Research Article

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Characterisation of proteolysis profile of Argentinean sheep cheeses made by two different production methods

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Abstract

BACKGROUND: In this work the proteolysis profiles of Argentinean sheep cheeses made by two different production methods were studied in order to develop products with typical and defined features. Cheeses with a starter of *Streptococcus thermophilus*, curd cut to corn grain size, washed and heated to 43 °C (S cheeses) and cheeses with a mixed starter of *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus bulgaricus*, curd cut to rice grain size, unwashed and heated to 47 °C (L cheeses) were manufactured. The cheeses were ripened at 12 °C and 80% relative humidity for 180 days and samples were taken throughout this period.

RESULTS: Gross composition and primary proteolysis were similar for both types of cheeses. Streptococci counts diminished from 10^9 to 10^7 colony-forming units g^{-1} during ripening in both S and L cheeses. Lactobacilli counts in L cheeses decreased during ripening and disappeared at 180 days. L cheeses had significantly lower pH values and showed higher peptidolysis than S cheeses. Triangle sensory evaluation indicated important differences between the two types of cheeses.

CONCLUSION: S cheeses had a low proteolysis level and a soft flavour, making them appropriate for consumption after a short ripening time. L cheeses had a higher proteolysis level and more intense sensory characteristics, making them appropriate for consumption after a longer ripening time.

Keywords: sheep cheeses; production methods; proteolysis profile; sensory characteristics

INTRODUCTION

Although Argentina is one of the world's largest cow cheese-producing countries, its cheese production from sheep milk is very low. Traditionally, sheep cheeses have been home-made, but more recently they have also been produced on a small industrial scale.[1] In many regions of Argentina, sheep cheeses are considered a tourist attraction and are frequently available in fine restaurants and shops in larger cities.

At present, about 75 000 sheep are used for milk production in Argentina, but only 3200 belong to registered dairy industries.[1,2] Data on home-made sheep cheeses and those made on a limited industrial scale and traded mainly at local or regional markets are very difficult to obtain. In 2002, 56 milk farms with an average flock size of 150 individuals were recorded, mainly in the provinces of Buenos Aires and Chubut. The duration of the milking period is about 180 days from September to February and the average daily production is 0.8 L per animal.[2]

The principal sheep breeds in Argentina are crosses among different breeds from Europe, Asia and Africa. Pampinta is a native breed developed from Frisian and Corriedale breeds by Instituto Nacional de Tecnología Agropecuaria (INTA), Anguil, La Pampa, Argentina, an institution with wide expertise in the management of sheep flocks.[3]

In the period 2001–2002, 553 000 L of milk was processed to obtain 73 500 kg of sheep cheese. This production was significantly higher than that in the period 1996–1997, when only 39 000 kg of cheese was produced.[1,2]

The sheep cheese industry in Argentina is in its infancy. Any future expansion will depend on appropriate policies being developed and commercialisation problems being solved. In this respect, many government institutions are working together with cheese producers in order to improve the quality of sheep milk and cheeses. In addition, the growing tourist activity in Argentina can be an important factor in developing the sheep cheese industry. As a consequence, the development of standardised products of good and consistent quality is necessary. In our institute we have designed two production methods for sheep cheeses in order to obtain Argentinean products with typical and defined

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Table 1. Production methods of S and L cheeses

	S cheeses	L cheeses
Direct-to-vat starter (added to reach 10^6 CFU mL^{-1} in milk)	*S. thermophilus* 100%	*S. thermophilus* 60% *L. bulgaricus* 20% *L. helveticus* 20%
Milk coagulant	Maxiren® 150 (0.014 g L^{-1})	Maxiren® 150 (0.014 g L^{-1})
Curd particle size	Corn grain	Rice grain
Curd washing	10% of whey replaced by hot water (60 °C)	None
Rate of heating/final temperature	1 °C min^{-1}/43 °C	1 °C min^{-1}/47 °C

features.[4] The aim of this work was to characterise the proteolysis profiles of Argentinean sheep cheeses made by these two different production methods in order to define their identity on a scientific basis.

