Microbiological profile in Serra ewes' cheese during ripening

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A.C. MACEDO, F.X. MALCATA AND T.A. HOGG. 1995. The microflora of Serra cheese was monitored during a 35 d ripening period at three different periods within the ewe's lactation season. After 7 d ripening, the numbers of micro-organisms reached their maximum, and lactic acid bacteria (LAB) and coliforms were the predominant groups. Pseudomonads were not detected after 1 week of ripening. At all stages of ripening, cheeses manufactured in spring exhibited the lowest numbers of LAB and yeasts, whereas cheeses manufactured in winter showed the lowest numbers of coliforms and staphylococci.

Leuconostoc lactis was the most abundant LAB found in Serra cheese whereas *Enterococcus faecium* and *Lactococcus lactis* spp. *lactis* exhibited the highest decrease in percentage composition. Numbers of both *Leuc. mesenteroides* and *Lactobacillus paracasei* tended to increase throughout ripening. The most abundant coliform was *Hafnia alvei. Klebsiella oxytoca* was found in curd but declined in number during ripening. Staphylococcal flora of curd was mainly composed of *Staphylococcus xylosus*, *Staph. aureus* and *Staph. epidermidis. Staphylococcus xylosus* was the major species found at the end of ripening. *Pseudomonas fluorescens* was the only *Pseudomonas* species isolated from the curd. Although a broad spectrum of yeasts were found in Serra cheese, *Sporobolomyces roseus* was the most abundant yeast isolated.

INTRODUCTION

Serra da Estrela cheese (or simply Serra cheese) is a semi-soft cheese variety manufactured at the farm level from raw ewes' milk using traditional methods in the mountainous centre region of Portugal. This cheese is made in batches twice daily (early morning and late afternoon) from October to May using the unpasteurized milk immediately after collection. Coagulation is catalysed by a crude vegetable rennet, which consists of a suspension of the dry flowers of *Cynara cardunculus*, prepared at room temperature and filtered through a piece of cotton cloth. Coagulation takes place at 27–30°C for 1–2 h. Once the right degree of consistency is attained, the curd is cut in irregularly shaped pieces, mainly by hand. After cutting, the curd is poured into perforated plastic moulds and lightly pressed by hand, and the cheese is salted by rubbing dry salt on the surface. Cheeses are ripened on wood shelves, namely in a basement for *ca* 40 d. The use of Bordaleira ewes' milk which possesses a high fat content (6–20%) (Macedo *et al.* 1993), coupled with the use of the vegetable rennet, which possesses a strong, unselective proteolytic activity (Barbosa *et al.* 1981), results in a cheese with unique bouquet and creamy texture.

Studies on the microbial flora of ripened Serra cheese have been reported by Hiscox *et al.* (1941), Cruz (1945) and Antunes and Santos (1943); however, very little was known until recently about the nature and the evolution of the main microbial groups during ripening. Studies focused on the changes in the numbers of the main groups of micro-organisms in the interior and on the surface of Serra cheese, throughout the lactation season, and during ripening have been conducted by Macedo *et al.* (1995). The aim of the present study was to complement such previous information via the identification of the species of lactic acid bacteria (LAB), yeasts, coliforms, staphylococci and pseudomonads in Serra cheese during ripening and throughout the lactation season; such work was developed hoping that a standardized starter suitable for Serra cheese manufacture will eventually be possible.

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MATERIALS AND METHODS

Manufacture and sampling of cheese

Three batches of 12 0·5 kg Serra cheeses were manufactured and ripened according to the traditional practice

(Macedo *et al.* 1993) in November, February and May in an attempt to adequately represent autumn, winter and spring manufactures. The 12 cheeses were randomly divided into four sets of three cheeses each; one set picked at random was sent to this laboratory on the day of cheese-making, and the others after 7 d, 21 d and 35 d of ripening, respectively. All samples were transported under refrigerated conditions (*ca* 4°C). Sampling of the interior of the cheese and all microbiological analyses thereafter were performed immediately upon receipt of the cheeses (< 1 h).

Microbiological analyses

Ten g of each cheese sample were homogenized with 90 ml of a sterile solution of 2% (w/v) sodium citrate (Merck, Darmstadt, Germany) at 45°C for 1 min in a Stomacher Lab-Blender 400 (Seward Medical, London, UK). Decimal dilutions were prepared in sterile 0·1% peptone water (Sigma Chemical, St Louis, MO, USA) and plated in duplicate. Lactic acid bacteria (LAB) were grown anaerobically (Gas-Pak anaerobic system BBL, Cockeysville, MD, USA) on M17 Agar (M17A; LabM, Bury, UK) and on Rogosa Agar (RA; Oxoid, Basingstoke, UK) at 30°C for 3 and 5 d. Cycloheximide (Sigma) was added (100 mg l^{-1}) to prevent the growth of yeasts (Kandler and Weiss 1986). Coliforms were determined on Violet Red Bile Agar (VRBA; Lab M) and staphylococci on Baird-Parker egg yolk tellurite medium (BPM; Lab M), at 37°C for 1 and 2 d, respectively. Pseudomonads were grown on Pseudomonas Base Agar (PSDA; LabM) supplemented with cephaloridine, fucidin and cetrimide (Lab M) and yeasts on Potato Dextrose Agar (PDA; LabM) acidified with 10% lactic acid (Merck), at 25°C for 2 d and 5 d, respectively. The technique of surface viable count was used for all media except VRBA for which the pour-plate and overlay technique was used. Results were expressed as cfu g^{-1} of cheese.

For the curd and 35-d-old cheese, as well as from each of the aforementioned agars, colonies with different morphologies were counted, picked (three colonies of each type), purified, and stored at 4°C as slope cultures until further characterization.

