Review - Pregledni rad UDK: 579.676

Psychrotrophic bacteria and milk and dairy products quality

Dubravka Samaržija, Šimun Zamberlin, Tomislav Pogačić*

University of Zagreb, Faculty of Agriculture, Department of Dairy Science, Svetošimunska 25, 10000 Zagreb, Croatia

Received - Prispjelo: 21.02.2012.
Accepted - Prihvaćeno: 24.05.2012.

Summary

The characteristics of microbial populations in raw milk at the time of processing has a significant influence on shelf life, organoleptic quality, spoilage and yields of the raw milk, processed milk as well as on the other dairy products. Unfortunately, cold and extended storage of raw milk, as a common practice in dairy sector today, favour the growth of psychrotrophic bacteria. Therefore, their count in the cooled milk is more than the ideal limit of 10% of the initial raw milk microbial population. Psychrotrophic bacteria are generally able to form extracellular or intracellular termoresistant enzymes (proteases, lipases and phospholipases) which can cause spoilage of milk and dairy product. In addition, besides exhibiting spoilage features, some species belonging to the psychrotrophs are considered as opportunistic pathogenic bacteria that carry inherent resistance to antibiotics and/or to produce toxins. In sense of quality, psychrotrophic bacteria have become major problem for today's dairy industry as leading causes of spoilage and significant economic losses. This review focuses on the impact of psychrotrophs on quality problems associated with raw milk as well as on the final dairy products. In addition, the most common species and means of controlling strategies were also discussed

Key words: psychrotrophic bacteria, contamination, biofilm, milk and dairy products quality, control

Introduction

With the introduction of cooling and cold storage of raw milk in the 1950s, as obligatory technological steps, its bacteriological quality has been significantly improved (Causin and Bramley, 1985). Since then, the acidification of raw milk caused by the growth of mainly lactic acid bacteria and/or other mesophilic bacteria was almost completely stopped. According to the official data of the Croatian Agriculture Agency (internal report HPA, 2011), 93 % of the total quantity of delivered raw milk in 2011 year, achieved the EU standards for the total count of aerobic mesophilic bacteria (≤100,000 cfu mL^{-1}) and for the somatic cells count (≤400,000 mL^{-1}).

However, the extended storage times of raw milk at low temperatures (2-6 °C) have a significant influence on the composition of the natural present microbial population. Thus, in cooled raw milk initially dominant Gram-positive mesophilic aerobic bacteria are replaced by Gram-negative and Gram-positive psychrotrophic bacteria (Causin and Bremley, 1985; Lafarge et al., 2004). The domination of psychrotrophic bacteria in the total microbial population is even more pronounced when milk is produced in poor hygiene conditions and/or contains increased numbers of somatic cells (Walstra et al., 1999a; Barbano et al., 2006). For these reasons, psychrotrophic bacteria usually account for more than 90% of the total microbial population in cooled raw milk (Magan et al., 2001). Also, Cempírková (2002) reported a high correlation (r=0.69) between the number of psychrotrophic bacteria and the total count of bacteria in bulk samples of raw milk in a two-year experiment (three stages).

*Corresponding author/Dopisni autor: Phone/Tel.: +385 1 2393 926; E-mail: samarzija@agr.hr

In addition to the ability to grow and multiply at low temperatures, psychrotophic bacteria have the ability to produce heat stable extracellular and/or intracellular hydrolytic enzymes (Causin, 1982; Chen et al., 2003). Many of these enzymes retain their activity even after the conventional heat treatment of milk. Furthermore, psychrotrophic bacteria are the most commonly isolated organisms which caused the spoilage of the heat treated milk and dairy products as the result of post-pasteurization contamination of the products (Larsen and Jørgensen, 1997; Eneroth et al., 2000; Santana et al., 2004). Due to these properties of psychrotrophs, the spoilage and reduced quality of milk and dairy products can be both the consequence of the presence of live organisms and/or their thermostable enzymes (Grosskopf and Harper, 1969; Fox and Stepaniak, 1983; Braun et al., 1999; Koka and Weimer, 2001). Spoilage is occurred as the change of flavour, undesirable coagulation of milk proteins, and the increased concentration of free fatty and amino acids. In addition, depending on the type of dairy product, the atypical texture and proportion of certain undesirable organic compounds are occurred (Cox, 1993; Boor and Murphy, 2002; McPhee and Griffiths, 2002; Cempírková and Mikulová, 2009). With regard to other quality aspects, such as suitability of milk for the production of dairy products, psychrotophs have a significant negative effects on yields as well as on limiting shelf life of dairy products (Causin, 1982).

The majority of psychrotrophic bacteria that causes milk and dairy product spoilage are not pathogenic. However, certain species of these bacteria have the ability to produce toxins and/or show resistance to antibiotics and therefore must be considered as opportunistic human pathogenic bacteria (Netten et al., 1990; Beattie and Wiliams, 2002; Finlay et al., 2002; Munsch-Alatossava and Alatossava, 2006; Hemalatha and Banu, 2010; Senesi and Ghelardi, 2010).

Almost all species of psychrotrophic bacteria have the capacity to adhere to solid surfaces so they may form a biofilm on the inner surfaces of dairy equipments. In relation to the planktonic cells of the same bacterial species, biofilm is difficult to remove by means of any conventional cleaning and antibacterial chemicals. For that reason, in the dairy industry, biofilm can be a persistent source of permanent product contamination by psychrotophs which causes spoilage and/or can be conditionally pathogenic (Sillankorva et al., 2008; Simões et al., 2010).

The EU standard for high raw milk quality requires that the total count of mesophilic aerobic bacteria is lower than 30,000 cfu mL^{-1}, and the count of psychrotrophic bacteria that is lower than 5,000 cfu mL^{-1}. In that sense, any increase of psychrotophs in the microbial population of raw milk will negatively influence the product quality to a certain extent and, indirectly, will reduce the incomes. Thus, it is estimated that modern dairy industry has losses of up to 30 % due to spoilage and reduced product quality caused by psychrotrophic bacteria (Varnam and Sutherland, 1996a; 1996b; Garbutt, 1997; Randolph, 2006).

In the context of the negative influence of psychrotrophic bacteria on milk and dairy product quality, this review aims to describe: characteristics and properties of the most important species of psychrotrophic bacteria associated with milk and dairy products and their potential for the occurrence of spoilage of raw milk, heat-treated milk, cream, butter and cheese. In addition, the most common sources of contamination and the importance of control of psychrotrophic bacteria in the dairy industry are also described.

Psychrotrophic bacteria

Psychrotrophic bacteria are not the specific taxonomic group of microorganisms, but are defined as group of different bacterial species that are able to grow at 7 °C or less regardless of their optimal temperature of growth (IDF Bulletins, 1976).

Psychrotrophic bacteria are ubiquitous in nature, primarily in water and soil, including vegetation. A small number of psychrotrophic bacteria can also be present in the air. Their optimal metabolic activity is expressed at temperatures between 20 and 30 °C. However, they can grow and multiply at low temperatures through an enrichment of polyunsaturated fatty acid in their membrane lipids. In other words, the altered cell membrane secures sufficient permeability for membrane fluidity and transport activity of metabolites necessary for growth and reproduction of bacteria at low temperatures (Schinik, 1999). Furthermore, in the microbiological sense, the genus *Pseudomonas* certainly includes the most diverse and ecologically significant group of bacteria on Earth. Ubiquitous distribution of these bacteria indicates a remarkable degree of their physiological and genetic adaptability (Spier et al., 2000).

Psychrotrophic bacteria isolated from cooled milk belong to Gram-negative and Gram-positive genera and are taxonomically classified into seven classes. *Gammaprotobacteria*, *Bacilli* and *Actinobacteria* are the dominant classes containing between 19 and 21 species, while *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria* are the four less significant classes (Hantsis-Zacharov and Halpern, 2007). Furthermore, Hantsis-Zacharov and Halpern (2007) isolated from the cooled raw milk approximately 20 % of isolates for which they assumed to be new species of psychrotrophic bacteria.

A significant number of bacterial strains isolated from cooled milk belong to the genera: *Bacillus*, *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas* and the species *Burkholderia cepacia* (previously called *Pseudomonas cepacia*) and same of them are considered potentially pathogenic bacteria. Namely, these bacterial species are associated with infections in humans and animals, particularly in cases of immune-repression, and they show pronounced resistance to antibiotics (Foght et al., 1996; Svensson et al., 2006.; Munsch-Alatossava and Alatossava, 2005; Beena et al., 2011).

The genera *Pseudomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter* and *Flavobacterium* with predominance of the genus *Pseudomonas* are mentioned as the most often representatives of the Gram-negative population isolated from raw milk (Lück, 1972; Murray and Stewart, 1978; Zall, 1985; Sørhaung and Stepaniak, 1997; Stepaniak, 2002). The species of the genus *Pseudomonas* show particular physiological and genetic adaptability. Many molecular studies have confirmed an astounding degree of polymorphism in the length of restriction fragments between strains of the same species, and even between strains that are very closely related (Spiers et al., 2000; Martins et al., 2006). In addition, *Pseudomonas* spp. have showed greater genetic variability within strains belonging to the same species then genetic diversity among different species of Gram-negative psychrotrophic bacteria have been estimated (Martines et al., 2006).

Pseudomonas spp., with predominance of *P. fluorescens*, are the most commonly isolated spoilage organisms in raw and pasteurized milk. The majority of these bacteria (58-91 %) have the ability to show distinct enzymatic extracellular proteolytic, lipolytic and phospholipolytic activity (Law, 1979; Stead, 1986; Wang and Jayarao, 1999; Wiedmann et al., 2000). The differences found in the extracellular enzymatic activity of *Pseudomonas* spp. are attributed to their extremely genetic variability among strains. However, the strains those have the same ribotipy usually have the same extracellular enzyme profile (Wiedmann et al., 2000; Dogan and Boor, 2003).

In comparison to other psychrotrophic bacteria, *Pseudomonas* spp. are characterised by a short generation time (<4 h), which implies that contamination with just a single microbial cell can lead to their numbers greater than 10^6 cfu mL^{-1} in milk after eight days of storage at temperatures of 4 °C (Langeveld and Cuperus, 1976). This fact corresponds to the coefficient of multiplication of $1x10^n$ mL^{-1}, meaning that within 48 hours, the number of bacteria will increase by approximately $4x10^{n+3}$ mL^{-1} when the generation time is 4 hours. At the same time, the increase in the number of bacteria will be only $1.6x10^{n+1}$ mL^{-1} if their generation time is 12 hours (Suhren, 1989).

The Gram-positive psychrotrophic bacteria isolated from raw milk include following genera: *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Arthobacter*, *Streptococcus*, *Staphylococus* and *Lactobacillus*. With the exception of *Arthobacter* and *Lactobacillus*, the other genera of that group belong to the thermoresistant psychrotrophic bacteria (Washam et al., 1977).

Among the microbial species that can survive the usual heat treatment of milk, *Bacillus* spp. are the most common isolated Gram-positive bacteria. According to their physiological characteristics, they belong to the mesophilic and thermophilic psychrotrophic strains (Grosskopf and Harper, 1969; Thomas and Druce, 1969; Collins, 1981; Sørhaung and Stepaniak, 1997; Kumarsan et al., 2007). *Bacillus* spp. are a very heterogeneous group of bacteria characterised by different nutritional requirements, the ability to grow in a wide range of temperatures and pH values, and show different resistance to osmotic pressure. Due to the different physiological properties they express, the standardisation of procedures to isolate these bacteria from milk and dairy products, as well as to define the conditions for their inactivation are made more difficult (McGuiggan et al., 1994; Francis et al., 1998).