MATERIALS AND METHODS

Cheesemaking

All cheeses were made in the pilot plant of Instituto de Lactología Industrial (INLAIN) using raw milk from Pampinta sheep, provided by INTA. The milk was collected and transported to our plant on the same day under refrigerated conditions (4 °C). On each cheesemaking day, 40 L of milk was batch pasteurised at 65 °C for 20 min. After cooling to 37 °C, $CaCl_2$ (Merck, Darmstadt, Germany) was added to a final concentration of 0.2 g L^{-1}. The milk was then divided into two vats of 20 L each in order to manufacture simultaneously two types of cheeses according to the following production methods (Table 1).

In production method 1 a commercial lyophilised direct-to-vat culture composed of selected strains of *Streptococcus thermophilus* (Chr. Hansen, Hørsholm, Denmark) was used as primary starter. The culture was dispersed in a small quantity of pasteurised milk and added to the cheese milk in order to obtain a count of 10^6 colony-forming units (CFU) mL^{-1}. After 15 min, 0.014 g L^{-1} chymosin obtained by fermentation (Maxiren® 150, Gist-Brocades, Seclin, France) was added to the vat. When the curd had reached the desired firmness, it was cut to corn grain size. The curd was then washed by replacing 10% of the whey with hot water (60 °C) under gentle stirring. The resultant mixture (~40 °C) was slowly heated (1 °C min^{-1}) by indirect steam to 43 °C in order to reduce the water content. When the curd particles had reached the desired moisture level, the stirring was stopped and the whey was drained and discarded. The curd was then placed in cylindrical moulds (9 cm height, 10 cm diameter) and pressed (0.2–0.3 kg cm^{-2}) for 24 h. Cheeses obtained by this method were identified as S cheeses.

In production method 2 a starter composed of a mixture of *S. thermophilus* (60%), *Lactobacillus bulgaricus* (20%) and *Lactobacillus helveticus* (20%) (Chr. Hansen) was used. The dose employed was sufficient to reach a final count of 10^6 CFU mL^{-1} in the cheese milk. The coagulation step was carried out as in production method 1. When the curd had reached the required firmness, it was cut to rice grain size, i.e. smaller than that for S cheeses. The mixture of curd particles and whey was then gently stirred and heated at a rate of 1 °C min^{-1} to 47 °C by indirect steam (without the washing step) in order to reduce the moisture content of the curd to the desired level. Finally, moulding and pressing of the curd were performed as in production method 1. These cheeses were identified as L cheeses.

Both S and L cheeses were salted by brining in 200 g L^{-1} NaCl solution (pH 5.4) at 12 °C for 7 h (each cheese weighed approximately 700 g). Ripening was carried out at 12 °C and 80% relative humidity for 6 months.

Six replicates of each cheese type were prepared during the period November 2005–May 2006.

Milk analysis

The cheesemaking milk was analysed for its protein and fat contents according to International Dairy Federation (IDF) standard methods.[5,6] All analyses were carried out in duplicate.

Cheese analysis

Proximate composition and pH of cheeses

Protein and fat contents were analysed according to IDF standard methods[5,6] in cheeses after 2 days of ripening. Dry matter content was determined according to the IDF standard method[7] in 2- and 180-day-old cheeses. pH was evaluated with a pH meter (Horiba, Kyoto, Japon) according to the American Public Health Association (APHA) standard method[8] at 2, 45, 90 and 180 days of ripening.

Microbiological analysis

Primary starter counts were determined at 2, 45, 90 and 180 days of ripening according to APHA standard methods.[9] *Streptococcus thermophilus* was enumerated on skim milk agar (SMA) after 48 h of incubation at 37 °C. Lactobacilli populations were determined on de Man–Rogosa–Sharpe (MRS) agar after 96 h of incubation at 37 °C.