Identification of micro-organisms

The API 50 CHL system (BioMérieux, Marcy-l'Etoile, France) was used to identify to the species level the following genera: (i) Lactobacillus (Gram-positive, catalase-negative rods); (ii) Leuconostoc (Gram-positive, catalase-negative, non-producers of ammonia from arginine and heterofermentative cocci); (iii) Lactococcus (Gram-positive, catalase-negative and homofermentative cocci that grow at 10° but not at 45°C). Identification of enterococci (considered here as Gram-positive, catalase-negative, homofermentative cocci that grow at both 10° and 45°C) was performed using the API STREP system (BioMérieux). Coliforms (considered here as Gram-negative, catalase-positive, glucose-fermenters, nitrate-positive and oxidase-negative short rods) were identified by the API 20E system (BioMérieux). Staphylococci (considered here as Gram-positive, glucose-fermenters under anaerobic conditions and catalase-positive cocci) were identified by the API STAPH system (BioMérieux). *Staphylococcus aureus* were confirmed by testing coagulation of lyophilized rabbit plasma (BioMérieux) at 37°C within 24 h. Results obtained from the different API systems were matched with the aid of a commercially available software for the automatic identification of bacteria (Anon. 1993). Identification of pseudomonads (considered here as Gram-negative and oxidase-positive rods) was according to the method of Collins and Lyne (1984). Biochemical and physiological characterization of yeasts included: (i) assimilation of carbon compounds such as L-arabinose, cellobiose, erythritol, galactose, gluconate, glucosamine, glucose, α-methyl-D-glucoside, glucuronate, glycerol, 2-keto-gluconate, DL-lactate, lactose, mannitol, maltose, melibiose, melezitose, raffinose, rhamnose, ribose, saccharose, sorbose, trehalose and D-xylose; (ii) assimilation of nitrogen compounds (nitrate); and (iii) temperature tolerance (growth at 25°, 30° and 37°C). In addition, morphological criteria such as budding/splitting of cells and colony pigmentation were taken into account. The computer program of Barnett *et al.* (1990) was used for automatic identification of yeasts.

Statistical analysis

ANOVA tables (not shown) were constructed with the replicated data pertaining to the numbers of micro-organisms *vs* the factors ripening time and period within the lactation season.

RESULTS

The data statistically significant on the 5% level encompassing the numbers of LAB on M17A, LAB on RA, coliforms on VRBA, staphylococci on BPM, and yeasts on PDA are plotted in Figs 1, 3, 5, 7 and 9, respectively, as the average of the three replicates considered in ANOVA tables. The percentage composition in terms of microbial species of each of the aforementioned groups for both the curd and the 35-d-old cheese are depicted in Figs 2, 4, 6, 8 and 10.

DISCUSSION

Inspection of Figs 1, 3, 5, 7 and 9 indicates that the growth patterns of the main groups of micro-organisms studied, as

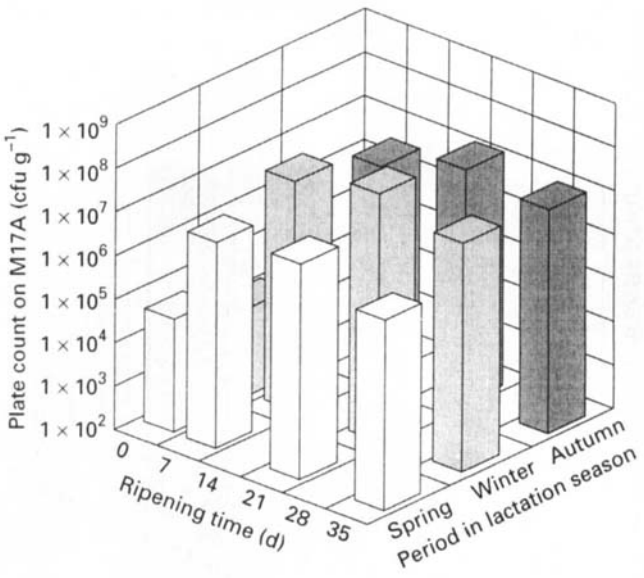

Fig. 1 Changes in numbers of lactic acid bacteria of Serra cheese (grown on M17 agar) with ripening time and period in ewes' lactation season. □, Spring; ▨, winter; ■, autumn