Among the bacteria belonging to genus Bacillus, from raw, heat treated milk and dairy products *B. stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis* and *B. circulans* are the most commonly isolated species. The spores of these thermoresistant psychrotrophic aerobic or facultative anaerobic bacteria are activated immediately after the heat treatment of milk by forming vegetative forms. In relation to *Pseudomonas* spp., the vegetative cells of *Bacillus* spp. have a greater capacity to form broad spectrum of thermostable extracellular and intracellular hydrolytic enzymes (Chen et al., 2003; 2004). In 40-84 % of cases, *Bacillus* spp. (with the predominance of the species *B. cereus*) isolated from milk expresses both proteolytic and lipolytic activities, and approximately in 80 % of cases also a phospholipolytic activity (Muir, 1996; Matta and Punj, 1999). Furthermore, certain species of the genus *Bacillus* can produce more than one type of proteinases simultaneously. However, according to their hydrolytic characteristics these enzymes are comparable to the hydrolytic enzymes formed by *Pseudomonas fluorescens*.

In addition to hydrolytic thermostable enzymes, *Bacillus* species such as *B. cereus*, *B, licheniformis* and *B. subtilis* are able to form different types of toxins which are implicated in food borne diseases (Griffiths, 1990; Salkinoja-Salonen et al., 1999; Svensson et al., 2006). It is particularly interesting that *Bacillus cereus*, which is a very common contaminant of milk and dairy products, can produce several different enterotoxins that are responsible for human infections or intoxication. For example, haemolytic BL (HBL), non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK) are associated with gastrointestinal diseases and/or other systematic infections in humans. These enterotoxins are released in the small intestine after consumption of the contaminated product. However, the emetic enterotoxins such as the cereulids that *B. cereus* secretes directly into food are responsible for intoxication that is manifested with the appearance of nausea and vomiting within 1-6 hours of consumption of the product (Brown, 2000; Senesi and Ghelardi, 2010).

Gram-positive spore-forming bacteria are present in raw milk in a much lesser extent than Gram-negative psychrotrophic bacteria (Suhren, 1898). The most likely reason for the lesser occurrence of these bacteria in raw milk is their longer generation time (~8.5 hours) and longer lag phase at temperatures between 2-7 °C. However, in milk, these bacteria become dominant when it is stored for longer periods at a temperature of 10 °C (which is often the case in shops), or when produced in improved technological conditions. Wong et al. (1988a) confirmed the presence of *B. cereus* in: 52 % of ice cream samples, 29 % powdered milk samples, 17 % of fermented milk samples and 2 % of pasteurized milk samples. According to the study by Griffiths and Phillips (1990), about 50 % of the *Bacillus* spp. strains isolated from milk are even capable of growing at a temperature of 2 °C. Therefore, *Bacillus* spp. are today considered the main microbial causes for the spoilage of milk and milk products, and the main reason for significant economic losses in the dairy industry (Meer et al., 1991; Brown, 2000).

Hydrolytic enzymes

The majority of psychrotrophic bacteria have the ability to form hydrolytic thermostable enzymes that break down the major constituents of milk; milk fat, protein and lecithin (Fox, 1981; McKellar, 1982; Rowe et al., 1990; Dogan and Boor, 2003). These enzymes retain from 30 to 100 % of their activity after conventional heat treatment of milk (pasteurisation: 72 °C/15 s; sterilisation 138 °C/2 s; 149/10 s). From the perspective of quality and economics, the hydrolytic thermostable enzymes formed by *Pseudomonas* spp. and *Bacillus* spp., i.e. proteases, lipase and phospholipase, have the most significant meaning in the dairy industry.

In general, the proteinases of psychrotrophic bacteria destabilise casein through hydrolysis, and the result is the formation of a gel structure or coagulation of sterilised milk during storage. In cheese making, proteinases (which are not extracted by whey) cause a significant yield loss (Adams et al., 1976; Law et al., 1977; Cousin, 1982; Cousin and Mirth, 1977; Mitchell and Marshall, 1989). Furthermore, proteolysis caused by psychrotrophic bacteria has a negative effect on the products flavour, which has been described as bitter, foreign, unclean, fruity, yeasty or metallic (Marshall, 1982; Ran et al., 1988).

With the hydrolysis of milk fat through the activity of the bacterial lipases of psychrotrophic bacteria, free fatty acids are released. These are the primary cause for the changes in product flavour that is described as rancid, unclean, soapy or bitter. The lipolytic flavours defects are particularly pronounced in cream, butter, cheese and sterilised (UHT) milk (Stead, 1986; Champagne et al., 1994).

Lecithinase and other phospholipases are important groups of lipases of psychrotrophic bacteria that are able to disrupt the native membrane structure of fat globules and milk fat become available to the native milk lipases resulting in physical degradation of the emulsion in milk (Shah, 1994). The lipases of Gram-negative and Gram-positive psychrotrophic bacteria have a molecular mass between 30 and 50 kDa and pH optimum between 7 and 9, and expressed certain preferential specificities with regard to the cleaving site and for hydrolysis of triacylglycerols, diacylglycerols and monoacylglycerols (Chen et al., 2003).

The activity of the hydrolytic enzymes of *Pseudomonas* spp. in cold storage raw milk is 100 %. However, several of these enzymes are able to keep their activity between 60-70 % after pasteurisation, and 30-40 % after sterilisation of milk. For those reasons, these bacterial enzymes have been the most studied and best described (Fox and Stepainak, 1983; Sørhaung and Stepaniak, 1997; Braun et al., 1999; Koka and Weimer, 2001). Particular strains of *Pseudomonas* spp. are capable of simultaneously forming all three types of hydrolytic enzymes (proteinase, lipase and phospholipase), while some form only lipases or proteinases (Matta and Punj, 1999). The differences in the extracellular enzymatic activity of individual strains are most likely associated with a belonging to a particular genetic group (Ercolini et al., 2009). For example, strains of *Pseudomonas* spp. with 55-S-6 ribotype exhibit the highest proteolytic activity than those belonging to the 50-S-8 ribotype. On the other hand, strains belonging to the 72-S-3 ribotype have the highest lipase activity (Dogan and Boor, 2003).

The proteinases of *Pseudomonas* spp. are primarily enzymes with a molecular mass between 40-50 kDa, which contains at least one Zn atom and up to 16 Ca atoms in their molecular structure. The optimum pH of the *Pseudomonas* spp. proteinases are neutral (~7) or alkaline (7-9). The majority of these proteinases are extremely thermostable, and some are even stable after the heating of milk to 100 °C/30 minutes (Fox, 1981; McKellar, 1982; Kumura et al. 1993). Also, in comparison with the spores of *B. stearothermophilus*, the proteinases of *P. fluorescens* are reported to have approximately 4000 times greater thermostability (Adam et al., 1976). Despite the exceptional thermostability they exhibit, it is interesting to note that the most of these proteinases are unstable at temperatures of 55-60 °C (McPhee and Griffiths, 2002). However, at those temperatures, the loss of the proteinase activity is not the consequence of autoproteolysis (which often occurs in the absence of proteins), but as a result of the formation of the enzyme-casein complex in milk (Chen et al., 2003). The proteinases of *Pseudomonas* spp. have the ability to degrade κ, α_{s1} and β-casein of milk, which is the consequence of the physical destruction of the colloidal system that is primarily manifested in the form of coagulation and the development of an intensively bitter flavour in the product (Muir, 1996).

The lipases and phospholipases of Gram-negative psychrotrophic bacteria hydrolyse milk fat and lecithin, thereby releasing free fatty acids (the normal content of free fatty acids in milk fat is between 0, 5-1, 2 mmol 100 g^{-1}). The increased level of free short-chain fatty acids (C4-C8) causes rancid flavour in dairy products. Medium-chain fatty acids (C10-C12) are the most common cause for the soapy, unclean or bitter product flavours. Free long-chain fatty acids (C14-C18) are not believed to influence a change in the flavour of milk and milk products (Al-Shabibi et al., 1964; Champagne et al., 1994). The necessary concentration of free fatty acids in the product for evident change in flavour is approximately 8.0 g/kg (C8) to 27.5 mg/kg (C4).

Bacillus spp. in comparison with *Pseudomonas* spp. show more diverse proteolytic activity, and many species are capable of forming more than one type of extracellular and intracellular proteinase (Nabrdalik et al., 2010). However, the intensity of protolytic changes is dependent on species and stain of *Bacillus* spp., as well as of the temperature. Thus, among the casein fractions, κ-casein is hydrolysed in fastest rate when temperature of milk is higher then 4 °C and the result is the formation of the para-κ-casein complex. The breakdown of α- and β-casein is somewhat less pronounced at the same temperatures, and is generally not observed at temperatures of 4 °C (Janštová et al., 2004). The result of the proteolytic changes caused by *Bacillus* spp. is a significant increase in the concentration of free tyrosine (Nabrdalik et al., 2010), which can increase in milk up to 2.13 mg mL^{-1} in comparison with their initial values of approximately 0.65 mg mL^{-1} (Janštová et al., 2006).

The lipolytic activity of *Bacillus* spp. is also significant at temperatures higher than 4 °C, and lipases of almost all species isolated from milk show a certain degree of specificity for the breakdown of mono- and diacylglycerols. Significant lipolytic changes, in the sense of increasing the concentration of free fatty acids in milk, have been confirmed for the presence of *B. licheniformis* and *B. cereus* (Chen et al., 2004; Janštová et al., 2006).

Unlike the bacteria *Pseudomonas* spp. which have the ability to form divergent lipases belonging to six different biochemical groups, the bacteria of the genus *Bacillus* form closely related extracellular and intracellular lipases that belong to two groups (Chen et al., 2004). In comparison to proteinases, the lipases of *Bacillus* spp. are more thermostabile. However, both types of enzymes are sufficiently thermostable at all temperatures of milk heat treatment and therefore remain active in the all dairy products, including milk powder (Chen et al., 2004).

Despite numerous researches dealing with the ability to form thermostable enzymes and their influence on the most important components of milk, the mechanisms of their synthesis has not yet been fully explained. This fact confirms their complexity on the one hand and makes setting objectives for their control impossible on the other (Sørhaug and Stepaniak 1997; McPhee and Griffiths, 2002; Němečková et al., 2009).

Biofilm

An important characteristic of psychrotrophic bacteria associated with milk and dairy products is their ability to form exopolysaccharides and/or lipopeptides. This components form structurally different groups of metabolites that enable bacteria to adhesion to solid surfaces upon which they can form a biofilm (Watnick and Kolter, 2000; Raaijmakers et al., 2010). The biofilm may be formed by a single bacterial species, including spoilage and pathogenic organisms. However, the biofilm is often consists of the mixture of microorganisms present in the actual environment. So, biofilm is generally defined as a complex structural, heterogeneous, genetically divergent community of microorganisms that exist on a solid surface in the form of an extracellular matrix composed of polymeric compounds. The nature of the extracellular three-dimensional matrix, the ratio of proliferation and interaction between cells within the biofilm is determined by the available conditions for growth, the medium and substrate (Watnik and Kolter, 2000; Constantin, 2009).