Proteolysis assessment

Proteolysis was assessed at 2, 45, 90 and 180 days of ripening by determination of soluble nitrogen at pH 4.6 (SN-pH 4.6), in 120 g L^{-1} trichloroacetic acid (SN-TCA) and in 25 g L^{-1} phosphotungstic acid (SN-PTA). Peptide profiling of water-soluble cheese extracts and electrophoresis of insoluble nitrogen fractions at pH 4.6 were also performed.

Soluble nitrogen. Cheese samples were treated to obtain crude citrate extracts and soluble fractions at pH 4.6 (SN-pH 4.6), in 120 g L^{-1} trichloroacetic acid (SN-TCA) and in 25 g L^{-1} phosphotungstic acid (SN-PTA) according to Hynes *et al.*[10] Grated cheese (10 g) was mixed with 20 mL of 0.5 mol L^{-1} sodium citrate solution and then homogenised using a mortar and pestle. Deionised water was added to a volume of about 90 mL and the pH was adjusted to 4.6. After centrifugation at 3000 × *g* for 15 min the soluble fraction volume was adjusted to 100 mL. SN-TCA and SN-PTA fractions were obtained from SN-pH 4.6 fractions according to

Table 2. Proximate composition and pH of S and L cheeses

	Dry matter (g kg^{-1})		Fat (g kg^{-1})	TP (g kg^{-1})	pH			
Cheeses	2 days	180 days	2 days	2 days	2 days	45 days	90 days	180 days
S	572 ± 10a	712 ± 16a	302 ± 17a	218 ± 13a	5.47 ± 0.28a	5.35 ± 0.06a	5.47 ± 0.08a	5.46 ± 0.08a
L	573 ± 23a	709 ± 14a	300 ± 26a	231 ± 9a	5.07 ± 0.17b	4.95 ± 0.05b	5.03 ± 0.09b	5.13 ± 0.10b

TP, total protein.
Means in a column with different letters differ significantly ($P < 0.05$).

Gripon *et al*.[11] The nitrogen content was determined in duplicate by the macro-Kjeldahl method.[5]

Electrophoresis. Primary proteolysis was assessed by electrophoresis. Samples of cheeses were prepared by precipitation at pH 4.6 and purified. The insoluble residue at pH 4.6 was analysed by urea polyacrylamide gel electrophoresis (urea-PAGE) in a Mini-Protean II cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Andrews.[12] The acrylamide concentration was 75 g L^{-1}.[13] Proteins were stained with Coomassie Blue G-250.

Peptide profiles. Peptide profiles were analysed by means of reverse phase high-performance liquid chromatography (RP-HPLC). The HPLC equipment consisted of a Series 200 chromatograph, quaternary pump, online degasser and UV–visible detector (Perkin Elmer, Norwalk, CT, USA). An interface module connected to a computer was used for acquisition of chromatographic data with the software Turbochrom (Perkin Elmer). A 220 mm × 4.6 mm Aquapore OD-300 C18, 5 nm, 300 Å analytical column (Perkin Elmer) was used. Water-soluble extracts of the cheeses were obtained by blending 5 g of cheese and 15 mL of distilled water with a mortar and pestle, then heated to 40 °C and maintained at that temperature for 1 h. The suspension was centrifuged at 3000 × *g* and filtered through fast flow filter paper. The filtered solution was adjusted to a final volume of 25 mL. Samples were filtered through 0.45 µm Millex membranes (Millipore, São Paulo, Brazil), and 60 µL aliquots were injected into the chromatograph. Detection was performed at 214 nm and the column temperature was 40 °C. The column was equilibrated initially with 100% solvent A (water/trifluoroacetic acid (TFA), 1000 : 1.1 v/v). After 10 min of injection a gradient was generated by increasing the concentration of solvent B (acetonitrile/water/TFA, 600 : 400:1 v/v/v) from 0 to 80% over 80 min, then from 80 to 100% over 1 min and finally maintaining 100% B for 4 min. The column was then returned to the starting conditions over 1 min. These last setting conditions were maintained for 10 min.

Sensory evaluation

A panel of 12 untrained subjects performed a triangle test to determine whether sensory differences existed between S and L cheeses at 45 days of ripening. This method is particularly useful in situations where treatment effects may have produced changes in a product that cannot be characterised simply by one or two attributes.