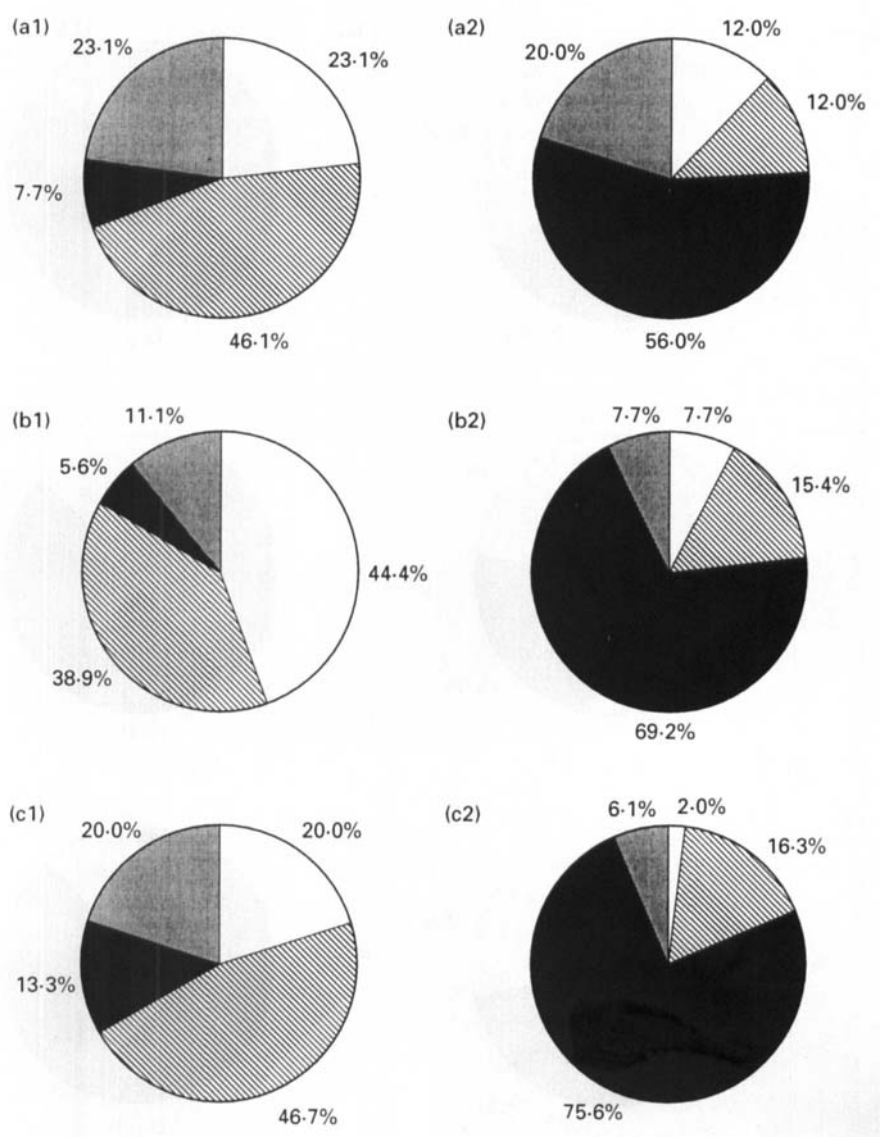

Fig. 2 Composition of LAB of Serra cheese (grown on M17A) in (1) curd and (2) 35-d-old cheese, produced in (a) autumn, (b) winter, and (c) spring, in terms of *Enterococcus faecium* (□), *Lactococcus lactis* ssp. *lactis* (▧), *Leuconostoc lactis* (■), *Leuc. mesenteroides* ssp. *mesenteroides/dextranicum* (▩)

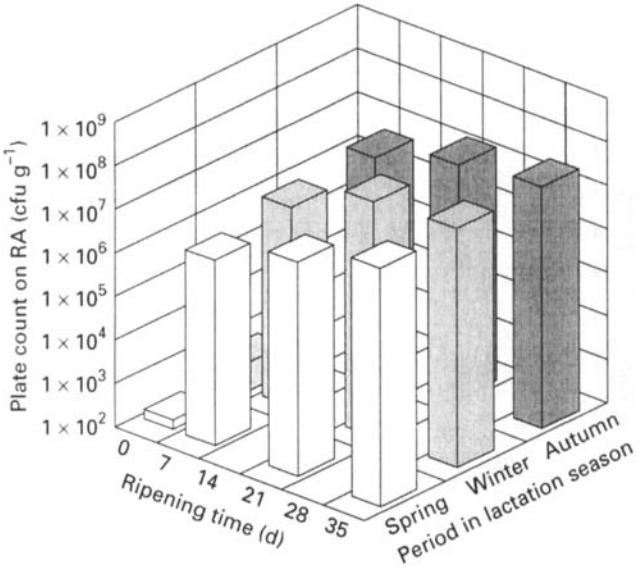

Fig. 3 Changes in numbers of LAB of Serra cheese (grown on Rogosa agar) with ripening time and period in ewes' lactation season. □, Spring; ▨, winter; ■, autumn

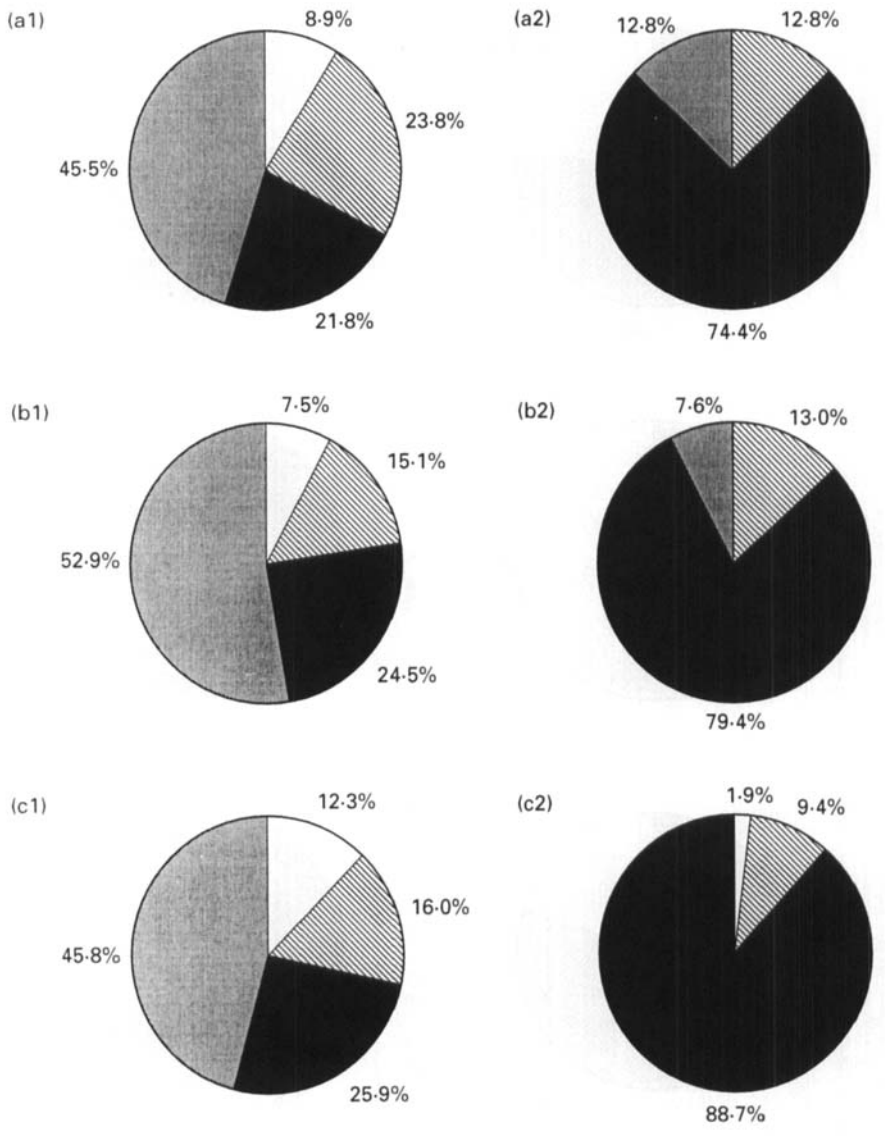

Fig. 4 Composition of LAB of Serra cheese (grown on RA) in (1) curd and (2) 35-d-old cheese, produced in (a) autumn, (b) winter, and (c) spring, in terms of *Lactobacillus plantarum* (□), *Lact. paracasei* ssp. *paracasei* (▧), *Leuconostoc lactis* (■) and *Leuc. mesenteroides* ssp. *mesenteroides/dextranicum* (▨)

