Effect of dairy farm and milk refrigeration on microbiological and microstructural characteristics of matured Serra da Estrela cheese

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Abstract

This work was aimed at enumerating the viable microorganisms in ripened Serra da Estrela cheeses, manufactured from both refrigerated and non-refrigerated milk, in various dairies located throughout the demarcated region. Scanning electron microscopy was used to analyze the microstructure, and thus aid in understanding possible differences in their microbiological profile. The cheeses were allowed to ripen under controlled conditions, and sampled at 60, 90, 120, 150 and 180 d following manufacture. Viable numbers of lactic acid bacteria, staphylococci, *Enterobacteriaceae* and yeasts were obtained following standard plate counting on a number of selective media. *Lactococcus* was the most abundant genus (above 10^8 cfu g^{-1} of cheese) up to 120 d of ripening. No significant microstructural differences were observed in cheeses manufactured in different dairies over the ripening process. However, microstructural differences were apparent between cheeses manufactured with refrigerated versus non-refrigerated milk.

Introduction

Traditional products are often considered a nuclear part of the cultural heritage of a nation, and they frequently account for the difference between positive and negative net profits of farmers in less developed, rural areas. However, empirical manufacturing practices and poor sanitary conditions associated with such practices are incompatible with the tighter control enforced by Public Health Offices, a situation that will eventually hamper the global trade of such products. One such example is Serra da Estrela cheese, which originates in the central region of Portugal and bears the name of the highest mountains in that country. This cheese is manufactured from raw ewes' milk and curdled with an aqueous extract of the wild thistle *Cynara cardunculus*, without addition of any commercial starter culture. Its manufacture follows traditional protocols (Macedo, Malcata, & Oliveira, 1993) and relies on the unique features of the milk imparted by the local feed, coupled with the proteolytic specificity of the plant rennet and the pattern of curd breaking by hand. The raw milk is heated to ca. 30 °C, before the coagulant is added (0.25 g L^{-1}, which is equivalent to 492.6 rennet units (RU) mL^{-1}; 1 RU is the amount of enzyme needed to coagulate 10 mL of milk at 30 °C in 100 s (FIL-IDF-International Dairy Federation, 1992). The curd is finally allowed to set for 30–45 min (note that the plant coagulant used is ca. 60-fold weaker than commercial rennet). Since no starter culture is added, the native microflora plays an important role during cheese ripening. Cutting of the curd (which occurs at ca. pH 6.5) is performed manually by stirring with bare hands, or with the help of a knife. The curd obtained, which is quite irregular in shape and size, is then moulded and pressed (ca. 2.45×10^5 N m^{-2}). Ripening takes place at 8 °C and 95% relative humidity (RH) for 21 d, and 11 °C and 85% RH thereafter. The cheese is typically sold after 60 d of ripening.

In attempts to overcome the aforementioned sanitary issues, several studies (Macedo, Costa, & Oliveira, 1996; Macedo & Malcata, 1997; Macedo et al., 1993; Sousa & Malcata, 1996, 1997; Tavaria & Malcata, 1998, 2000) have

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characterized the physico-chemical, microbiological and biochemical profiles of this cheese variety. However, evidence that relates evolution of the microbiological profile with physical features such as microstructure is still scarce.

Milk refrigeration has been tested in attempts to avoid microbial proliferation between the milking and cheese-making steps. However, refrigeration raises concerns pertaining to growth and proliferation of psychrotrophic bacteria, which are known to produce very active (and thermostable) proteolytic and lipolytic enzymes. These enzymes catalyze reactions that increase the levels of free fatty acids causing potential off-flavours, and partial solubilization of β-caseins. This causes a decreased diameter and an increased hydration degree of casein micelles, both of which promote a greater stability. As a result, a less compact and more fragile final coagulum is attained (Manfredini & Massari, 1989).

Chemical composition, rheological behaviour and sensory attributes in foods, including cheese, are closely related to their microstructure. Although light microscopy has for long been used in microstructure studies, because it is rapid, and enables viewing of such cheese components as protein, fat and bacteria following specific staining, it is nevertheless constrained by its very shallow depth of focus (Kaláb, 1993). This constraint results in a lack of detail in the case of larger particles (e.g. fat globules and bacteria), so poor resolution is the result. Scanning electron microscopy (SEM) is able to overcome such limitations, and is able to visualize three-dimensional details, which include the protein network and the microbial inventory in cheese.