Cheese samples were prepared in equal numbers of the six possible combinations (LSS, SLL, LLS, SSL, LSL and SLS) and presented at random to the subjects. The subjects were asked to examine the samples in order from left to right and select the different sample.

Statistical analysis

Statistical analysis was performed with SPSS 10.0 (SPSS Inc., Chicago, Il, USA). Data were processed by one-way analysis of variance (ANOVA), and means were compared by the least significant difference (LSD) test when differences were found.[14]

Peptide profiles were analysed by principal component analysis (PCA), a multivariate technique that reduces the number of original variables to a smaller number of unobservable variables called principal components (PCs), which are linear combinations of the original variables.[15] Chromatograms were preprocessed by a fuzzy approach in order to reduce the number of peaks for subsequent PCA. Processed data consisted of classes of retention time wherein peak areas were accumulated by using the distance from the centre of the class as a weight. The fuzzy approach provides a fast, reliable and objective methodology for selecting variables from chromatograms for chemometric analysis.[16]

RESULTS AND DISCUSSION

Properties of cheese milk

The mean composition of the raw milk used in cheesemaking was total protein 53.7 ± 3.5 g kg^{-1}, fat 76 ± 22 g kg^{-1}, total solids 193 ± 34 g kg^{-1}, pH 6.4 ± 0.1 and Dornic acidity 22.4 ± 2.5 °D. Levels of total protein, fat and total solids were similar to the mean values of the average composition of different sheep milks.[17]

Proximate composition and pH of cheeses

Values of dry matter, fat, total protein and pH for the cheeses manufactured by both production methods are presented in Table 2.

As can be seen, no differences ($P > 0.05$) were found in dry matter, fat and total protein between the cheeses made by different production methods. Nevertheless, both types of cheeses showed an important loss of moisture (around 68%) during ripening, which has also been observed in other sheep cheeses with a long ripening time, such as Idiazabal,[18] Manchego[19] and Roncal.[20]

Regarding pH, L cheeses showed values significantly lower than those of S cheeses throughout ripening. These results suggest a higher acidifying activity of lactobacilli of the starter in L cheeses.

Dry matter content and pH of S and L cheeses at the beginning as well as at the end of ripening were comparable to those reported for other sheep cheeses such as Manchego,[19] Roncal[21] and Idiazabal,[18] while fat and total protein contents were lower than those reported for these cheeses. However, fat content was similar to levels reported in Kefalotiri and Halloumi cheeses at the end of ripening.[21]

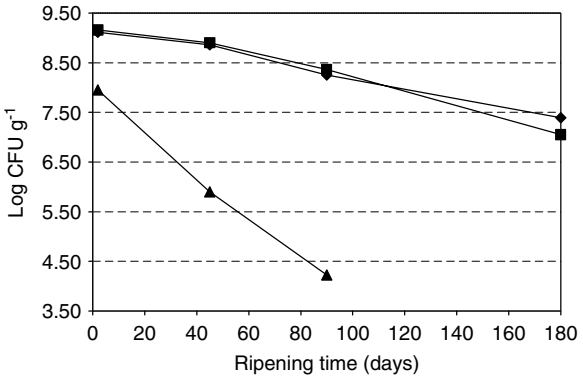

Figure 1. Evolution of primary starter in S and L cheeses during ripening: ■, *Streptococcus thermophilus* in S cheeses; ♦, *S. thermophilus* in L cheeses; ▲, lactobacilli in L cheeses.

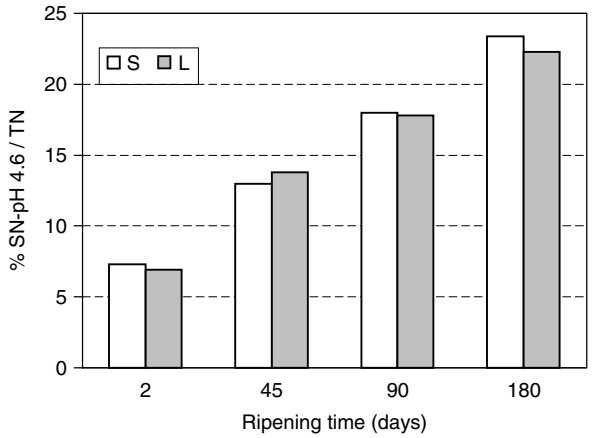

Figure 2. Soluble nitrogen at pH 4.6 (SN-pH 4.6), expressed as % of total nitrogen (TN), in S and L cheeses during ripening.

