mature propeptide across the cytoplasmic membrane. Depending of the bacteriocin, the cleavage of the leader peptide may take place prior to, during, or after the export from the cell (McAuliffe *et al.* 2001).

Most Class II bacteriocins are synthesized as a prepeptide containing a conserved N-terminal leader, but unlike lantibiotics, they do not undergo extensive post-translational maturation. The prepeptide is processed to remove the leader peptide concomitant with export from the cell through a specific ABC transporter and accessory proteins (Ennahar *et al.* 2000).

The biosynthesis of bacteriocins is usually regulated through a two-component regulatory system that consists of signal-producing proteins, a membrane histidine protein kinase, and a cytoplasmic regulator. The histidine kinase senses a certain concentration of bacteriocin in the environment, phosphorilate the regulator that activate the transcription of the genes present in the bacteriocin operon (Nes *et al.* 1996). For nisin, the bacteriocin itself acts as an external signal to regulate its own biosynthesis (Guder *et al.* 2000), but class II bacteriocin production is regulated by a bacteriocin-like peptide with no antimicrobial activity (Ennhar *et al.* 2000).

Bacterial immunity to their own lantibiotics are mediated by immunity protein strongly associated outer part of the cell membrane, and ABC-transport proteins. Conversely, class II bacteriocins immunity is determined by dedicate proteins that are loosely associated to the cell membrane, and usually found in higher concentration in the cytoplasm (Quadri *et al.* 1995). According to Tichazek *et al.* (1993) and Diep *et al.* (1996), the immunity proteins are generally slightly hydrophobic, with pI values between 7 and 10, and a size range of 51-257 amino acids. In most cases, the immunity gene of Class II bacteriocins is located next to, and in the same operon as the pre-bacteriocin gene, and the immunity protein is produced in high amounts (Holo *et al.* 1991; Nissen-Meyer *et al.* 1993; Venema *et al.* 1995).

In the enterocin B and the carnobacteriocin A clusters,
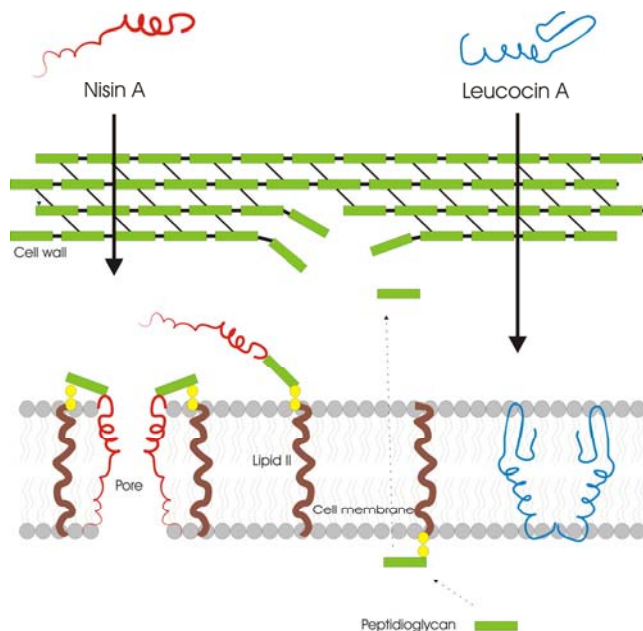
Fig. 1 Mode of action of bacteriocin class I (nisin) and class IIa (leucocin). Nisin (class I), can bind to lipid II, the main transporter of peptideglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell death. They can also use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. Leucocin A (class II) has an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarisation and death (adapted from Cotter *et al.* 2005).

the pre-bacteriocin and immunity genes are transcribed in separate, oppositely oriented units (Franz *et al.* 2002). Although the immunity and bacteriocin genes reside in separate transcription units, they are not necessarily transcribed independently (Worobo *et al.* 1994; Casaus *et al.* 1997; Franz *et al.* 2000).

Most LAB bacteriocins are single molecules, however it has been shown that some strains secrete two peptides, both of which are needed for full bacteriocin activity. The mature peptides are undistinguishable from the one-peptide bacteriocins except when it comes to antimicrobial activity. Amusingly, both the one-peptide and two-peptide bacteriocins depend on a single protein for immunity (Wood and Warner 2003).

Bacteriocins have bactericidal or bacteriostatic activity, inducing pore formation in the membrane of the sensitive cell, which leads to an ionic imbalance and flow of phosphate ions (Rosa and Franco 2002). The consequence of pore formation is the dissipation of a proteomic force, which is involved directly with adenosine triphosphate (ATP) synthesis, protein phosphorylation, flagella synthesis and rotation, protein transportation, etc. (Bruno and Montville 1993). With protonic force dissipation, 98.9% of ATP is hydrolyzed, in an attempt to dissipate protonic force maintenance. The active transport of amino acids is interrupted and reserve amino acids are released from the cell by the formed pores. This prior disturbance can cause other disorders like cellular lyses (Garcerá *et al.* 1993). The first stage in pore formation by bacteriocins involves the electrostatic interaction between the positive charge and the polar residues of bacteriocins with the anionic phospholipids presents in the lipidic bilayer of target membrane (Chikindas *et al.* 1993; Abee *et al.* 1995). At this stage the bacteriocin is sensitive to proteolytic enzymes. The second stage is irreversible and involves lethal changes in bacteriocin-sensitive strains (Desmazeaud 1997).