In this study, classical microbiological data (reflecting microbiological activity) were correlated with SEM images in attempts to validate differences among cheeses manufactured in four dairies along the Appélation d'Origine Protegée (AOP) region of Serra da Estrela, as well as between cheeses manufactured in the same dairy farm from non-refrigerated and refrigerated milk.

Materials and methods

Cheese manufacture and sampling

Experimental cheeses were produced on the same day within the typical ewes' lactation period (January–May), according to the traditional protocol (Macedo et al., 1993), in four selected certified dairies located in the AOP region. Five batches of cheese were produced in each dairy farm, and three cheese replicates were picked up at random from each batch throughout ripening (at 60, 90, 120, 150 and 180 d). This resulted in a total of 75 independent cheeses, with ca. 1 kg each (3 replicates $\times$ 5 ripening times $\times$ 4 dairies + 15 cheeses from refrigerated milk). After sampling for microbiological analyses, cheeses were stored as 2 cm^3 blocks in a formal saline solution (10 mL of 40% (w/v) formalin and 90 mL of 0.94% (w/v) sodium chloride), for at least 1 month, as described by Dean, Berridge, and Mabbitt (1959), until microstructural observations were carried out.

Microbiological analyses

Ten grams of cheese were mixed with 90 mL of sterile 2% (w/v) aqueous sodium citrate solution (Merck, Darmstadt, Germany) as extraction buffer, and homogenized in a Stomacher Lab-Blender 400 (Seward Medical, London, UK). One-mL samples were taken, decimally diluted in sterile 0.1% (w/v) aqueous peptone (Sigma Chemical, St. Louis, MO, USA), and then plated in duplicate on several media as described by Tavaria and Malcata (1998).

Microstructural analyses

Cheese blocks (1 $\times$ 1 $\times$ 2 cm) from the outermost, middle and bulk parts of the cheese were taken from each sample, and immersed in formal saline solution. Samples of formalin-fixed cheese were fractured by hand and dehydrated in a graded series of ethanol (10–100%), each for 10 min. The dehydrated samples were mounted on SEM stubs with conductive cement, and placed in the holder of a JSM-5600LV SEM (JEOL, Tokyo, Japan), operated at an accelerating voltage of 20 kV. Scanning electron micrographs were obtained under several magnifications.

Statistical analyses

Analyses of variance (ANOVA) were conducted for the viable numbers of the various microbial groups; preliminary logarithmic transformation of the data was required, to guarantee that they were independent and normally distributed (as required for validity of this analysis). The first ANOVA was conducted to assess significance of the dairy farm, whereas a second ANOVA was aimed at assessing significance of milk refrigeration. Fisher's protected least significant difference tests were used to make pairwise comparisons. The procedures were performed using the Statistical Software package from StatSoft (2001).

Results and discussion

Effect of dairy farm

The average counts of viable lactobacilli, lactococci, leuconostoc, enterococci, yeasts, *Enterobacteriaceae* and staphylococci in the cheeses manufactured in the four independent dairies are plotted in Fig. 1.

The ANOVA results (Table 1) indicated that both the dairy farm and the ripening time are statistically significant factors ($p < 0.05$) in determining the viable numbers of bacteria, irrespective of the microbial group. Lactic acid bacteria (i.e. lactobacilli, lactococci, leuconostoc and enterococci) were quantitatively the dominant groups at all ripening times and for all dairies, with viable numbers