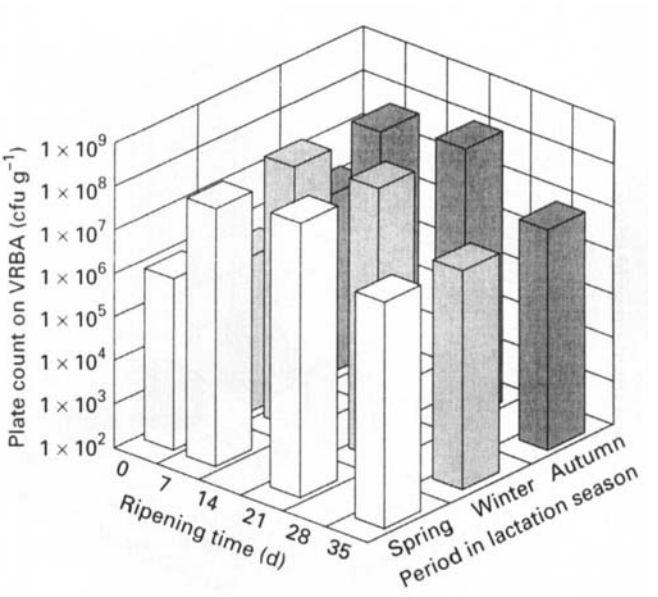

Fig. 5 Changes in numbers of coliforms of Serra cheese (grown on Violet Red Bile Agar) with ripening time and period in ewes' lactation season. □, Spring; ▨, winter; ■, autumn

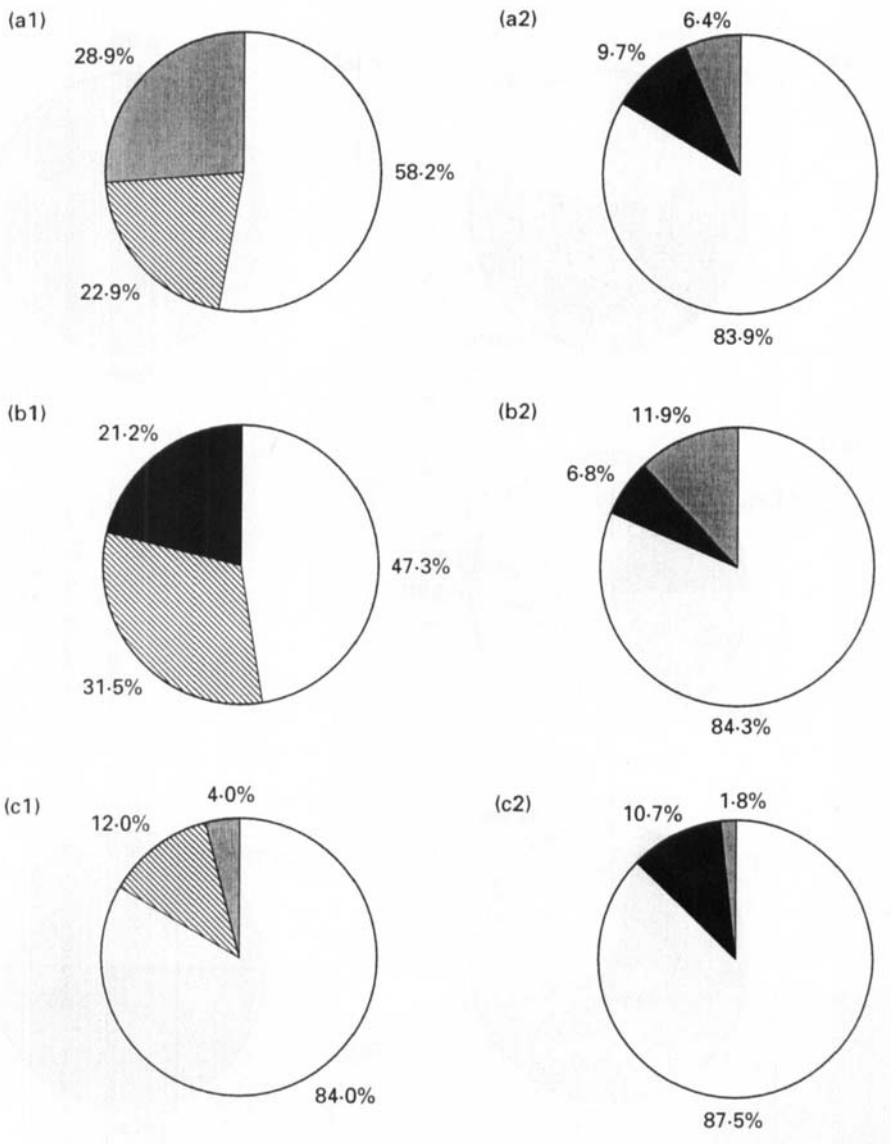

Fig. 6 Composition of coliforms of Serra cheese (grown on VRBA) in (1) curd and (2) 35-d-old cheese, produced in (a) autumn, (b) winter, and (c) spring, in terms of *Hafnia alvei* (□), *Klebsiella oxytoca* (▧), *Escherichia coli* (■) and *Citrobacter freundii* (▨)

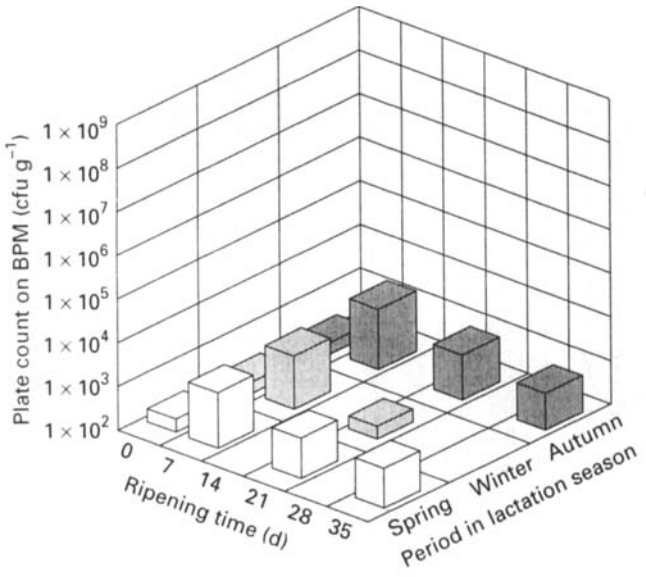

Fig. 7 Changes in numbers of staphylococci of Serra cheese (grown on Baird-Parker Medium) with ripening time and period in ewes' lactation season. □, Spring; ▨, winter; ■, autumn