Lantibiotics act on membrane potential independently of the presence of a receptor (Sahl *et al.* 1987). Nisin (**Fig. 1**), although not requiring a membrane receptor, is more active when it is energized. Lipid II molecules present in the target bacterial membrane can serve as membrane receptors in the sensitive bacteria (Breukink *et al.* 1999), enhancing the conductivity and stability of the pores made by lantibiotics (Rosa and Franco 2002).

The class II bacteriocins, like leucocin, do not have modified amino acids and, regardless of the electric gradient, can interact with the receptors to insert themselves in the cytoplasmic membrane (**Fig. 1**).

Bacteriocin production in LAB is growth associated: it usually occurs throughout the growth phase and ceases at the end of the exponential phase (or sometimes before the end of growth) (Parente *et al.* 1997; Lejeune *et al.* 1998). Bacteriocin production is affected by type and level of the carbon, nitrogen and phosphate sources, cations surfactants and inhibitors (Savadogo *et al.* 2006).

Most lantibiotics have a broad inhibitory spectrum, inhibiting not only closely related bacteria form the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, but also other less closely related bacteria, as *L. monocytogenes*, *S. aureus*, *B. cereus*, and *C. botulinum*. Conversely, class II bacteriocins show a narrow activity spectrum, and only inhibit closely related gram-positive species. In general, class II bacteriocins are effective against *Enterococcus*, *Lactobacillus*, and *Pediococcus*, while *Lactococcus* species are resistant.

Bacteriocins in cheese production

The first direct references to bacteriocins in cheesemaking were published in the early 30th. These report the inhibitive effect of nisin produced by dairy *Lc. lactis* on NSLAB *Lb. bulgaricus*. Since them, bacteriocinogenic bacteria were found to be common among cheese LABs, and nisin gained widespread application in the food industry.

Cheese can be supplemented with *ex situ* produced bacteriocin preparations, or by inoculation with bacteriocinogenic strains under conditions that favor the production of bacteriocin *in situ* (Stiles 1996). In the first case, bacteriocin preparations obtained by cultivation of the producer strain in controlled industrial scale can be added as concentrates. *In situ* bacteriocin production offers legal and costs advantages compared to *ex situ* production. However, the use of bacteriocinogenic cultures requires careful selection of strains well-adapted to cheese environment and that grow and produce enough bacteriocin amounts to inhibit the target pathogenic, spoiling or other undesirable bacteria. In this sense, both conventional breeding and genetic engineering approach can contribute to improve bacteriocinogenic strains (Rodrigues *et al.* 2003; Zhou *et al.* 2006).

Several authors showed the effectiveness of the addition of nisin and other bacteriocins for the control of harmful bacteria during cheese making. For example, Ferreira and Lund (1996) found that the addition of nisin and the inoculation with a nisin-resistant strain into long-life cottage cheese, significantly reduce *L. monocytogenes* population. Similar results were obtained in ricotta and other cheeses (Davies *et al.* 1997). Addition of nisin suppressed total plate and spore counts, including *B. stearothermophilus*, *B. cereus* and *B. subtilis*, in processed cheese during 3 months of storage nisin (Plockova *et al.* 1996).

The industrial viability of lacticin 3147-based powder is being evaluated as a cheese biopreservative. Morgan *et al.* (2001) found that a 10% lacticin 3147 powder was extremely effective for the inhibition of Listeria in yoghurt and cottage cheese.

Another commercially exploited bacteriocin is pediocin PA-1, produced by *Pediococcus*. This bacteriocin is mainly used in meat products, but its application has been evaluated in dairy products due its wide pH range activity, thermal stability and antilisterial activity (Nes *et al.* 1996).

As pointed out by Bagenda and Yamazaki (2007), although generally regarded as safe (GRAS), regulatory limitations still represent one of the most important challenges for the commercial application of bacteriocins as an ingre-

dient in cheese production.