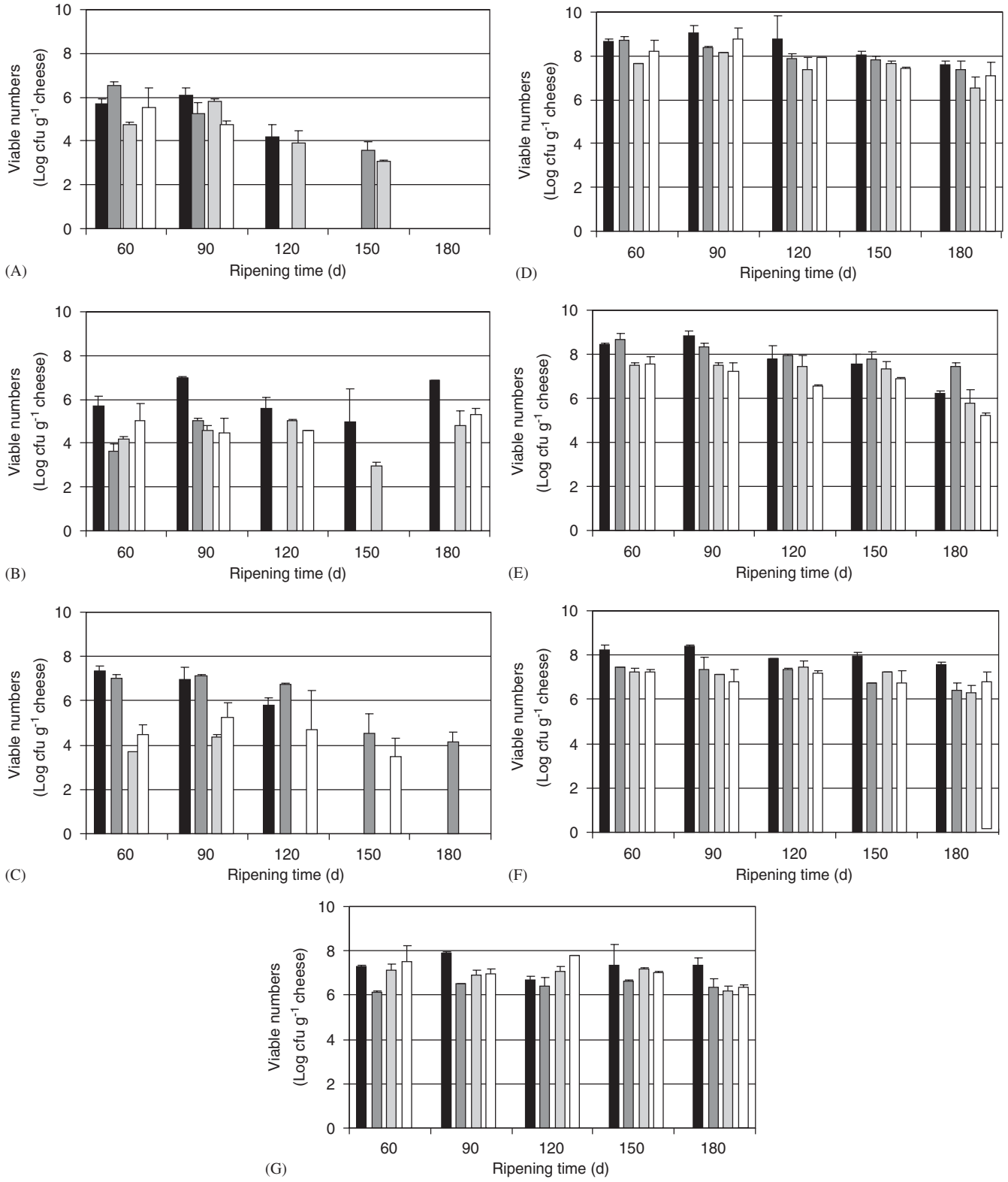

Fig. 1. Mean values, and corresponding standard deviations (error bars), of viable counts of *Enterobacteriaceae* (A), staphylococci (B), yeasts (C), lactococci (D), lactobacilli (E), leuconostoc (F) and enterococci (G) in cheeses throughout ripening, from the four dairies: dairy A (■), dairy B (▩), dairy C (▨) and dairy D (□).

ranging from 10^7 to 10^9 cfu g^{-1} of cheese and increasing with ripening time. These results are consistent with those reported for other artisanal cheeses produced in Southern Europe from small ruminants' milk (Fernández-del-Pozo, Gaya, Medina, Rodríguez-Marín, & Núñez, 1988; Fontecha et al., 1990; Tzanetakis, Litopoulou-Tzanetaki,

Table 1
Analysis of variance results for various microbial groups in cheeses from all dairy farms: two main effects (dairy farm and ripening time) and their interactions