Microbiological properties of cheeses

The evolution of primary starter populations during ripening is presented in Fig. 1. In both types of cheeses, initial streptococci counts were about 10^9 CFU g^{-1}, but this population decreased by one and two log orders at 90 and 180 days respectively. The streptococci population did not show significant differences between S and L cheeses ($P > 0.05$).

As for the starter lactobacilli population in L cheeses, it diminished significantly from the beginning (10^8 CFU g^{-1}) to 90 days (10^4 CFU g^{-1}), while no lactobacilli were detected at 180 days. Although we did not study autolysis, these results suggest that this decrease in lactobacilli population in L cheeses probably took place along with their autolysis. In fact, several strains of thermophilic lactobacilli have shown autolytic properties in the cheese matrix during ripening.[22,23]

Proteolysis assessment

Soluble nitrogen

SN-pH 4.6 increased during ripening, but there were no significant differences ($P > 0.05$) between S and L cheeses (Fig. 2). This was not surprising, because the SN-pH 4.6 fraction reflects mainly primary proteolysis, especially that due to plasmin and possibly residual or reactivated chymosin. This fraction is composed of peptides and smaller-sized nitrogen compounds such as amines/urea and ammonium.[24,25] On the other hand, although different strains of *L. helveticus* have demonstrated the ability to attack α_s and β caseins through their cell wall proteases, this activity was only verified *in vitro*.[26,27]

In contrast, the SN-TCA and SN-PTA fractions showed significant differences ($P < 0.05$) between L and S cheeses throughout the ripening period. Values of both fractions were higher in L cheeses than in S cheeses. This difference was more noticeable for the SN-PTA fraction, whose level in L cheeses was twice that in S cheeses from 45 days until the end of ripening (Fig. 3).

The diverse components of the SN-TCA and SN-PTA fractions, such as small peptides and free amino acids, are related to the proteolytic activity of the starter bacteria. Thus the results obtained reveal a stronger peptidase activity of lactobacilli of the starter in L cheeses in comparison with streptococci added to S cheeses. Generally, it is assumed that bacterial peptidases can work only after being released into the cheese matrix as a result of cell lysis.[22] Although we did not investigate bacterial lysis, this result reinforces the hypothesis that a decrease in lactobacilli population would come along with cell lysis. Nevertheless, further research on this subject is needed.