The physiologically similar cells within the biofilm indicate significantly different properties and changes in the bacterial physiology in comparison with their planktonic counterparts (O'Toole et al., 2000; Vlková et al., 2008). Due to the changes, the bacterial communities within the biofilm are more resistance to the majority of antibiotics and disinfectants then planktonic cells (López et al., 2010). Also, significant is the fact that the bacterial species *B. subtilis*, *B. cereus* (facultative anaerobic bacteria) and *P. fluorescens* (aerobic bacteria) are capable of jointly forming biofilm. In the biofilm, these bacteria develop different physiological characteristics than then have planktonic cells (Constantin, 2009). For example, in a binary biofilm, the survival of the bacteria *P. fluorescens* after exposure to chlorine dioxide (ClO_2) is higher in the presence of the species *B. cereus* (Lindsay et al., 2002). Furthermore, Bester et al. (2005) confirmed that the biofilm serves as a source of free bacterial cells with varying characteristics in relation to the planktonic cells and cells of the same species within the biofilm. Namely, the bacterial cells within the biofilm develop a greater cell biomass and have the ability to temporarily separate from the formed biofilm. For these reasons, in the dairy industry, biofilm formation can be a difficult to resolve and long-lasting source of permanent product contamination with psychrotrophic bacteria that cause spoilage and/or can be opportunistic pathogenic (Mosteller and Bishop, 1993; Sillankorva et al., 2008; Simõoes et al., 2010).

Sources of contamination

Psychrotrophic bacteria are not part of the natural microbial population of the udder, and therefore their presence in raw milk is exclusively the result of milk contamination after milking (Gaunot, 1986; Suhren, 1989; Munsch-Alatossava et al., 2005). The most commonly stated sources of Gram-negative psychrotrophic bacteria are: residual water in milking machines, milk pipelines or coolers, dirty udders and teats, inadequate cleaning of surfaces of dairy equipment for reception, transport and storage of milk, and biofilm (Santana et al., 2004; Simõoes et al., 2010).

In terms of the sources of psychrotrophic spore-forming bacteria, such as those from the genus *Bacillus*, there is no universally accepted opinion. In general, the appearance of *Bacillus* spp. in raw milk is usually attributed to seasonal effects (Sutherland and Murdoch, 1994). Hay and dust are considered to be sources of these bacteria during winter months, while teats dirtied by soil are sources during the humid summer months. Christiansson et al. (1999) confirmed that the number of spores in milk is significantly correlated to the degree of soil contamination of teats. On the contrary, Lukaševa et al. (2001) and Foltys and Kirchenerová (2006) did not confirm a significant seasonal influence on the presence of *Bacillus* spp. in raw milk, based on a one-month analysis of raw milk samples collected over the course of one year from different farms. A high correlation with incidence of *Bacillus* spp. in raw milk was confirmed in August and October by Lukaševa et al. (2001), who explained this as being due to changes in meals and poor udder hygiene during milking, and not due to a seasonal influence.

The bulk milk storage tanks, pipelines and filling machines are a significant source of contamination of milk and dairy products with *Bacillus* species (Mosteller and Bishop, 1993; Eneroth et al., 1998; Sillankorva et al., 2008; Simõoes et al., 2010). Wijman et al. (2007) confirmed that within 24 hours, *B. cereus* forms a biofilm in all systems that are partially filled during technological operations or where residual liquid are reminded after the end of the process. Depending on the conditions, biofilm can consist of up to 90 % spores, and probably is the favourable site for the formation of spores. The spores of *B. cereus* are able to separate out of the biofilm into the production environment. For this reason, in most cases, the presence of *Bacillus* spp. spores in pasteurised milk is not the result of post-pasteurisation contamination, as was previously thought (Griffiths and Phillips, 1990).

Raw milk

Immediately after hygiene performed milking, fresh raw milk contains less than 5000 cfu mL^{-1}. The dominant microbial population is mainly consisted of bacteria belonging to genera *Micrococcus*, *Streptococcus*, *Lactococcus* and *Corynebacterium*, and a negligible number of other Gram-positive and Gram-negative bacteria (Causin and Bremley, 1985). In the comparison with the total bacterial count, the occurrence of psychrotrophic bacteria in raw milk is less than 10 % (Gehringer, 1980). In practice, that means that milk produced under clean milking conditions contains between 5,000-20,000 cfu mL^{-1} of psychrotrophic bacteria. However, cooling and the longer held raw milk at approximately 4 °C prior to processing, causes the changes in microbial population. Under those conditions, the dominant Gram-positive bacteria are replaced by Gram-negative and Gram-positive psychrotrophic bacteria. These bacteria in most of the cases have considerable negative effects on the quality of milk as well as dairy products (Causin and Bremley, 1985; Lafarge et al., 2004; Samaržija et al., 2009). Among psychrotrophic bacteria that are associated with milk and dairy products, *Pseudomonas* spp. and *Bacillus* spp. are the most common isolated organisms in raw or heat treated milk at the time of spoilage (Mc Phee and Griffiths, 2002).

In a study conducted on the seven farms over the period of 18 months, Cempíriková (2002; 2007) confirmed a high correlation ($p<0.001$) between psychrotrophic Gram-negative bacteria (PB) and total count of mesophilic aerobic bacteria (TB) with a proportional PB/TB index of 0.18. In the microbiological quality assessment of raw milk in Denmark, Holm et al. (2004) confirmed that psychrotrophic bacteria were dominant in 28 % of cases where the TB exceeded 30,000 cfu mL^{-1}. Although, that is the case of relatively low level of milk contamination. Contrary to this, under conditions of unhygienic milking, the number of psychrotrophic Gram-negative bacteria in raw milk accounted for 75-99 % of the total microbial population, with the predominance of the *P. fluorescens* (Marshall, 1982; Cox, 1993; Muir, 1996; Magan et al., 2006).

Gram-positive spore-forming psychrotrophic bacteria in raw milk are present in negligible numbers <1 mL^{-1} (Boor and Murphy, 2002). However, due to their psychrotrophic properties, with cooling and extended cold storage of raw milk, they become present in 27-58 % of cases (Johnston and Bruce 1982; Griffiths and Phillips, 1990). Among spore-forming bacteria, incidences of *Bacillus* spp. in raw milk are approximately 95 % (Mirth and Steele, 2001). The incidence of *Bacillus* spp. in the number of 10^5 cfu mL^{-1} after seven days of storage of raw milk at 6 °C are confirmed by Griffiths and Phillips (1990). Also, the same authors found that 50 % of the *Bacillus* spp. strains have ability to survive even at a temperature of 2 °C. The incidences of mesophilic aerobic psychrotrophic spore-forming bacteria in cold raw milk in the number of 2.5-340 cfu mL^{-1} are estimated by Foltys and Kirchenerová (2006). This result is based on the analysis of 294 bulk raw milk samples that they have been collected from 14 farms in Slovakia over one year.

Potential sources of the bacteria *B. cereus* in raw milk were investigated over two years during the grazing season by Christiansson et al. (1999). Depending on the degree of contamination of the teats with soil, they established that the number of spores of *B. cereus* in raw milk can range between <10 to 880 L^{-1}. In the raw milk collected in western Scotland (from 1040 farms), Johnston and Bruce (1982) confirmed the presence of *Bacillus* spp. in 27.2 % of samples. *B. cereus* was isolated in 65.7 %, *B. licheniformis* in 19.9 %, and *B. coagulan* in 10.1 % of samples, while the other species of the genus *Bacillus* were present in milk in negligible numbers. Of 100 samples of raw milk, Matt and Punj (1999) confirmed the presence of *Bacillus* spp. in 48 % of samples. In a relative comparison of the presence of particular species of this genus in raw milk, this study confirmed that *B. cereus* can be considered the most common species (32.2 %). On the contrary, in a one-year study of raw milk quality based on the 276 samples that has been conducted in the Czech Republic by Lukaševa et al. (2001), the absolute domination of *B. licheniformis* (85 %) was confirmed, while other *Bacillus* spp. including *B. cereus* were less frequently present. Based on these results and results of other studies, it can be assumed that the dominant species of the genus *Bacillus* in raw milk is largely regionally specific.

The rate at which psychrotrophic bacteria will achieve a level of contamination of $\geq 10^6$cfu mL^{-1}, i.e. the level in most cases necessary for the evident appearance of spoilage, is dependent on the initial number of bacteria immediately after milking and on time of cold storage of raw milk. For example, the number of psychrotrophic bacteria in milk prior to pasteurization will be greater than 10^7 cfu mL^{-1} even in cases of a low initial level of contamination (<50,000 cfu mL^{-1}) when it is held for 3-4 days at a temperature of 4-5 °C (Causin, 1982; Sørhaug and Stepaniak, 1997). However, by monitoring the growth dynamics of the microbial population of cooled raw milk (at 4 °C) using DNA-based molecular methods, Lafarge et al. (2004) confirmed that the psychrotrophic microbe population becomes the dominant population in milk within 24 hours, regardless of the initial level of contamination.

The consequences of enzymatic activity of psychrotrophic bacteria in cooled raw milk include undesirable changes of milk fat, protein and lecithin, which negatively influence its quality and processing capacity (Fox and Stepainak, 1983; Braun et al., 1999; Koka and Weimer, 2001). In addition, the thermostability of those enzymes, including some which are thermostable after heating to a temperature of 100 °C/30 minutes, may be a reason that heat treated milk is unacceptable for consumption and/or producing of high quality dairy products (Fox, 1981; McKellar, 1982; Dogan and Boor, 2003; Hantsis-Zacharov and Halpern, 2007).

The foreign flavour of raw milk that is described as bitter, fruity, yeasty or metallic is the result of the growth of non-spore forming psychrotrophic bacteria of the following genera *Proteus*, *Pseudomonas* (with the predominance of the species *P. flourescens*), *Acinetobacter*, *Flavobacterium*, *Alcaligenes* and *Seratia* (Marshall, 1982; Muir, 1996; Magan et al., 2001).

Unlike Gram-negative bacteria, the spores of Gram-positive psychrotrophic bacteria present in raw milk can survive pasteurisation and sterilization. For that reason, the shelf life of heat processed milk is reduced, off flavour as well as sweet clotting and gelation of milk are often observed (Johnston and Bruce, 1982; Svensson et al., 2004). In addition, compared with Gram-negative psychrotrophic bacteria, the incidence of *Bacillus* spp. can become predominant in raw and heat treated when it is processed under hygienic conditions (Ledford, 1998; Eneroth et al., 2001). Namely, in the absence of competitive microbial populations, the surviving

Table 1. Influence of the growth of psychrotrophic microbial populations in raw milk on dairy product quality

Product	Kind of defect	Cause
Pasteurized milk	Precipitation when milk added to hot beverage (bitty cream)	Activity of phospholipases and proteinases from *Bacillus* spp., fat destabilization
	Gelation	Thermoresistant proteinases [Gram-positive and Gram- negative of psychrotophic bacteria (~10^6-10^8 cfu mL^{-1})]
	Fruity flavour	Syntesis of esters; *P. fragi*
	Shorter shel-life	Stimulated by presence of etanol
	Sweet curdle	Protein hydrolysis
	Fouling in heat exchangers	Proteases decrease heat stability of proteins
Sterilized milk	Rancidity, off-flavour, bitterness, soapy	High concentration of free fatty acids due to activity of thermostabile lipases; protein hydrolysis due to activity of heat stabile proteinases
	Gelation after 1 week	Thermostabile proteinases [Gram-positive and Gram-negative psychrotrophic bacteria (~10^8 cfu mL-^{1})]
	Sweet curdling after 1-2 mjeseca	Thermostabile proteinases [Gram-positive and Gram-negative psychrotrophic bacteria (~10^4 cfu mL^{-1})]
Cream, butter	Rancidity, off-flavour, fruity bitterness, soapy	High concentration of lipases and proteinases in milk (cream) prior pasterization; post-pasterisation contamination (*Pseudomonas* spp. *Bacillus* spp) High concentrations of free fatty acids (C_4-C_6; $C1_{10}$-C_{12})
Cheese	Rancidity, off-flavour	Activity of lipases and proteinases remain in curd that ongoing hydrological changes during ripening
	Lower yields	Proteolysis cause mostly by proteinases of *Pseudomonas* spp.
	Shorter coagulating time	Higher concentration of free amino acids (bacterial proteinases) which stimulate the growth of starter culture
	Longer coagulation time	Higher concentration of free fatty acids (bacterial lipases) which inhibite the growth of starter culture

(modified from Champagne et al., 1994)

spores in favourable condition can form vegetative cells and proliferate in milk rapidly. Therefore, for milk and dairy product quality, spores represent a much greater problem than their vegetative cells (Brown, 2000; Barbano et al., 2006).