Microbial group	Effect of dairy farm (p-value)	Effect of refrigeration (p-value)	Effect of dairy farm × ripening time (p-value)	Effect of refrigeration × ripening time (p-value)
Enterobacteriaceae	0.010	0.005	0.038	0.452
Staphylococcus	0.000	0.710	0.001	0.175
Yeasts	0.000	0.133	0.002	0.000
Lactobacillus	0.000	0.004	0.039	0.000
Lactococcus	0.004	0.410	0.919	0.590
Leuconostoc	0.000	0.690	0.251	0.060
Enterococcus	0.000	0.190	0.009	0.360

& Manolkidis, 1987). In La Serena cheese, manufactured from raw ewes' milk and coagulated with *C. cardunculus* (Medina, 1996), levels of viable microorganisms were similar to those reported in this study, whereas lactococci were reported to dominate during the first month of ripening in Manchego cheese and to be outnumbered thereafter by homofermentative lactobacilli. In ripened Fiore-Sardo cheese, an Italian variety also produced from raw sheep's milk without addition of starter, enterococci were reported to be the dominant microflora (Ledda, 1996).

Examples of scanning electron micrographs of cheeses manufactured from non-refrigerated milk, in two different dairies, are shown in Fig. 2. Small and large void spaces, which are scattered uniformly over the cheese matrix, can be observed, indicating the probable location of fat globules within the matrix. The average size of these globules range from 3 to 25 μm, in agreement with the report by Fontecha, Kaláb, Medina, Peláez, and Juárez (1996) for ewes' milk cheeses. Pérez-Munuera, Estévez, and Lluch (1999) reported that a similarly compact proteinaceous structure, in which the fat globules were uniformly distributed, is typical of enzymatic coagulation, and is often present in such ripened and pressed cheeses as Mahón and Manchego.

Such a microstructure is believed to impart a smooth, creamy texture to this cheese variety. Micrographs Fig. 2A and B show cheeses from the same dairy farm, whereas micrographs 2C and D show cheeses originated at a different dairy farm. Although substantial microstructural differences between these cheeses were expected (owing to the large variability of raw materials, coupled with such different manufacturing practices as cutting and pressing of curd), this was not observed. No apparent differences could be seen between Figs. 2A and C, probably due to the late time of ripening (60 d). In these two dairies, different patterns of coagulum cutting were used; in dairy farm A, the curd was cut manually via stirring with a knife, thus obtaining very irregular curd pieces; whereas in dairy farm B, the curd was broken manually into very small pieces, before moulding. In the latter case, this should have resulted in a more compact matrix, as syneresis was more efficient due to the larger pores, which present lower resistance to the outflow of whey (Aichinger et al., 2003); however, this was not observed. Cutting procedures may influence microbial levels, because microorganisms tend to grow and develop as colonies along curd junctions (Parker, Gunning, Macedo, Malcata, & Brocklehurst, 1998). Therefore, if the exposed surface area is increased by cutting finer curd pieces, potentially more microorganisms will grow on these fissures. A rise in numbers of lactic acid bacteria during the maturation process can be observed in Figs. 2E and F. By 180 d (micrograph 2F), the numbers of cocci have increased significantly when compared to their counterparts by 60 d of maturation (micrograph 2E).

Effect of milk refrigeration

The mean values (and corresponding standard deviations) of viable counts in cheeses manufactured with refrigerated and non-refrigerated milk in one of the dairies are shown in Fig. 3. The ANOVA results (Table 1) revealed that refrigeration produced significant differences in viable numbers ($p<0.05$) only for *Enterobacteriaceae* (i.e. viable numbers were higher in cheeses produced from non-refrigerated milk) and for lactobacilli (i.e. viable numbers were lower in cheeses obtained from non-refrigerated milk). Similarly to reports pertaining to other cheese varieties such as Manchego (Núñez & Martinez-Moreno, 1976) and La Serena (Núñez, Medina, & Gaya, 1989), the numbers of *Enterobacteriaceae* dropped to negligible levels by 120 and 150 d of ripening, respectively, in cheeses manufactured from refrigerated and non-refrigerated milk. This reinforces the view that refrigeration is effective in control of certain undesirable microorganisms, such as the enteric microflora. Conversely, milk refrigeration seems to favour yeast growth (Figs. 3C and 4C). Yeasts are believed to play important roles, such as utilization of lactic acid and increased rate of protein and triglyceride breakdown (Fernández-del-Pozo et al., 1988), which contribute to development of specific flavours (Dahl, Tavaria, & Malcata, 2000).