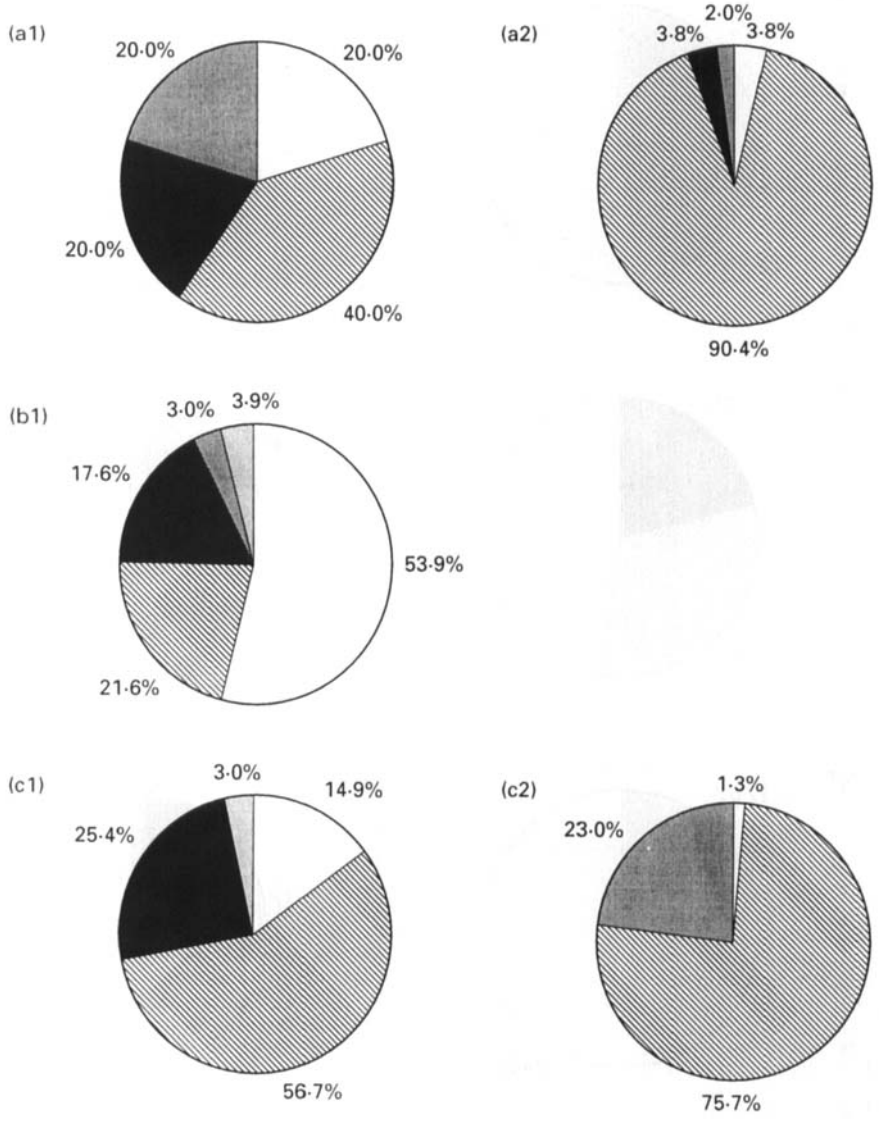

Fig. 8 Composition of staphylococci of Serra cheese (grown on BPM) in (1) curd and (2) 35-d-old cheese, produced in (a) autumn, (b) winter, and (c) spring, in terms of *Staphylococcus aureus* (□), *Staph. xylosus* (▧), *Staph. epidermidis* (■), *Staph. simulans* (■) and *Staph. hominis* (▨)

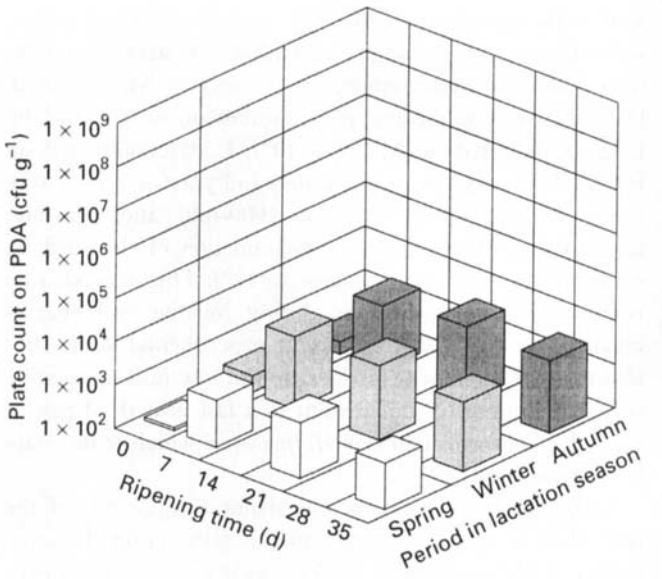

Fig. 9 Changes in numbers of yeasts of Serra cheese (grown on Potato Dextrose Agar) with ripening time and period in ewes' lactation season. □, Spring; ▨, winter; ■, autumn