The influence of contamination of raw milk with psychrotrophic bacteria prior to heat processing on dairy product quality is shown in Table 1.

Pasteurized and sterilized milk

Defects due to excessive number of psychrotrophic bacteria in raw milk also have a negative impact on the quality of pasteurized (HTST) and sterilized (UHT) milk. Namely, properly conduced heat treatment procedures of raw milk eliminate Gram-negative psychrotrophic bacteria (that are the most common in cooled milk), but their heat stabile enzymes survive as well as the spore of Gram-positive psychrotrophic bacteria (Rogelj and Bogović, 1990; Elmher, 1998; Eneroth et al., 2000). Therefore, the spoilage of HTST and UHT milk is the consequence of the growth of these bacteria in raw milk prior to heat treatment and/or the activation of their thermoresistant enzymes or spores after heat treatment (Nelson, 1985; Craven et al., 1994; Eneroth et al., 2000; Budová et al., 2002). In addition, it is not unusual that Gram-negative psychrotrophic bacteria in pasteurized milk may be the result of post-pasteurization contamination.

Among the Gram-negative psychrotrophic bacteria, *Pseudomonas* spp. causes spoilage of pasteurized milk in 15-33 % cases, and those incidence are in high correlation (r=0.857) with the total count of bacteria in milk (Samaržija, 2003; Kumaresan and Annalvilli, 2008). So, even very law initial number of these bacteria in pasteurized milk during cooled storage will reach the critical level of 10^6-10^7 cfu mL^{-1} within 10 days and contribute to milk spoilage (Eneroth et al., 1998). This level of bacterial contamination causes spoilage of pasteurized milk, which are associated with occur of bitter components, unclean flavour and shortening of shelf life. For HTST milk, the expected shelf life can be less than 14 days (Sørhaug and Stepainak, 1997; Aaku et al., 2004). In comparison with HTST milk, the atypical flavour and negative proteolysis of milk that can appear after the fifth day of storage is more pronounced in UHT milk (Nörnberg et al., 2010). It seams that use of the higher temperature during processing lead to the exposure of new enzyme substrate sites on protein molecules (McKeller, 1982).

Unlike Gram-negative, the Gram-positive spore-forming psychrotrophic bacteria survive heat treatment of milk and continue their growth in cooled milk (Larsen and Jorgensen, 1997; Huck et al., 2007). Of the Gram-negative psychrotrophic bacteria, *Bacillus* spp. with the predominance of the species *B. cereus*, *B. subtilis* and *B. licheniformis* are the most commonly isolated bacteria in spoiled HTST and UHT milk. The combinations of thermoresistant and psychrotrophic properties allow them to better withstand stresses then would be the case of their vegetative form. However, due to physical activation of spores by the temperature of heat treatment of milk they will grow again as active vegetative bacteria (Brown, 2000). Thus, *B. cereus*, *B. subtilis* and *B. licheniformis* in heat treated and cold storage of milk, where there is no contamination by other organisms will have favourable conditions to grow and multiply rapidly. Therefore, in modern dairy industry these bacteria are represented the major causes of spoilage of HTST and UHT milk (Meer et al. 1991; Muir, 1996; Lin et al., 1998; Matta and Punj, 1999).

Similar to most Gram-negative psychrotrophic bacteria, the vegetative cells of *Bacillus* spp. are able to produce thermoresistant enzymes that are comparable to those of *Pseudomonas* spp. For example, *Bacillus* spp. like *Pseudomonas* spp. exhibited marked biochemical activities such as hydrolyses of casein in 84 %, lecithin in 77 %, fat in 52 % and lactose in 8 % of cases that are responsible for milk spoilage (Johanton and Bruce, 1982).

The ability of 108 bacteria species of the genus *Bacillus* isolated from milk to induce proteolysis in sterilized milk is studied by Murugan and Villi (2009). The authors measured total proteins in milk and established a reduced ratio of casein nitrogen of 4.82 % when contamination is caused by *B. subtilis*, 4.15 % for the species *B. cereus*, and 4.08 % in the case of *B. licheniformis*. All three species caused a foreign flavour and coagulation of milk after 30 hours of incubation at a temperature of 37 °C. In the sense of proteolytic and lipolytic changes caused by the occurrence of *Bacillus* spp. in sterilized milk, Janštová et al. (2004) observed the reduction of total protein from 34.60 g/L to 29.46-32.86 g/L, and casein ratio reduction by 7.3 %. At the same time, the ratio of β-casein within the casein micelles was decreased by 27.53 % and α-casein by 43.95 %.

The significant increase of free fatty acids concentration in sterilized milk, as a consequence of lipolysis caused by *Bacillus* spp. is confirmed by Janštová et al. (2006). Namely, after three weeks of storage of UHT milk at a temperature of 24 °C, the initial concentration of free fatty acids of 41.97 mmol kg^{-1} was increased to the 1617.22 mmol kg^{-1}. The largest increase in the concentration of free fatty acids was confirmed in the case of the simultaneous contamination of UHT milk with *B. licheniformis* and *B. cereus*. In addition, due to the capacity of extensive lipolysis and proteolysis, it is considered that *Bacillus* spp. reduced the shelf-life of UHT milk by 25 %. Furthermore, *Bacillus subtilis* and/or the thermostable proteolytic enzymes of Gram-negative bacteria hydrolyse casein, thereby destabilising the casein micelles and coagulation of milk similar to coagulation by rennin is occurred (Law et al., 1977; Mitchell and Marshall, 1989; Meer et al., 1991; Hamamoto et al., 1993; Rukure and Bester, 2001).

The incidences of the bacteria *B. cereus* in pasteurized milk produced in three Danish dairies were monitored by Larsen and Jørgensen (1997) over a period of one-year. Of the total of 458 samples analysed, *B. cereus* was isolated in 257 (56 %) of samples, and its presence in pasteurized milk was significantly higher during the summer (72 %) than during the winter (28 %) months. The estimated number of *B. cereus* in pasteurized milk was between 10^3 and $3x10^5$ cfu mL^{-1}, and no significant differences were observed between dairies in the percentage of *B. cereus* positive samples. Likewise, López-Pedemonte et al. (2003) also observed the higher occurrence of this bacterial species in pasteurized milk during the summer (May - October) than in the winter period.

B. cereus is the dominant microbial species in pasteurized milk in cases when the cooling temperature is above 7 °C and the cold storage time is longer (McGuiggan et al., 1994; TeGiffel et al., 1997; Larsen and Jørgensen, 1999; Eneroth et al., 2000). Namely, 53 % of strains of *B. cereus* are able to grow at these temperatures with a generation time of approximately 8-9 hours (Dufrenne et al., 1994; TeGiffel et al., 1997). At the end of the shelf-life of naturally contaminated pasteurized milk, Larsen and Jørrgensen (1999) estimated the occurrence of *B. cereus* in 24 of the total 27 samples. This study was conducted on three different dairies in Denmark, and the samples taken during the experiment were stored at temperatures of 7±0.5 °C and analysed daily over 9 days. Immediately after pasteurization, *B. cereus* was confirmed in only 2 of 27 samples, but after 5 days of storage, the number of this bacterium started to increase significantly. Of the total of 24 positive samples, after 9 days of storage 11 samples contained *B. cereus* more than 10^3 mL^{-1}. Although this level of contamination does not cause spoilage in HTST or UHT milk, it does represent a level which may be hazardous to human health if the strains are pathogenic (IDF Bulletin, 2000; Christinsson, 2002; Svenson et al., 2005). The presence of *B. cereus* in pasteurized milk in number greater than 10^6 cfu mL^{-1} causes a change of the characteristic flavour of HTST or UHT into unclean, fruity, bitter, rancid or yeasty flavour.

Within the genus *Bacillus* spp., the spore-forming, non-pathogenic bacteria *B. sporothermodurans* and *B. stearothermophilus* are the most tolerant to the temperature and time conditions of milk sterilization (Huemer et al., 1998). Unlike *B. cereus*, *B. subtilis* and *B. licheniformis*, the presence of these bacteria in sufficient numbers caused an acidic coagulation and a cheese-like flavour and aroma of sterilized milk.

Fermented milks

In general, due to the lower pH value (~4.2-4.6), fermented milks is not a suitable environments for the majority of spoilage-causing bacteria (Rašić and Kurman, 1978; Robinson et al., 2002; 2006). However, in the production of fermented milks, the problem lies in the raw milk quality that is stored at low temperatures for longer period of time prior to processing (Rossland et al., 2005). Under those conditions, psychrotrophic bacteria hydrolyze the proteins and milk fat. The consequences of these hydrolytic changes on the quality of fermented milks are manifested, first of all, in changes of texture and flavour. For example, due to the hydrolysis of κ-casein in raw milk, yogurt has a more firm gel and higher viscosity, while syneresis is more pronounced (Gassem and Frank, 1991). It is interesting to note that the proteolytic changes favour the growth of microbial cultures by increasing the concentrations of the free amino acids. However, at the same time due to the lipolytic changes caused by the lipases of psychrotrophic bacteria, absent of typical aroma in final product is very often occurred. Instead, an atypical flavour is formed which can be described as bitter, rancid, unclean and fruity (Sørhaug and Stepaniak, 1997).

Cream and butter

Considering that cooled milk is almost exclusively used in the present day production of cream and butter, in approximately 25 % of cases, psychrotrophic bacteria are the main causes of spoilage and reduced shelf-life of those products (Cox, 1993; Larsen and Jørgensen, 1997; Spreer, 1998b). The defects can be caused directly by microbial contamination of the product, or indirectly as the result of prior growth of psychrotrophic bacteria in raw milk (Causin, 1982; Cox, 1993; Mirth, 1998; Spreer, 1998a; McPhee and Grffiths, 2002).

Since the cream and butter are products with high fat content they are more prone to lipolytic than to proteolytic spoilage caused by the thermostable enzymes of psychrotrophic bacteria (Kornacki and Flowers, 1998). Although, bitterness and off flavour also can be developed by their ability to hydrolyse milk protein (Causin, 1982; McPhee and Grffiths, 2002).