Bacteriocinogenic strains can be used either directly as starter cultures, as adjunct or co-cultures in combination with a starter. When used as a starter culture, the bacteriocinogenic strain must be able to carry out the desirable fermentation besides being able to produce bacteriocin to afford protection, and in some cases, to production increase the competitiveness and stability of the starter. Adjunct cultures do not need to contribute to the fermentation process, but they must not interfere with the primary function of the starter or the desirable NSLAB strains. For these reason, bacteriocin resistance or tolerance of the starters and other LAB that participate in cheese ripening should be ensure in order to guarantee the proper fermentation and the physico-chemical characteristics of the final product (Hui *et al.* 2004; Smid and Gorris 2007). Moreover, the production of bacteriocins and bacteriocin activity are influenced by several factors as pH, fat content, water activity, temperature, among others (Gálvez *et al.* 2007), that should be optimized in order to obtain the desire effect.

The use of an enterocin producer strain of *Enterococcus* as adjunct culture in combination with a commercial starter culture had no effect on the growth of the starter or the physicochemical characteristics of nonfat hard cheese, but was able to drastically inhibit the development of the pathogenic bacteria *B. cereus* and *S. aureus* (Muñoz *et al.*, 2004, 2007). This bacteriocin has been proved efficient in the control of *L. monocytogenes* in Manchego cheese (Nuñez *et al.* 1997). Conversely, the addition of a lacticin 481 *Lc. lactis* adjunct culture increase starter lysis while inhibiting NSLAB proliferation during Cheddar cheese ripening but do not compromise acid production and cheese quality (O'Sullivan *et al.* 2003). Moreover, the addition of lacticin *L. lactis* producing strains during Cheddar cheese production reduces NSLAB populations contributing for cheese quality (Ross *et al*. 1999). To manipulate microbial flora during cheese production and ripening, Ryan *et al.* (2001) used a *Lc. lactis* lacticin-producer starter associated with a previously selected *Lb. paracasei* lacticin-resistant adjunct strain. Lacticin was produced and remained stable during ripening, with levels of either 1,280 or 640 AU/g detected after 6 months of ripening. The more-resistant adjunct culture survived and grew in the presence of the bacteriocin in each trial, reaching high counts during ripening, in contrast to the sensitive strain, which was present at levels 100-to 1,000-fold lower.

In other examples of bacteriocinogenic LAB utilization in cheese making, Zottola and Sasahara (1994) used nisin-producer stater cultures in pasteurized process cheese showing a significant increase in shelflife with a drastic reduction of *L. monocytogenes*, *S. aureus*, and *C. sporogenes*. Efficient reduction of *L. monocytogenes* was also obtained inoculating a *Lc. lactis* strain with the pediocin 1A coding plasmid pMC117 as starter cultures (Buyong *et al.* 1998) or *Lb. plantarum* strain-producing pediocin AcH add as adjuvant on red smear cheese manufacture (Loesner *et al.* 2003).

Recently, Gálvez *et al.* (2007) discuss the synergistic effect of bacteriocins and/or bacteriocinogenic strains when used combined with other antimicrobial agents, including chemical preservatives, natural phenolic compounds, and other antimicrobial proteins.

CONCLUDING REMARKS

The interaction between food, nutrition and health is a new challenge for both food science and food industry, and it is also important that claims on health-promoting effects have sufficient scientific substantiation. In this context, the new trend on food technology consist in attend certain items like: demands of consumers due to their changing lifestyles and expectations for fresher, more natural foods, which are less severely processed, contain less preservatives, or are even free from "artificial" additives; nutritionally more advantageous food; safer food; and at the same time are foods convenient to handle; needs for less energy requirement of

processing; the necessity for lower impact on the environment.

This challenge is particularly difficult in cheese production, as cheese is not a product but a generic name use to designate a very large number of products made from milk (cow, buffalo, goat, sheep) involving its coagulation, and in general, its fermentation by LAB. As each cheese has its own process and problems, specific solution should be developed and implemented in order to obtain safety and high quality products.

As exemplified in this review, many studies have been developed to describe microbial communities and understand the dynamics of these organisms during the processing and ripening of different types of cheese. The individual and associated effects of physical factors on harmful and useful bacterial populations as well as the interaction between the most important microorganisms have been established. However, the transfer of this knowledge to cheese production has been difficult due to the particularities of each cheese type: industrial or artisanal production, origin and quality of milk, technological disparities among producers, among other factors that affect milk borne microorganisms, contamination during processing, and consequently, the dynamics of microbial populations.

In this context, bacteriocinogenic LAB represent the most promising biological tools to reduce harmful microorganisms and to control LAB populations during cheese processing and ripening. Advances in bacteriocins and bacteriocinogenic LAB research including the prospection of new bacteriocins, molecular and biochemical aspects, mode of action and spectrum, bacteriocin biosynthesis, and bacteriocins interaction with physic-chemical factors, are opening the possibility to use bacteriocinogenic LAB in commercial cheese production. Due to these advances, regulatory limitations are disappearing, and bacteriocinogenic starters and adjuvant cultures are commercialized. However, to be largely and properly used in commercial cheese production, studies at the industrial level and in the production of different cheeses with their peculiar microbial communities and physic-chemical properties, are still necessary.

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