Other microbial groups (mostly LAB) exhibited higher viable numbers in cheeses produced from non-refrigerated

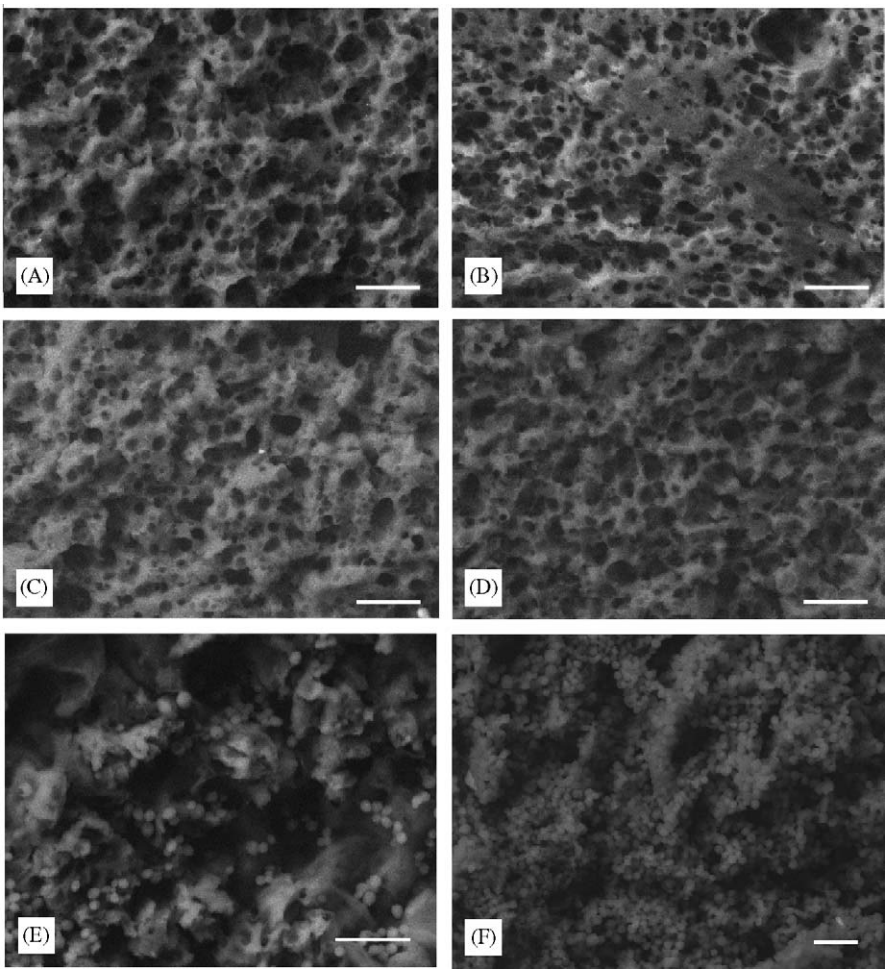

Fig. 2. Scanning electron micrographs showing microstructure in cheeses produced from non-refrigerated milk in two different dairies [A (A, B) and B (C, D, E, F)] and ripened for 60 d (A, C) and 180 d (B, D) (scale bar = 20 μm), showing distribution of microorganisms in cheeses by 60 d (E; scale-bar = 10 μm) and 180 d (F; scale-bar = 20 μm).

milk up to 90 d of ripening. After this time, their numbers were higher in cheeses made from refrigerated milk. Micrographs of cheeses manufactured from refrigerated milk (Fig. 4) show that they have a coarser structure with larger spaces, thus reflecting a less compact coagulum, which prevails until the end of the ripening period. In samples of cheeses produced from refrigerated milk, larger voids of up to 200 μm in diameter were observed (Figs. 4A and B), and the resulting cheeses were creamier with a softer texture. Furthermore, the higher temperature of the non-refrigerated milk may induce faster acidification by the native bacteria, thus producing a coarser gel structure (which leads to a higher porosity) and favouring whey expulsion (Aichinger et al., 2003). Refrigeration, in turn, enhances microbial-mediated lipolytic activity, thus disrupting the fat globule network with a concomitant decrease in the number of these globules, as found by Juven, Gordin, Rosenthal, and Laufer (1981) and Núñez, Chavarri, and Núñez (1984). These trends can be observed in Figs. 4A and B. Refrigeration also affects the colloidal phase of the milk by partially solubilizing β-casein which tends to separate from the micelles, hence resulting in a less compact coagulum (Manfredini & Massari, 1989). This effect is rather accentuated in the case of ewes' milk, owing to the higher β-casein percentage (ca. half of the total casein), when compared to cows' milk (only ca. one third), and due to the smaller size of ovine casein micelles.