P. fragi or, more rarely *P. fluorescens* have been reported as major organisms associated with rancidity of these products due to their lipolytic capacity of fat digestion to free fatty acids (Stead, 1986). In addition, if the distribution of moisture in butter is incorrect they may grow within the butter. In such cases, P. *fragi* develops apple-like esters taints first and consequently rancidity will be detected later by taste. *P. putrefaciens* more commonly grows on the surface of butter at usual storage temperatures between 4-7 °C. Due to release of certain organic acids, in particular isovaleric acid, after 7-10 days, putrid odour of butter can be recognised. Both of them are able to produce greenish pigments and various taints that can give unpleasant discoloration on the surface of butter. Less common spoilage defects as unpleasant flavour and black discoloration are usually caused by P. *mephitica* and *P. nigrifaciens* (Jay, 1992; Koka et al., 2001; Munsch-Alatossava et al., 2005).

Cheese

Every increase in the number of psychrotrophic bacteria in milk for the cheese making above ~10^3 cfu mL^{-1} always has a larger or a smaller negative impact on the overall quality of the cheese. The occurrence of psychrotrophic bacteria even in relatively small numbers in raw milk will increase rapidly to the contamination levels of 10^5-10^8 cfu mL^{-1} after just two days of cold storage. This level of bacterial contamination significantly reduces the suitability of milk for cheese production. The first negative consequence is the destabilization of the natural plasmin system of milk. Namely, the proteinases of psychotropic bacteria stimulate the release of plasmin and plasminogen from the casein micelles. In cheese production, that means that plasmin and plasminogen will be largely removed into whey fraction of milk. That process, in generally, could affect the quality of cheese, as plasmin is important for flavour and texture development as well as cheese yield (Fajardo-Lira and Nielsen, 1998). Consequently, the rennet coagulation time of raw milk decrease significantly which is opposite to pasteurised milk where coagulating time of milk is significantly increased. This can be explained by possible interaction between the psychrotrophs and the rest of the native microbial populations in raw milk that have an effect on rennet. In addition, since α- and β-caseins are attacked by psychrotophs proteinases, κ-casein may become more accessible to attack by rennet. So, the coagulation time in raw milk is reduced (Cousin and Mirth, 1977). On the other hand, growth of psychrotrophic bacteria in raw milk may affect milk protein to modify or irreversibly change its structure during heat treatment of milk. Namely, the changes of protein that has happened in raw milk may cause it to become sensitive to the temperatures, so different protein complexes can be formed such as the complex between β-lactoglobulin and casein that extended time of coagulation in heat treated milk.

The problems with proper coagulating time are also frequently associated with partial solubilization of ß-casein in raw milk and later its tendency to leave the casein micelle. This leads to reduction in diameter and an increased hydration capacity of the casein micelle, both of which promote a greater stability. However, as a result, the curd becomes more fragile and less compact. The problems with proper coagulating time are even more pronounced in the production of cheeses from sheep's milk, which contains a higher ratio of β-casein (~1/2 CN) in comparison with cow's milk, where the ratio of β-casein in the casein micelles is about ~1/3 (Manfredini and Massari, 1989; Tavaria et al., 2006). Regardless of the reason, coagulation time and the quality of curd are significantly changed.

In addition, it is important to stress that milk contamination with only 10^3 cfu mL^{-1} of psychrotrophic bacteria, after cold storage at low temperatures for 48 hours will reduce the yield of the curd by ~4 % (Leitner et al., 2008). The *Pseudomonas* spp. and/or their thermostable proteolytic enzymes are most often associated with this kind of fault (Fox, 1981; McSweeney, 1997; McPhee and Griffiths, 2002). Moreover, these hydrolytic enzymes remain in the cheese curd and later cause an atypical flavour during ripening, particularly in semi-hard and hard cheeses that have long ripening (Fox, 1981).

The activity of the proteinases of *Pseudomonas* spp., and especially *P. fluorescens* leads to the occurrence of sliminess and pigmentation in fresh cheeses, greater losses in the production of semi-hard and hard cheeses and defects in the flavour, texture and stability of cheese (Walker, 1988; Mitchell and Marshall, 1989). For example, it is confirmed that the gelatinion and/or slimy texture of fresh cheeses (with 55-80 % moisture content) is a direct result of the contamination of cheese with Gram-negative psychrotrophic bacteria, usually the species *P. flourescens*, *P. fragi* and *P. putida* (Brocklehurst and Lund, 1985; Fox et al., 2000). Indirectly, the poor texture of fresh cheeses is the result of the activity of the thermostable proteolytic enzymes of those bacteria.

In comparison with the proteinases which are extracted in significantly large part by whey, the lipases of psychrotrophic bacteria are adsorbed on the fat globules and remain in the cheese (Fox and Stepaniak, 1983; Stead, 1986; Cox, 1993). Thus, undesirable lipolysis takes place by the end of cheese ripening and primarily is responsible for causing off-flavours in cheese due to realised of free fatty acids from the fat globules. In the case of lipases produced by *P. fluorescens*, rancidity taste in hard cheeses will be appeared after two months of ripening. With respect to cheeses where desirable lipolysis takes place, undesirable lipolysys raises the concentration of free fatty acids by 3-10 times (Law, 1979; Corsetti et al., 2001).

Bacillus spp. are bacteria that can survive the usual temperatures of milk pasteurization (72 °C/15 seconds) that are used in the manufacturing of a large number of different cheeses varieties. These bacteria are therefore considered the next microbial group that can cause an extended time of coagulation and have a negative impact on the quality of the curd (Johnson, 1998; Caceres et al., 1997; Rukure and Bester, 2001). Because these bacteria grow slowly in milk, they are the cause of cheese defects when their numbers in milk reach a level of contamination of $\geq 10^6$ cfu mL^{-1}. Most often, these bacteria cause an undesirable texture of the cheeses, and the appearance of defect is specific for different varieties of cheese.

Due to the fact that there are numerous different cheese varieties, each significantly different than other, the occurrence and appearance of defects caused by psychrotrophs are not uniform for all cheeses (Spreer, 1998c; Walstra, 1999b; Walstra, 1999c; Corsetti et al., 2001). However, the defects that are common to all cheese varieties are improper coagulation time and a reduction of the quality of the cheese curd.

Controls

The negative impacts of psychrotrophic bacteria on the quality of milk and dairy products are unquestionable. Their ubiquitous nature in the production environment and ability for rapid growth under low temperatures have made this group of bacteria the leading direct and/or indirect cause of spoilage of milk and dairy products. Contamination of raw milk with psychrotrophs, even under the best manufacturing practices, cannot be completely avoided. However, cooling of milk at a temperature of 2 °C, instead of at temperatures between 4 and 6 °C (which is the most commonly applied cooling temperature), can significantly slow their growth as well as their proteolytic and lipolytic activity (Kumarsan et al., 2007). However, the slowing of the growth of psychrotrophic bacteria at lower milk storage temperatures has an effect only in cases where their initial number is $\leq 10^3$ cfu mL^{-1}. The heat treatment of raw milk (65-69 °C/15 s) in the dairy plant prior to pasteurization can reduce the number of Gram-negative psychrotrophic bacteria by 77-97 %, but has no effect on spore-forming Gram-positive psychrotrophic bacteria (Champagne et al., 1994).

Filling machines in the dairy industry represent the most common site for product recontamination by psychrotophs. Therefore, permanent hygiene controls of filling machines are recommended.

It is assumed that Gram-positive spore-forming psychrotrophic bacteria can contaminate heat treated milk and dairy products during the entire production process via inaccessible corners in the production system and via the biofilm on the surface of dairy plant equipment (Eneroth et al., 1998; Poulsen, 1999). Considering that spore-forming bacteria, especially *Bacillus* spp. comprise a very heterogeneous group of bacteria bearing different physiological properties, it is very difficult to standardize the procedures to isolate these bacteria from milk and dairy products and to define the conditions for their activation (McGuiggan et al., 1994; Francis et al., 1998). In that sense, the combination of cooling temperatures and heat treatment of milk, and its bactofugation can be of significant advantage. However, the problem of subsequent contamination of the final product with these bacteria is not easy to control. So, it is believed that well designed dairy plant equipments that are easy to clean can largely reduce the occurrence of spore-forming psychrotrophic bacteria in the product. Also, based on the research of this issue, it can be assumed that the domination of certain *Bacillus* species in dairy products appears to be largely regionally specific. Therefore, it is important for the dairy producers to determine which of these species has the highest predisposition for contamination of their particular dairy products.

Regardless of whether the psychrotrophic bacteria are Gram-negative or Gram-positive, controls of the production process by adoption of HACCP or similar quality control systems is still considered to be the most effective form of control. However, depending on the type of dairy product, further controls on the growth of psychrotrophic bacteria can be recommended, such as controlling water activity, pH, and the usage of permitted antimicrobial compounds as well as through ensuring conditions for rapid cooling of finished products to temperatures ≤2 °C.

Instead of a conclusion

Over the past fifty years, numerous studies on psychrotrophic bacteria have been conducted, though many issues relating to psychrotrophic populations in milk and dairy products remain unanswered (Hantsis-Zacharov and Halpern, 2007). On the other hand, psychrotrophic bacteria remain a significant economic problem for the dairy industry (Varnam and Sutherland, 1996; Garbutt, 1997; Randolph, 2006). These facts suggest that the identification and control of psychrotrophic bacteria associated with milk and dairy products will continue to be the subject of many scientific and expert studies.

Due to the facts that psychrotrophic bacteria are the main microbial causative agents of spoilage of milk and dairy products, and that some of them are considered the opportunistic pathogenic bacteria, effective control of processing conditions have to be required. Only by regular controls of the presence of psychrotophs would significantly contribute to improving the quality of milk and dairy products, and to increasing profits.

Based on the previous research in terms of preventing measures, it seems to be the most acceptable to maintain the recommended standard for the quality of raw milk of ≤30,000 cfu mL^{-1} for the total number of aerobic mesophilic bacteria, and <5,000 cfu mL^{-1} for the number of psychrotrophic bacteria.

Psihrotrofne bakterije i njihovi negativni utjecaji na kvalitetu mlijeka i mliječnih proizvoda

Sažetak

Osobine i svojstva mikrobnih populacija sirovog mlijeka u momentu prerade presudne su za pojavu kvarenja, vrijeme održivosti, organoleptičku kvalitetu i randman mlijeka i mliječnih proizvoda. Hlađenje i vremenski duža pohrana sirovog mlijeka na niskim temperaturama, uobičajena u današnjim uvjetima proizvodnje, pogoduje rastu psihrotrofnih bakterija. Zbog toga, broj tih bakterija u ohlađenom sirovom mlijeku u odnosu na broj mezofilnih aerobnih bakterija značajno je viši od njihovog idealnog omjera od 10 %. Većinu psihrotrofnih bakterija karakterizira sposobnost tvorbe ekstracelularnih i/ili intracelularnih termostabilnih enzima (proteaze, lipaze, fosfolipaze) koji mogu uzrokovati kvarenje mlijeka i mliječnih proizvoda. Određene vrste psihrotrofnih bakterija pokazuju i prirodnu otpornost na antibiotike i/ili mogu stvarati toksine te se istovremeno smatraju i uvjetno patogenim bakterijama. U smislu kvalitete sirovog mlijeka i mliječnih proizvoda psihrotrofne bakterije postale su ozbiljan problem s kojim se suočava današnja mljekarska industrija. Svrha ovog preglednog rada bila je opisati negativan utjecaj koji psihrotrofne bakterije imaju na kvalitetu sirovog mlijeka i gotovih mliječnih proizvoda. Također, u radu su opisane najznačajnije vrste, te značenje kontrole kojom se može umanjiti kontaminacija mlijeka i mliječnih proizvoda psihrotrofnim bakterijama.