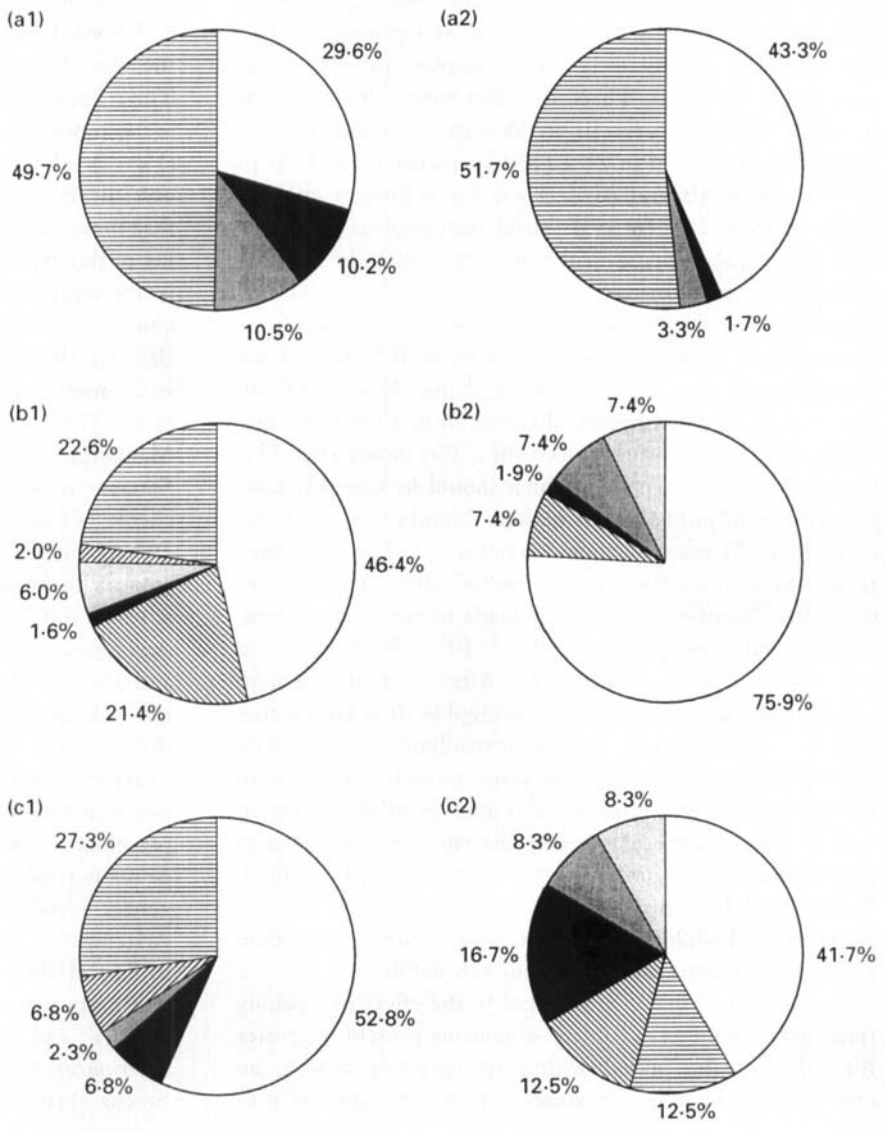

Fig. 10 Composition of yeasts of Serra cheese (grown on PDA) in (1) curd and (2) 35-d-old cheese, produced in (a) autumn, (b) winter, and (c) spring, in terms of *Sporobolomyces roseus* (□), *Kluyveromyces marxianus* (▧), *Rhodotorula aurantiaca* (■), *Yarrowia lipolytica* (■), *Pichia membranaefaciens* (▨), *Trichosporum beigelii* (▨), *Leucosporidium scottii/Debaryomyces hansenii* (▤)

well as the growth rates of LAB, staphylococci and yeasts, with ripening time and period within the lactation season were similar to those reported previously by Macedo *et al.* (1995). However, the initial contamination of the curd by LAB enumerated on M17 (*ca* 10^4), LAB enumerated on RA (*ca* 10^2), staphylococci (*ca* 10^2) and yeasts (*ca* 10^2) were lower by 1000-fold, 100-fold, 1000-fold and 100-fold, respectively, whereas initial contamination of the curd by coliforms tended to be the same (*ca* 10^6). This suggests that better standards of hygiene during milking and cheese-making were used in the present experimental study, but also that the inefficient refrigeration of raw milk is possibly still contributing to some extent to a fast growth of psychrotrophic coliforms (such as *Hafnia alvei* which is the main coliform in curd).

LAB and coliforms were the major components of the microflora during ripening throughout the entire lactation season. LAB enumerated on M17 agar (Fig. 1), LAB enumerated on RA (Fig. 3) and coliforms (Fig. 5) increased their numbers during the first week of ripening by 10^3, 10^4 and 10^2, respectively, with respect to the initial numbers in the curd. Thereafter, the tendencies exhibited by these bacteria were: (i) to increase at a much lower growth rate from 7 d to 21 d (10-fold increase); and (ii) to stabilize for LAB and to decrease for coliforms (10-fold decrease) from 21 d to 35 d. Strict microbiological regulations concerning cheese made from raw milk do not formally exist in Portugal; however, Spanish regulations pertaining to some cheese varieties manufactured from pasteurized milk specify maximum counts of 10^4 cfu g^{-1} for Enterobacteriaceae and 10^3 cfu g^{-1} for *Escherichia coli* (Gaya *et al.* 1983). Cheeses obtained from all batches utilized in the present work exceed those thresholds after 35 d of ripening (Fig. 5), figures which should be carefully considered due to public health reasons. Numbers of staphylococci (Fig. 7) reached their maximum at 7 d, and they tended to decrease thereafter at higher rates than LAB and coliforms. Numbers of pseudomonads in curd for autumn, winter and spring, were $1{\cdot}2 \times 10^3$, $2{\cdot}6 \times 10^3$ and $2{\cdot}3 \times 10^3$ cfu g^{-1}, respectively. After 7 d of ripening, numbers of pseudomonads were negligible. It is known that certain pseudomonads exhibit proteolytic and lipolytic extracellular activities that may cause undesirable effects in texture and flavour; however, the number of this group of micro-organisms present during the ripening process suggests that their role in flavour development is quite limited. Yeasts (Fig. 9) tended to increase during the first 2 weeks of ripening (10-fold increase), but much more slowly than LAB and coliforms, and then tended to stabilize.