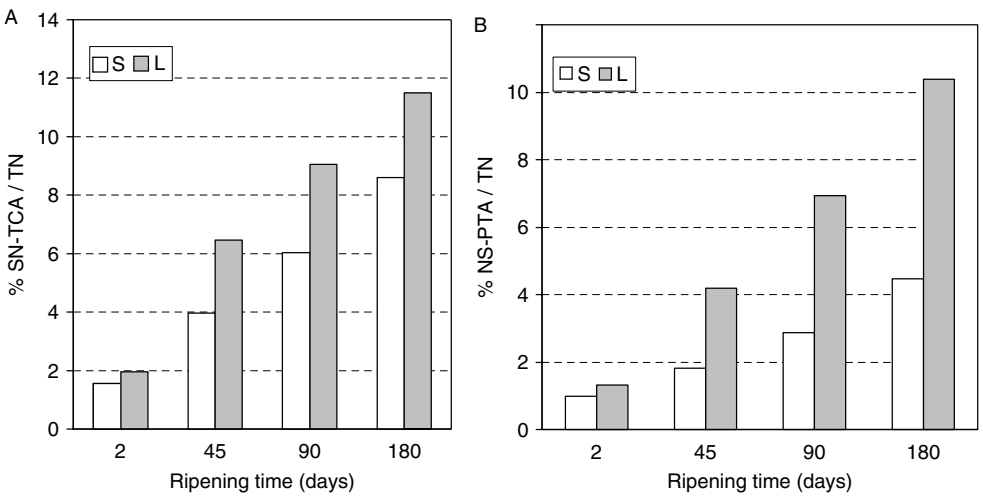

Figure 3. Soluble nitrogen in (A) 120 g L^{-1} trichloroacetic acid (SN-TCA) and (B) 25 g L^{-1} phosphotungstic acid (SN-PTA), expressed as % of total nitrogen (TN), in S and L cheeses during ripening.

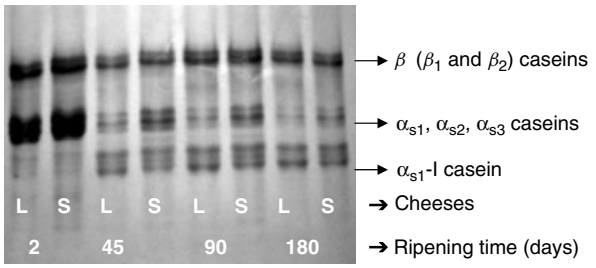

Figure 4. Urea polyacrylamide gel electrophoresis of insoluble residue at pH 4.6 in L and S cheeses at 2, 45, 90 and 180 days of ripening.

Electrophoresis

Typical electrophoretic patterns for the insoluble nitrogen fraction at pH 4.6 are presented in Fig. 4. It can be seen that β (β_1 and β_2) caseins did not differ significantly between L and S cheeses, and this fraction remained almost intact throughout ripening.

Mendia *et al.*[18] and Ferreira *et al.*[28] obtained the same result for Idiazabal and Terrincho cheeses respectively. However, different levels of β casein hydrolysis have been reported for others types of sheep cheeses. This situation has been mainly attributed to the kind and origin of the milk coagulant used and to the activity of plasmin, which increases after heating of the curd.[20,29] In our experience the action of plasmin (milk alkaline protease) is negligible, because the pH of cheeses (5.0–5.4) is considerably lower than its optimal pH (7.5).[30]

In contrast to other protein fractions, α_{s1} casein showed extensive hydrolysis, because it is easily attacked by chymosin.[31] Effectively, the primary site of chymosin action on α_{s1} casein is the Phe_{23}–Val_{24} bond, releasing the peptide α_{s1} CN (f1-23) and α_{s1}-I casein,[32] whose presence was detected at 2 days of ripening in both types of cheeses (Fig. 4). The hydrolysis of α_{s1} casein was higher in L cheeses from the beginning of ripening. This might be explained on the basis of three hypotheses: (i) it is probable that a lower amount of coagulant remained in S