Ključne riječi: psihrotrofne bakterije, kontaminacija, biofilm, kvaliteta mlijeka i mliječnih proizvoda, kontrola

Literatura

1. Aaku, E.N., Collison, E.K., Gashe, B.A., Mpuchane, S. (2004): Microbiological quality of milk from two processing plants in Gaborone Botswana. *Food Control 15*, 181-186.
2. Adams, D.M., Barach, J.T., Speck, M.L. (1976): Effect of Psychrotrophic Bacteria from Raw Milk on Milk Proteins and Stability of Milk Proteins to Ultrahigh Temperature Treatment. *Journal of Dairy Science 59*, 823-827.
3. Al-Shabibi, M.M.A., Langer, E.H., Tobias, J., Tuckey, S.L. (1964): Effect of Addet Fatty Acids on the Flavour of Milk**.** *Journal of Dairy Science 3*, 295-296.
4. Barbano, D.M., Ma, Y., Santos, M.V. (2006): Influence of Raw Milk Quality on Fluid Milk Shel Life. *Journal of Dairy Science 89* (E. Suppl) E15-E19.
5. Beattie, S.H., Williams, A.G. (2002): Growth and diaroeagenic enterotoxin formation by stains of Bacillus cereous in vitro in controlled fermentations and in situ in food products and a model food system. *Food Microbiology 19*, 329-340.
6. Beena, A.K., Ranjini, A.R., Riya, T.G. (2011): Isolation of Psychrotrophic multiple drug resistant Pseudomonas from Pasteurised milk. *Veterinary World 4*, 349-352.
7. Bester, E., Wolfaardt, G., Joubert, L., Garny, K., Saftic, S. (2000): Planktonic-Cell Yield of a Pseudomonad Biofilm. *Applied and Environmental Microbiology 71*, 7792-7798.
8. Boor, K.J., Murphy, S.C. (2002): Microbiology of market milks. U knjizi: Dairy Microbiology Handbook, The microbiology of Milk and Milk Products, edited by Robinson, R.K., third edition, Wiley Interscience, New York, 91-122.
9. Braun, P., Fehlhaber, K., Klug, C., Kopp, K. (1999): Investigations into the activity of enzymes produced by spoilage causing bacteria: a possible basis for improvet shelf life estimation. *Food microbiology 16*, 531-540.
10. Brocklehurst, T.H., Lund, B.N. (1985): Microbiological changes in cottage cheese varieties during storage at +7 °C. *Food microbiology 2*, 207-233.
11. Brown, K.L. (2000): Control of bacterial spores. *British Medical Bulletin 56*, 158-171.
12. Budová, O., Baranová, M., Lauková, A., Różańska, H., Rola, J.G. (2002): Hygiene of pasteurised milk depending on psychrotrophic microorganisms. *Bulletin of the Veterinary Institute in Pulawy 46*, 325-329.
13. Caceres, P., Castillo, D., Pizarro, M. (1997): Secondary Flora of Casar de Caceres Cheese: Characterization of *Micrococcaceae*, *International Dairy Journal 7*, 531-536.
14. Causin, M.A. (1982): Presence and activity of psihrotrophic microorganisms in milk and dairy products: a review. *Journal of Food Protection 45*, 172-207.
15. Causins, C. M., Bramley, A.J. (1985): The Microbiology of Raw Milk. U knjizi: Dairy Microbiology, The Microbiology of Milk, (vol.1) edited by Robinson, R.K. Alsevier Applied Science Publishers, New York, 119- 164.
16. Cempíriková, R. (2002): Psychrotrophic vs.total bacterial counts in balk milk samples. *Veternary Medicine Czech 47*, 227-233.
17. Cempíriková, R. (2007): Contamination of cow's milk by psychrotrophic and mesophilic microflora in relation to selected factor. *Czech Journal of Animal Science 52*, 378-393.
18. Cempírková, R., Mikulová, M. (2009): Incidence of psychrotrophic lipolytic bacteria in cow's milk. *Czech Journal of Animal Science 54*, 65-73.
19. Cempírková, R., Mikulová, M., Trávniček, J. (2009): Counts of psychrotrophic lipolytic bacteria in cow's raw milk samples from the aspect of technological quality. *Journal of Agrobiology 26*, 113-121.
20. Champagne, C.P., Laing, R.R., Roy, D., Mafu, A. A. (1994): Psychrotrophs in Dairy Products: Their Effects and Their Control critical reviews *Food science and Nutrition 34*, 1-30.
21. Chen, L., Coolbear, T., Daniel, R.M. (2004): Characteristics of proteinases and lipases produced by seven *Bacillus* sp. Isolated from milk powder production lines. *International Dairy Journal 14*, 495-504.
22. Chen, L., Daniel, R.M., Coolbear, T. (2003): Detection and impact of protease and lipase activities in milk and milk powder (review). *International Dairy Journal 13*, 255-275.
23. Christiansson, A. (2002): *Bacillus cereus*. *Encyclopedia of Dairy Sciences*, Vol. 1., Roginski, H., Fuquay, W.J., Fox, F.P., Academic Press, 123-140.
24. Christiansson, A., Bertilsson, J., Svensson, B. (1999): *Bacillus cereus* Spores in Raw Milk: Factor Affecting The Contamination of Milk During the Grazing Period. *Journal of Dairy Science 82*, 305-314.
25. Collins, E.B. (1981): Heat Resistant Psychrotrophic Microorganisms. *Journal of Dairy Science 64*, 157-160.
26. Constantin, O.E. (2009): Bacterial biofilms formation at air liquid interfaces. *Innovative Romanian Food Biotechnology 5*, 18-22.
27. Corsetti, A., Rossi, J., Gobbetti, M. (2001): Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *International Journal of Food Microbiology 69*, 1-10.
28. Cousin, M.A., Marth, E.H. (1977): Changes in milk proteins caused by psychrotrophic bacteria. *Milkwissenschaft 32*, 377-341.
29. Cousin, M.A. (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. *Journal of Food Protection*, *45* 172-207.
30. Cox, M.J. (1993): The significance of psychrotrophic *Pseudomonas* in dairy products. *The Australian Journal of dairy Technology 48*, 108-113.
31. Craven, H.M., Forsyth, S.R., Drew, P.G., Macauley, B. J. (1994): A new technique for early detection of Gram-negative bacteria in milk. *The Australian Journal of Dairy Technology 49*, 54-56.
32. Dogan, B., Boor, K.J. (2003): Genetic Diversity and Spoilage Potentials among *Pseudomonas* spp. Isolated from Fluid Milk Products and Dairy Processing Plants. *Applied and Mnvironmental Microbiology 69*, 130-138.
33. Dufrenne, J., Soentoro, P., Tatini, S., Day, T., Notermans, S. (1994): Characteristics of *Bacillus cereus* related to safe food production. *International Journal of Food Microbiology 23*, 99-109.

34. Elmher, H.M. (1998): Extended Shelf Life Refrigerated Foods: Microbiological Quality and Safety. *Food technology 52*, 57 - 62.

35. Eneroth, A., Ahrne, S., Molin, G. (2000): Contamination of milk with Gram negative spoilage bacteria during filling of retail containers. *International Journal of Food Microbiology 57*, 99-106.

36. Eneroth, A., Christiansson, A., Brendehang, J., Molin, G. (1998): Critical Contamination Site in the Production Line of Pasteurised Milk, with Reference to the Psychrotophic Spoilage Flora. *International Dairy Journal 8*, 829-834.

37. Ercolini, D., Russo, F., Ferrocino, I., Villani. F. (2009): Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology 26*, 228-231.

38. Fajardo-Lira, C., Oria, M., Hayes, K.D., Nielsen, S.S. (2000): Effect of Psychrotrophic Bacteria an of an Isolated Protease from Pseudomonas fluorescens M3/6 on the Plasmin System of Fres Milk. *Journal of Dairy Science 83*, 2190-2199.

39. Fajardo-Lira, C., Nielsen, S.S. (1998): Effect of psychrotrophic microorganisms on the plasmin system in milk. *Journal of Dairy Science 81*, 901-908.

40. Finlay, W.J.J., Logan, N.A., Sutherland (2002): *Bacillus cereus* emetic toxin production in relation to dissolved oxygen tension and sporulation. *Food Microbiology 19*, 423-430.

41. Foght, J.M., Westlake,W.S., Johnson, W.M., Ridgway, H.F. (1996): Environmental gasoline-utilizing isolates and clinical isolates of *Pseudomonas aeruginosa* are taxonomically indistinguishable by chemotaxonomic and molecular techniques. *Microbiology 142*, 2333-2340.

42. Foltys, V., Kirchnerová, K. (2006): Mesophilic and psychrotrophic aerobe sporulating microorganisms in raw cow's milk. *Arhiva Zootechnica 9*, 41-55.

43. Fox, F.P. (1981): Proteinases in Dairy Technology. *Netherlands Milk and Dairy Journal 35*, 233-253.

44. Fox, P.F., Stepaniak, L. (1983): Isolation and some properties of extracellular heat-stable lipases from *Pseudomonas flourescens* strain AFT36. *Journal of Dairy Research 50*, 77-89.

45. Fox, P.F., Guinee, T. P., Cogan, T.M., McSweeney, P.L.H. (2000): Microbiology of Cheese Ripening. U knjizi Fundamentales of Cheese Science, An Aspen Publication, Aspen Publishers, Inc. Gaithersburg, Maryland, 206-232.

46. Francis, K.P., Mayer, R., von Stetten, F., Gordon, S.A. Stewart, B., Scherer, S. (1998): Discrimination of Psychrotrophic and Mesophilic Strains of *Bacillus cereus* Group by PCR Targeting of Major Cold Shock Protein Genes. *Applied and Environmental Microbiology 64*, 3525-3529.

47. Garbutt, J. (1997): Factor affecting the growth of microorganisms. U knjizi Essentionals of Food Microbiology, Garbutt, J., Arnold, London, 54-86.

48. Gassem, M.A., Frank, J.F. (1991**)**: Physical properties of yogurt made from milk treated with proteolytic enzymes. *Journal of Dairy Science 74*, 1503-1511.

49. Gaunot, A-M. (1986): Psychrophilic and psychrotrophic microorganisms. *Experimentaria 42*,1192-1197.

50. Gehringer, G. (1980): Multiplikation of bacteria during farm storage. In Factor influensing the bacteriological-quality of raw milk. *International Dairy Federation Bulletin*, Document 120.

51. Griffiths, M.W. (1990): Toxin production by psychrotrophic *Bacillus* spp. present in milk. *Journal of Food Protection 53*,790-792.

52. Griffiths, M.W., Phillips, J.D.(1988): Modelling the Relation Between Bacterial Growth and Storage Temperature in Pasteurised Milk of Varying Hygienic Quality. *Journal of the Society of Dairy Technology 41*, 96-102.