As shown in Figs 1, 3, 5, 7 and 9, the effect of ripening time on the numbers of micro-organisms present is greater than that of the period within the lactation season, an observation which is consistent with results reported previously by Macedo *et al.* (1995). LAB and yeasts were favoured by the environmental conditions prevailing in the maturation rooms during winter and autumn, i.e. lower temperatures and higher relative humidities than in spring; conversely, staphylococci and coliforms survived better in spring. Fernandez del Pozo *et al.* (1988) reported that lower counts of coliforms were obtained in La Serena cheese made in spring than in winter. Following the consideration of the inhibitory effect of LAB on the growth of coliforms in Manchego cheese (Gaya *et al.* 1983), the higher numbers of LAB recorded in winter might explain the apparent accelerated decrease of coliforms during this period. The initial contaminations by coliforms and staphylococci in Serra cheese are also in agreement with seasonal variations of coliform counts in raw ewes' milk which apparently decrease from autumn to spring (Gaya *et al.* 1987), and with seasonal variations of staphylococci counts in raw ewes' milk which have been reported as highest in spring and lowest in winter (Bautista *et al.* 1986).

Among LAB, *L. lactis* ssp. *lactis* and *Ent. faecium* were the most abundant bacteria found in curd (Figs 2 and 4). These bacteria showed the largest decrease in their percentage composition from the curd to the fully ripened cheese (Fig. 2). However, the presence of lactococci and enterococci during the late stages of maturation (Fig. 2) suggests that these bacteria may play an important role in the ripening of this type of cheese. It has been reported that *L. lactis* is the most abundant *Lactococcus* species in La Serena cheese (Fernandez del Pozo *et al.* 1988), Hrudkovy and Bryndza cheeses (Prekoppova 1990), and *L. lactis* ssp. *lactis* is the most abundant subspecies in Roncal cheese (Arizcun *et al.* 1992). *Enterococcus faecium* is commonly found in Manchego cheese (Nuñez and Martínez-Moreno 1976), La Serena cheese (Fernandez del Pozo *et al.* 1988) and Teleme cheese (Tzanetakis and Liptopoulou-Tzanetaki 1992). During ripening, however, the bacterium that grows faster appears to be *Leuc. lactis* (Figs 2 and 4) because it dominates in the fully ripened cheese. *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* was also found in both curd and 35-d-old cheese. *Leuconostoc* species can play an important role in aroma development and eye formation through their ability to metabolize citrate and ferment carbohydrates, respectively. The predominance of the *Leuconostoc* genus in Serra cheese is consistent with results presented elsewhere: Nuñez *et al.* (1984) reported that this genus is predominant in the Gram-positive psychrotrophic genera obtained from refrigerated raw ewes' milk, and Arizcun *et al.* (1992) reported high numbers of *Leuconostoc* in Roncal cheese. (In this latter case, *ca* 20% of the *Leuconostoc* species found were *Leuc. mesenteroides* ssp. *mesenteroides* and *ca* 24% were *Leuc. mesenteroides* ssp. *dextranicum*.) The main *Leuconostoc* species found in La Serena cheese was *Leuc. mesenteroides* (Fernandez del Pozo

et al. 1988). *Leuconostoc* species have been also deliberately added to Manchego cheese as a part of the microbial starter with good results (Ramos *et al.* 1990). Among the genus *Lactobacillus*, *Lact. plantarum* and *Lact. paracasei* ssp. *paracasei* could be identified in the curd (Fig. 4). During ripening, *Lact. paracasei* ssp. *paracasei* tended to grow while *Lact. plantarum* tended to disappear, indicating a possible role for *Lact. paracasei* ssp. *paracasei* in Serra cheese maturation. It has been asserted that *Lact. plantarum* and *Lact. casei* were the dominant lactobacilli in La Serena cheese (Fernandez del Pozo *et al.* 1988), Hrudkovy and Bryndza cheeses (Prekoppova 1990), and Roncal cheese (Arizcun *et al.* 1992), and that *Lact. plantarum* was the predominant lactobacillus in Feta and Teleme cheeses (Tzanetakis and Liptopoulou-Tzanetaki 1992). The data presented in the present study also indicate that lactation season has an influence on the initial contamination of the curd and the growth of each group of LAB throughout ripening. The level of enterococci has been correlated with the level of hygiene during milk processing (Thompson and Marth 1986); higher values of the percentage composition of *Ent. faecium* in the curd (Fig. 2) in winter than in autumn or spring might be explained by the poor housing conditions in winter, when the animals spend most of their time in stables due to adverse weather. The ripening conditions in spring contributed preferentially to the growth of *Leuc. lactis* (Figs 2 and 4) with respect to such other lactic cocci as *Leuc. mesenteroides* ssp. *mesenteroides/dextranicum* and *Ent. faecium*. The lowest percentage compositions of *L. lactis* ssp. *lactis* (Fig. 2) and *Lact. paracasei* ssp. *paracasei* (Fig. 4) were obtained in autumn and spring, respectively.

As a result of the absence of good sanitary conditions during milking and cheesemaking, coliforms are, like enterococci, important elements of the bacterial inventory of raw ewes' milk. The levels of coliforms found in the present work (which are of the same order of magnitude as those of LAB) are of considerable public health concern and technological relevance for Serra cheese since this cheese variety is made directly from raw ewes' milk without any type of thermal processing. *Hafnia alvei* was the most abundant and proliferating coliform found both in the curd and the 35-d-old cheese (Fig. 6). Gaya *et al.* (1983) also isolated *H. alvei* in very high proportions in 60-d-old Manchego cheese, and Kleeberger *et al.* (1980) stated that *H. alvei* is the typical coliform isolated from milk and dairy products. Nuñez *et al.* (1984) concluded that refrigeration of raw ewes' milk had effects of decreasing or increasing the counts of psychrotrophs depending on whether the refrigeration was made at 4° or 7°C, respectively. Additionally, Juven *et al.* (1981) reported that psychrotrophic Enterobacteriaceae exhibited proteolytic and lipolytic activities during refrigeration. Considering that the raw ewes' milk used for Serra cheesemaking is lightly refrigerated below room temperature, one concludes that the predominance of *H. alvei* (as a psychrotrophic bacterium) may play an important role in ripening of Serra cheese. Gaya *et al.* (1987) reported that *H. alvei* and *Klebsiella oxytoca* were the predominant species in dairy farm samples. *Klebsiella oxytoca* was found in the curd but not in the 35-d-old cheese (Fig. 6). This fact may be partly accounted for by the weak resistance of this species to low pH; Tornadijo *et al.* (1993) also found *Kl. oxytoca* in the curd but not in 1- or 2-week-old cheeses made from raw goats' milk which undergo pH variations similar to those in Serra cheese (Macedo *et al.* 1995). *Escherichia coli* was isolated from all ripened cheeses but only from the curd during the winter period; since it is able to ferment lactose, it may be responsible for the development of pin eyeholes in cheese. Failure to isolate *E. coli* until virtual completion of ripening may be probably attributed to the fact that the initial contamination of milk by these bacteria is very low when compared with contamination by other micro-organisms. Since *E. coli* is particularly resistant to such adverse conditions as the low pH which tends to develop towards the end of the ripening period, this bacterium is likely to increase its percentage composition importance as time elapses. Gaya *et al.* (1983) reported that *Enterobacter aerogenes*, *E. coli* and *H. alvei* were the most resistant species against the acidic environment generated by lactic acid bacteria. *Citrobacter freundii* was found in both curd and 35-d-old cheese, and its percentage composition was lowest in spring and highest in winter (Fig. 6).