Among the microorganisms detected, lactobacilli (Fig. 4C) and yeasts (Fig. 4D) were frequently found in our samples. Yeast cells are believed to prevail in the smear covering the outer surface of the cheese, as initially reported by Parker et al. (1998), where they play an active role despite their relatively low numbers. These findings are similar to the report of Pérez-Munuera et al. (1999) on non-cured, fresh cheeses (e.g. Herreño cheese).

The distribution of microorganisms within the cheese mass is well documented for cheeses such as Camembert (Kaláb, 1993), Gouda (Kaláb, 1977), St. Nectaire (Marcellino & Benson, 1992), Cheddar (Brooker, 1979) and St. Paulin (Rousseau, 1988). Although the microbiological profile of Serra da Estrela cheese has been established, the species present as colonies within the microstructure cannot be elucidated before antibodies for each species are developed. In situ immunolabelling would then allow

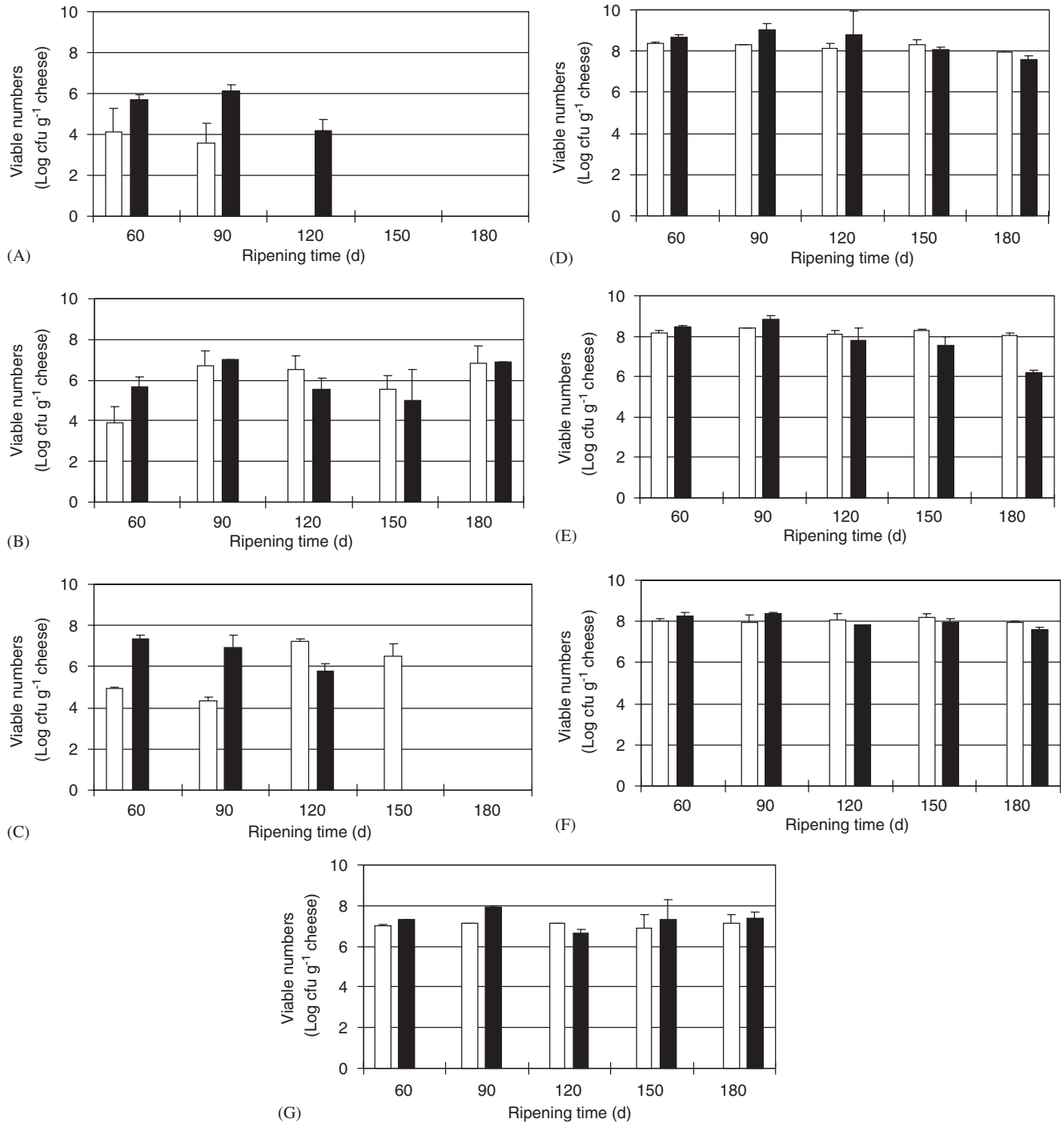

Fig. 3. Mean values (from a single dairy farm), and corresponding standard deviations even less, of viable counts of *Enterobacteriaceae* (A), staphylococci (B), yeasts (C), lactococci (D), lactobacilli (E), leuconostoc (F) and enterococci (G) in cheeses produced from non-refrigerated (■) and refrigerated (□) milk.