53. Griffiths, W.M., Phillips, J.D. (1990): Incidence, source and some properties of psychrotrophic *Bacillus* spp. found in raw and pasteurized milk. *International Journal of Dairy Technology 43*, 62-66.

54. Grosskopf, J., Harper, W.J. (1969): Role of Psychrophilic Sporeformers in Long - life Milk. *Abstr. Journal of Dairy Science 52*, 897**.**

55. Hamamoto, T., Kaneda, M., Horikoshi, K., Kudu, T. (1993): Characterization of a Protease from a Psychrotroph, *Pseudomonas fluorescens* 114. *Applied and Environmental Microbiology 60*, 3878-3880.

56. Hantsis-Zacharov, E., Halpern, M. (2007): Culturable Psychrotropcih Bacterial Communities in Raw Milk and their Proteolytic and Lipolytic Traits. *Applied and Environmental Microbiology 73*, 7162-7168.

57. Hemalatha, S., Banu, N., (2010): DNA fingerprinting of *Bacillus cereus* from diverse sources by restriction fragment lenght polymorphism analysis. *Advances in Bioscience and Biotechnology 1*, 136-144.

58. Holm, C., Jepsen, L., Larsen, M., Jespersen, L. (2004): Predominant microflora of Downgraded Danish Bulk Tank Milk. *Journal of Dairy Science 87*, 1151-1157.

59. Huck, J.R., Hammond, B.H., Murphy, S.C., Woodcock, N. H., Boor, K.J. (2007): Tracking Spore-Forming Bacterial Contaminantes in Fluid Milk-Processing Systems. *Journal of Dairy Science 90*, 4872-4883.

60. Huemer, I.A., Klijn, N., Vogelsang, H.W.J., Langeveld, L.P.M. (1998): Termal Death Kinetics of Spores of *Bacillus sporothermodurans* Isolated from UHT Milk. *International Dairy Journal 8*, 851-855.

61. IDF Buletin (1976): Psychrotrophs in milk and milk products. International Dairy Federation, Brussels, E-Doc 68.

62. IDF Bulletin (2000): *Bacillus thermodurans*. Detection and Identification of Spore-Forming Bacteria., International Dairy Federation, Brussels, 357/2000.

63. Janštová, B., Dračková, M., Vorlová, L. (2006): Effect of *Bacillus cereus* Enzymes on the Milk Quality following Ultra High Temperature Processing. *Acta Veterinaria Brno 75*, 601-609.

64. Janštová, B., Lukášová, J., Dračková, M., Vorlová, L. (2004): Influence of *Bacillus* spp. Enzymes on Ultra High Temperature-treated Milk Proteins. *Acta Veterinaria Brno 73*, 393-400

65. Jay, J.M. (1992): Characteristics and Growth of Psycrotrophic Microoroganisms. U knjizi Modern Food Microbiology, edited by Jay, J.M., Van Nostrand-Reinhold, New York, 415-441.

66. Johnson, M.E. (1998): Cheese Products. U knjizi Applied Dairy Microbiology, edited bv Marth, E.H., Steele, J.L., Marcel Dekker, Inc., New York, 213-249.

67. Johnston, D.W., Bruce, J. (1982). Incidence of thermoduric psychrtrophs in milk mproduced in West Scotland. *Journal of Applied Bacteriology 52*, 333-337.

68. Koka, R., Weimer, B.C. (2001): Influence of growth conditions on heat stable phospholipase activity in *Pseudomonas*. *Journal of Dairy Research 68*, 109-116.

69. Kornacki, J.L., Flowers, R.S. (1998): Microbiology of Butter and Related Products. U knjizi Applied Dairy Microbiology edited by Marth, E.H., Steele, J.L. Marcel Dekker, Inc., New York, 109-130.

70. Kumaresan,G., Annalvilli, R. (2008): Incidence of *Pseudomonas* species in pasteurised milk. *Tamilnadu Journal of Veterinary and Animal Science 4*, 56-59.

71. Kumarsan, G., Annalvilli, R., Sivakumar, K. (2007): Psychrotrophic Spoilage of Raw Milk at Different Temperatures of Storage. *Journal of Applied Science Research 3*, 1383-1387.

72. Kumura, H., Mikawa, K., Saito, Z. (1993): Purification and some properties of proteinase from *Pseudomonas fluorescens* No. 33. *Journal of Dairy Research 60*, 229-237.

73. Lafarge, V., Ogier, J.L., Girard, Maladen, V., Leveau, J.Y., Gruss, A., Delacroix-Buchnet, A. (2004): Raw Cow Milk Bacterial Population Shifts Attributable to Refrigeration, *Applied and Environmental Microbiology 70*, 5644-5650.

74. Langeveld, L.P.M, Cuperus, F. (1976): The Relation Between Temperature and Growth Rate in Pasteurized Milk of Difference Types of Bacteria Which are Important to the Deteriation of that Milk. *Nethelands Milk Dairy Journal 34*, 106-125.

75. Larsen, H.D., Jørgensen, K. (1997): The occurrence of *Bacillus cereus* in Danish pasteurized milk. *International Journal od Food Microbiology 34*, 179-186.

76. Larsen, H.D., Jørgensen, K. (1999): Growth of *Bacillus cereus* in pasterized milk product. International *Journal of Food Microbiology 46*, 173-176

77. Law, B.A. (1979): Review of the progress of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. *Journal of Dairy Research 46*, 573-578

78. Law, B.A., Andrews, A.T., Sharpe, M.E. (1977): Gelation of UHT Milk by Proteases from a Stain of *Pseudomonas fluorescens* Isolated from Raw Milk. *Journal of Dairy Research 44*, 145-148.

79. Ledford, R.A. (1998): Raw Milk and Fluid Milk Products. *Applied Dairy Microbiology 41*, 55-64.

80. Leitner, G., Silanikove, N., Jacobi, S., Weisbilt, L., Bernstein, S., Merin, U. (2008): The influence of storage on the farm and in dairy silos on milk quality for cheese production. *International Dairy Journal 18*, 109-113.

81. Lin, S., Schraft, H., Odumeru, J.A., Griffiths, M.W. (1998): Identification of contamination sources of *Bacillus cereus* in pasteurized milk. *International Journal of Food Microbiology 43*, 159-171.

82. Lindsay, D., Brözel, V.S., Mostert, J.F., von Holy, A. (2002): Differential efficasy of a chlorine dioxide-containing sanitizer against single and binary biofilms of a dairy associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. *Journal of Applied Microbiology 92*, 352-361.

83. López, D., Vlamakis, H., Kolter, R. (2010): Biofilms. Cold Spring Harbor *Perspectives in Biology* 2:a000398, 1-11.

84. López-Pedemonte, T.,J., Roig-Sague, A., X., Trujillo, A., J. (2003): Inactivation of Spores of *Bacillus cereus* in Cheese by High Hydrostatic Pressure with the Addition on Nisin or Lysozyme. *Journal of Dairy Science 86*, 3075-3081.

85. Lück, H. (1985): Quality Control in the Dairy Industry. U knjizi Dairy Microbiology, vol.2. ed. Robinsom, R. K. Elsevier Applied Science Publishers, London, 279-324.

86. Lukašová, J., Vyhnálková, J., Pácová, Z. (2001): *Bacillus* species in raw milk and in the farm environment. *Milchwissenschaft 56*, 609-611

87. Magan, N., Pavlou, A., Chrysanthakis, I. (2001): Milk - sense: a: volatile sensing system recognises spoilage bacteria and yeasts in milk. *Sensors and Actuators B 72*, 28-34.

88. Manfredini, M., Massari, M. (1989): Small ruminant milk. Technological aspects: Storage and processing. *Options Méditerranées 6*, 191-198.

89. Marshall, R.T. (1982): Relation ship between the bacteriological quality of raw milk and the final products a rewiew. *Kieler Milchwirtschaftliche Forschungsberichte 34*, 149-157.

90. Marth, E.H. (1998): Extended Shelf Life Refrigerated Foods: Microbiological Quality and Safety. *Food technology 52*, 57-62.

91. Martins, M.L., Pinto, C.L.O., Rocha, R. B., de Araújo, E.F., Vanetti, M.C.D. (2006): Genetic diversity of Gram-negative, proteolitic bacteria isolated from refrigerated raw milk. International *Journal of Food Microbiology 111*, 144 -148.

92. Matta, H., Punj, V. (1999): Isolation and identification of lipolytic, psychrotrophic spore forming bacteria fram raw milk. *International Journal of Dairy Technology*, *52*, 59-62.

93. McGuiggan, J.M., Gilmour, A., Lawrence, M.L.(1994): Factors influencing the recovery of psychrotrophic, mesophilic and thermophilic *Bacillus* spp. from bulk raw milk. *Journal of the Society of Dairy technology 47*, 111-116.

94. McKellar, R.C. (1982): Factor in Fluencing the Prodaction of Extracellular Proteinases by *Pseudomonas fluorescens*. *Journal of Applied Bacteriology 53*, 305-316.

95. McPhee, J.D. & Griffiths, M.W. (2002): *Pseudomonas spp*. *Encyclopedia of Dairy Sciences*, Vol. 4, Edited by Roginski, H., Fuquay W.J., Fox, F.P., Academic Press, 2340-2350.

96. McSweeney, P.L.H. (1997): The flavour of milk and dairy products: III. Cheese taste. *International Journal of Dairy Technology 50*, 123 - 128.

97. Meer, R.R., Baker, J., Bodyfelt, F.W., Griffiths, M.W. (1991): Psychrotrophic *Bacillus* spp. in fluid milk products: a review. *Journal of Food Protection 54*, 969-979.

98. Mitchell, S.L., Marshall, R.T. (1989): Properties of Heat-stabile Proteinases of *Pseudomonas fluorescens*: Characterization and Hydrolysis of Milk Proteins. *Journal of Dairy Science 72*, 864-874.

99. Mosteller, T.M., Bishop, J.R. (1993): Sanitizer efficacy against attached bacteria in milk biofilm. *Journal of Food Protection 56*, 34-41.

100. Muir, D. D. (1996): The shelf life of dairy products: 1. Factors influencing raw milk and fresh products. *Journal of the Society of Dairy Technology 49*, 24 - 32.

101. Munsch-Alatossava, P., Alatossava, T. (2006): The darker side of raw milk spoiling psychrotrophs. *Maataloustieteen Päivät www.smts.fi*

102. Munsch-Alatossava, P., Alatossava, T. (2005): Phenotypic characterization of raw milk associated psychrotrophic bacteria. *Microbiological Research 161*, 334-346.

103. Murray, J.G., Stewart, D. B. (1978): Advances in the Microbiology of Milk and Dairy Products. *Journal of the Society of Dairy Technology 31*, 243-248.

104. Murugan, B., Villi, R. A. (2009): Lipolitic activity of *Bacillus* species isolated from milk and dairy products. *The Indian Veterinary Journal 86*, 80-81.

105. Nabrdalik, M., Grata, K., Latala, A. (2010): Proteolytic activity of *Bacillus cereus* streins. *Proceedings of ECOpole 4*, 273-276.

106. Nelson, F.E. (1985): The microbiolohy of Market Milk. U knjizi: Dairy Microbiology vol.1., edited by Robinson, R.K., Elsevier Applied Science, Publishers, London, 165-207.

107. Nemečková, I., Pechačová, M., Roubal, P. (2009): Problems with Detection of Proteolytic Microorganisms and Their Undesirable Activities in Milk. *Chech Journal of Food Science 27*, Special Issue 2: S2-82-S2-89.