Due to their low numbers during ripening (*ca* 10^2), staphylococci are not likely to play as important a role in the maturation process as LAB (Fig. 7). The predominant *Staphylococcus* species found in curd were *Staph. xylosus*, *Staph. aureus* and *Staph. epidermidis*, and at lower proportions, *Staph. simulans* and *Staph. hominis* (Fig. 8). Similar staphylococci species have been reported by Bautista *et al.* (1986) and Nuñez *et al.* (1989) for ewes' milk, and by Fernandez del Pozo *et al.* (1988) for ewes' cheese. *Staphylococcus aureus* showed a tendency to disappear as ripening time elapses (Fig. 8), so it is less likely that it causes health hazards by the normal time of cheese consumption (i.e. 45 d). For ewes' milk, Bautista *et al.* (1986) reported higher values for the percentage composition of coagulase-positive staphylococci in spring than in the rest of the year; coagulase-positive staphylococci were not detected in La Serena cheese after 45 d (Fernandez del Pozo *et al.* 1988). The highest percentage composition of coagulase-positive strains among the staphylococci in curd was observed in winter. However, these species were less resistant in this period than in either spring or autumn since they could not be found in the 35-d-old cheese. This observation probably can be explained by the fact that more acidic cheese matrices, which are unfavourable to the growth of

coagulase-positive strains, are observed in winter (Macedo *et al.* 1995). *Staphylococcus xylosus* is the most abundant of the staphylococci in curd in the autumn and spring, whereas *Staph. aureus* is the most abundant species in winter; *Staph. xylosus* is also the most resistant and fastest growing bacteria during ripening (Fig. 8). *Staphylococcus epidermidis* was found in the curd and tended to disappear as ripening progressed; the percentual compositions associated with this strain in the curd and in the three periods within the lactation season were similar (Fig. 8). Initial contamination of the curd by *Staph. simulans* was highest in the autumn and lowest in the spring. However, this bacterium proved to be more resistant in spring than in autumn (Fig. 8). This fact probably can be correlated with the higher pH in cheese and higher ripening temperatures observed in spring (Macedo *et al.* 1995). *Staphylococcus hominis* was found in the curd at very low proportions and could not be detected in the 35-d-old cheese.

Among the *Pseudomonas* species, *Ps. fluorescens* was the only one found in Serra cheese. Nuñez *et al.* (1984) have stated that *Pseudomonas* are the predominant psychrotrophic genus in refrigerated raw ewes' milk, although *H. alvei* was also found. For Serra cheese, *H. alvei* is more abundant than *Ps. fluorescens.*

Although the numbers of yeasts are 10^4-fold lower than those of LAB, they deserve special attention because some yeasts are able to synthesize lipolytic and proteolytic enzymes which may eventually contribute to the development of aroma and flavour during ripening. It is, however, well established that their major contribution to the ripening process is the utilization of lactic acid, which, by increasing the pH, encourages growth of the bacteria sensitive to acidic environments, and thus helps initiating the second stage of maturation. Since yeasts are ubiquitous in agricultural environments, a broad spectrum of yeasts was, as expected, found in the cheese samples analysed (Fig. 10). *Sporobolomyces roseus* and *Leucosporidium scottii*/*Debaryomyces hansenii* (lactose-utilizing yeasts) were the predominant yeasts in both the curd and the 35-d-old cheese, but their proportions were dependent on lactation season (Fig. 10); the highest proportions occurred in the spring and the autumn, respectively. Predominance of lactic acid-utilizing yeasts over lactose-utilizing yeasts in La Serena cheese were reported by Fernandez del Pozo *et al.* (1988). *Sporobolomyces roseus* was, on the other hand, more resistant and more actively growing in winter than in autumn and spring. This observation is in agreement with previous data (Macedo *et al.* 1995) indicating that the lowest pH values in cheese occur in winter; therefore, higher growth rates of lactic acid-utilizing yeasts possibly can be expected in winter because the pH variations in Serra cheese are due mainly to the formation of lactic acid. *Rhodotorula aurantiaca* was present in both the curd and the 35-d-old cheese although at low proportions, and was more resistant and proliferating in spring than in autumn. *Yarrowia lipolytica* was isolated in all curds and ripened cheeses except for the curd obtained in the autumn. The proportions of *Kluyveromyces marxianus* (a lactose-utilizing yeast) and *Pichia membranaefaciens* both in the curd and the 35-d-old cheese exhibited variations within the lactation season; these yeasts could be isolated in the 35-d-old cheeses made in the winter and spring but not in curds made in the autumn and spring. *Trichosporum beigelii* was found only in the curd, and at very low proportions (Fig. 10).

As final remarks, considering that *Leuc. lactis*, *L. lactis* ssp. *lactis* and *Lact. paracasei* ssp. *paracasei* were the most resistant and proliferating LAB found in this study, further research on the possibility of including these bacteria as part of a starter and on their effects on the organoleptic characteristics of Serra cheese seems very promising. Refrigeration of ewes' raw milk on the farm level should also be optimized in order to control the growth of *H. alvei.* The temperature and relative humidity conditions of ripening must also be set and controlled because they have an influence on the growth/death rates of various microorganisms which may impart relevant organoleptic characteristics to the final Serra cheese.

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