bacterial species located as colonies throughout the cheese matrix to be properly identified.

Conclusions

The viable counts of microorganisms in Serra da Estrela cheese were greatly dependent on the farmhouse where manufacture took place. Lactic acid bacteria were quantitatively the dominant group, throughout ripening in all dairies, with numbers of viable cells ranging in 10^7–10^9 cfu g^{-1} of cheese. Microstructural differences were not obvious in cheeses produced in different dairies, using distinct manufacturing practices. Milk refrigeration prior to cheese making appeared to control growth of *Enterobacteriaceae,* and to promote formation of a coarser protein matrix. Although the microstructural observations

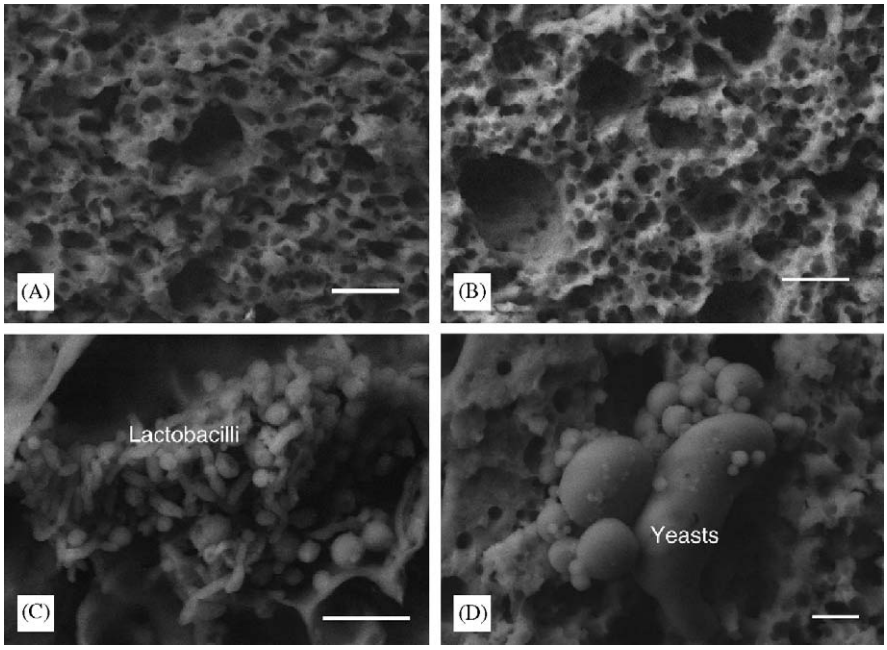

Fig. 4. Scanning electron micrographs showing microstructure in cheeses produced from refrigerated milk and ripened for 60 d (A) and 180 d (B) (scale bar = 20 μm), showing mixed colonies of bacilli and cocci (C; scale bar = 10 μm) and colonies with yeasts (D; scale bar = 20 μm).

did not permit detection of significant differences during ripening, differences were detected in cheeses manufactured from refrigerated milk. These cheeses had a looser matrix, where lactobacilli and yeasts predominated.

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