108. Netten Van, P., Moosdijak Van de, A., Hoensel Van, P., Mosel, D.A.A., Perales, I. (1990): Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology 69*, 73-79.

109. Nörnberg, M.F.B.L., Friedrich, R.S.C., Weiss, R.D.N., Tondo, E.C., Brandelli, A. (2010): Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. *International Journal of Dairy Technology 63*, 41-46.

110. O'Toole, G., Kaplan, H.B., Kolter, R. (2000): Biofilm formation as microbial developement. *Annu Rev Microbial 54*, 49-79.

111. Poulsen, V.L. (1999): Microbial Biofilms in Food Processing. *LWT-Food Science and Technology 32*, 321-326.

112. Raaijmakers, J.M., De Bruijn, I., Nybroe, O., Ongena, M. (2010): Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfacttants and antibiotics. *FEMS Microbiology Rewiews 34*, 1037-1062.

113. Randolph, H. (2006): Identifying spoilage bacteria and potential shelf- life problems. *Dairy Food*/Find Article 1-3

114. Rašić, J.Lj., Kurman, J.A. (1978): Defects of Yoghurt. U knjizi: Yoghurt Scientific Grounda, Technology, Manufacture and Preparations, Published by the Authors, Copenhagen, 297-301

115. Ren, T.J., Frank, J.F., Christen, G.L. (1988): Characterization of Lipase of *Pseudomonas flourescens* 27 Based on Fatty Profiles. *Journal of Dairy Science 71*, 1432-1438.

116. Robinson, R.K., Lucey, J.A., Tamime, Y. (2006): Manufacture of Yoghurt. U knjizi Fermented milk edited by Tamime, Y., Blackwell Science Ltd, Oxford, 53-71.

117. Robinson, R.K., Tamime, A.Y., Wszolek, M. (2002): Microbiology of fermented milks. U knjizi: Dairy Microbiology Handbook, The microbiology of Milk and Milk Products, edited by Robinson, R.K., third edition, Wiley Interscience, New York, 376-430.

118. Rogelj, I., Bogović, B. (1990): Utjecaj veličine populacije Gram negativnih psihrotrofa na njihovo preživljavanje pri pasterizaciji. *Mljekarstvo 40*, 323-329.

119. Rossland, E., Langsrud, T., Sorhaug, T. (2005): Influence of controlled lactic fermentation on growth and sporulation of *Bacillus cereus* in milk. *International Journal of Food Microbiology 103*, 69-77.

120. Rowe, M.T., Johanston, D.E., Kilpatric, D.J., Dunstall, G., Murphy, R.J. (1990): Growth and Extracellar Enzymes Production by Psychrotrophic Bacteria in Raw Milk Stored at Law Temperature. *Milchwissenchaft 45*, 459-499.

121. Rukure, G., Bester, B.H. (2001): Survival and growth of *Bacillus cereus* during Gouda cheese manufacturing, *Food Control 12*, 31-36.

122. Salkinoja-Salonen, M. S., Vuorio. R., Andersson, M.A., Kämpfer, P., Andersson, M.C., Honkanen-Buzalski T., Scoging,A.C. (1999): Toxigenic strains of *Bacillus licheniformis* related to food poisoning. *Applied and Enviromental Microbiology 65*, 4637-4645.

123. Samaržija, D., Podoreški, M., Sikora, S., Skelin, A., Pogačić, T. (2007): Mikroorganizmi - uzročnici kvarenja mlijeka i mliječnih proizvoda. *Mljekarstvo 57*, 251-273.

124. Samaržija, D. (1993): Upotreba Dip-slide metode za brzo određivanje ukupnog broja bakterija i broja *Pseudomonas* spp. u pasteriziranom mlijeku. Magistarski rad, Agronomski fakultet Sveučilišta u Zagrebu, 1-65.

125. Santana, E.H.W., de. Beloti, V., Müller, E.E., Ferreira, M.A.de., Morales, L.B.de., Pereira, M.S., Gusmão, V.V. (2004): Milk contamination in different points of dairy process. ii)mesophilic, psychrotrophic and proteolytic microorganisms. *Semina: Ciências Agrárias; Londrina 25*, 349-358.

126. Schinik, B. (1999): Habitats of Prokaryotes. U knizi: Biology of Prokaryotes Ed by Joseph W. Lengeler. Gerhard Drews i Hans G.Schlegel, Blackwell Science, New York, 763-801.

127. Senesi, S., Ghelardi, E. (2010): Production, Secretion and Biological Activity of *Bacillus cereus* Enterotoksins. *Toxins 2*, 1690-1703.

128. Shah, N.P. (1994): Psychrotrophs in milk: a rewiew. *Milchwissenchaft 49*,432-437.

129. Sillankorva, S., Neubauer, P., Azeredo, J. (2008): *Pseudomonas fluorescens* biofilms subjected to phage philBB-PF7A. *BMC Biotechnology 78*, Open Access 1-12.

130. Simões, M., Simões, L.C., Vierira, M.J. (2010): A rewiew of current and emergent biofilm control strategies. *Food Science and Technology 43*, 573-583.

131. Sørhaug, T., Stepaniak, L. (1997): Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. *Trends in Food Science and Technology 8*, 35-41.

132. Spiers, J.S., Buckling, A., Rainey, P.B. (2000): The couses of *Pseudomonas* diversity. *Microbiology 146*, 2345-2350.

133. Spreer, E. (1998a): Market Milk, Milk Drinks and Cream Products. U knjizi: Milk and Dairy Product Technology, Spreer, E., Marcel Dekker, Inc., New York, 155-198.

134. Spreer, E. (1998b): Butter Manufacture. U knjizi: Milk and Dairy Product Technology, Spreer, E., Marcel Dekker, Inc., New York, 203-241.

135. Spreer, E. (1998c): Cheese Manufacture. U knjizi: Milk and Dairy Products Technology , Spreer, E., Marcel Dekker, Inc., New York, 243-334.

136. Stead, D. (1986): Microbial lipases: their characteristic, role in food spoilage and industrial uses. *Journal of Dairy Research 53*, 481-505.

137. Stepaniak, L. (2002): Psychrotrophic Bacteria, Bacteria Other than *Pseudomonas* spp. *Encyclopedia of Dairy Sciences*, Vol. 4, Roginski, H., Fuquay, W.J., Fox, F.P., 2345-2351.

138. Suhren, G. (1989): Enzymes of Psychrotrophs in Raw Food. U knjizi: Producer Microorganisms. Edited by McKellar, R.C CRC Press, Inc, Boca Raton, Florida, 4-34.

139. Sutherland, A.D., Murdoch, R. (1994): Seasonal occurrence of psychrotrophic *Bacillus* species in raw milk, and studies on the interaction with mesophilic *Bacillus* spp.. *International Journal of Food Microbiology 21*, 279-292.

140. Svenson, B., Monthan, A., Shaheen, R., Andersson, A.M., Salkinoja-Salonen, M., Christiansson, A. (2005): Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain, *International Dairy Journal 16*, 740-749.

141. Svensson, B., Ekelund, K., Ogura, H., Christiansson, A. (2004): Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *International Dairy Journal 14*, 17-27.

142. Tavaria, F.K., Reis, P. J.M., Malcata, F.X. (2006): Effect of dairy farm and milk refrigeration on microbiological and microstructural chaeacteristics of matured Serra da Estrela cheese. *International Dairy Journal 16*, 895-902.

143. TeGiffel, M.C., Beumer, R.R., Granum, P.E., Rombouts, F.M. (1997): Isolation and characterisation of *Bacillus cereus* from pasteurised milk in household refrigerators in the Netherland. *International Journal of Food Microbiology 34*, 307-318.

144. Thomas, S.B., Druce, R.G. (1969): Psychrotrophic Bacteria in Refrigerated Pasteurized Milk - A review. *Dairy Industries 34*, 351-355.

145. Varnam, A.H., Sutherland, J.P. (1996.a): Cream and cream-based products. U knjizi: Milk and milk Products Technology, Chemistry and Microbiology, Chapman and Hall, London, Marcel Dekker, Inc., New York, 183-216.

146. Varnam, A.H., Sutherland, J.P. (1996.b): Butter, margarine and spreads. U knjizi: Milk and milk Products Technology, Chemistry and Microbiology, Chapman and Hall, London, Marcel Dekker, Inc., New York, 224-268.

147. Vlková, H., Babák, V., Seydlová, R., Pavlík, I., Schlegelová, J. (2008): Biofilms and Hygiene on Dairy Farms and in the Dairy Industry: Sanitation Chemical Products and their Effectiveness on Biofilms- a review. *Czech. J.Food Sci. 26*, 309-323.

148. Walker, S.J. (1988): Major Spoilage Microorganisms in Milk and Dairy Products. *Journal of the Society of Dairy Technology 41*, 91-92.

149. Walstra, P., Geurts, T.J., Noomen, A., Jellema, A., Van Boekel, M.A.J.S. (1999a): Microbiology of Milk U knjizi: Dairy Technology, Principles of Milk Properties and Processes, edited by Marcel Dekker, Inc, New York, 149-170.

150. Walstra, P., Geurts, T.J., Noomen, A., Jellema, A., Van Boekel, M.A.J.S. (1999b): Microbial Defects U knjizi: Dairy Technology, Principles of Milk Properties and Processes, edited by Marcel Dekker, Inc, New York, 639-649.

151. Walstra, P., Geurts, T.J., Noomen, A., Jellema, A., Van Boekel, M.A.J. S. (1999 c): Cheese Varrieties. U knjizi: Dairy Technology, Principles of Milk Properties and Processes, Marcel Dekker, Inc, New York, 651-708.

152. Wang, L., Jayarao, B.M. (2001): Phenotypic and Genotypic Characterization of *Pseudomonas fluorescens* Izolated from Bulk Tank Milk. *Journal od Dairy Science 84*, 1421-1429.

153. Washam, C.J., Olson, H.C., Vedamuthu, E.R. (1977): Heat-resistant psychrotrophic bacteria isolated from pasteurised milk. *Journal of Food Protection 40*, 101-108.

154. Watnick, P., Kolter, R. (2000): Biofilm, City of Microbes. *Journal of Bacteriology 182*, 2675-2679.

155. Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R., Boor, K.J. (2000): Molecular and Phenotypic Characterization of *Pseudomonas* spp. Isolated from Milk. *Applied and Environmental Microbiology 66*, 2085-2095.

156. Wijman, J.G.E., de Leeuw, P.P.L.A., Moezelaar, R., Zwietering, M.H., Abee, T. (2007): Air-Liquid Interface Biofilms of Bacillus cereus: Formation, Sporulation and Dispersion. *Applied and Environmental Microbiology 73*, 1481-1488.

157. Wong, H. C., Chang, M. H., Fan, J.Y. (1988a): Incidence and Characterization of *Bacillus cereus* Isolates Contaminating Dairy Products. *Applied and Environmental Microbiology 54*, 699-702.

158. Wong, H.C., Chen, L.Y., Chen, C.L. (1988): Growth germination and toxigenic activity of *Bacillus cereus* in milk products. *Journal of Food Protection 51*, 707-710.

159. Zall, R.R. (1985): Control and Destruction of Microorganisms. U knjizi: Dairy Microbiology vol 1.ed. Robinson, R.K. Elsevier Applied Science Publishers, London, 77-117.