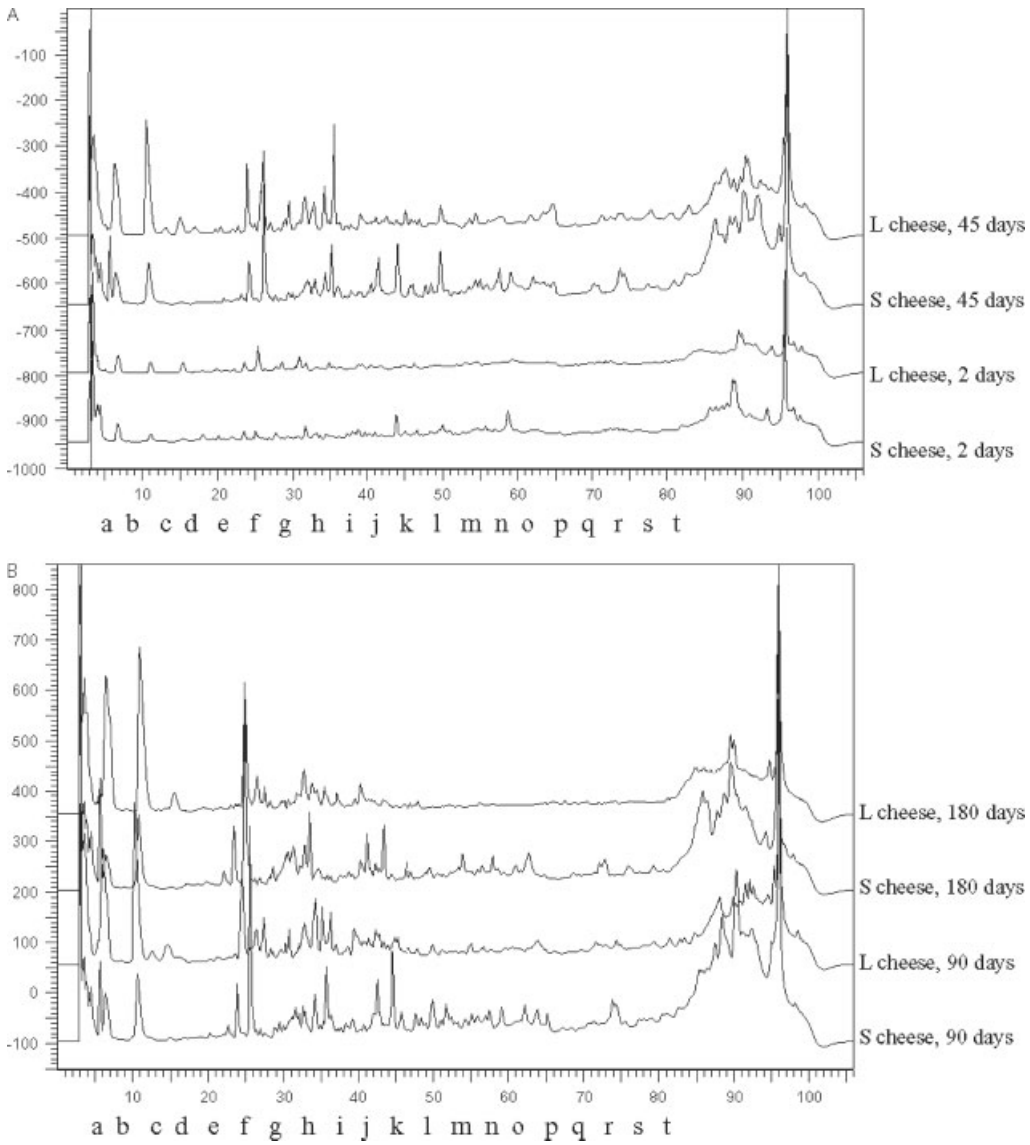

Figure 5. Reverse phase liquid chromatography profiles of water-soluble extract of L and S cheeses during ripening: A, 2 and 45 days; B, 90 and 180 days. Letters a–t denote the peaks selected for principal component analysis and correspond to the following retention times: 5.0, 9.1, 13.1, 17.2, 21.2, 25.3, 29.3, 33.4, 37.4, 41.5, 45.5, 49.6, 53.6, 57.7, 61.7, 65.8, 68.8, 73.9, 77.9, 82.0 min respectively.

cheeses than in L cheeses owing to the washing step; (ii) the pH of L cheeses was lower and thus closer to the optimal pH of chymosin (pH∼4.0); (iii) the accumulation of α_{s1}-I casein in S cheeses, as a result of it not being broken down by peptidases of lactic bacteria, could inhibit or delay the hydrolysis of α_{s1} casein.[24]

Peptide profiles

S cheeses showed a gradual increase in peptide profile peaks during ripening. In contrast, most peaks in the peptide profiles of L cheeses showed an increase followed by a diminution at 180 days of ripening, probably due to peptide hydrolysis and the consequent release of free amino acids (Fig. 5).

In general, the area of peaks in the middle and final regions of the chromatographic profiles of L cheeses was lower than that of S cheeses. The opposite situation was seen in the initial region, where higher peaks were observed in the peptide profiles of L cheeses.

In addition to their visual analysis, the chromatographic profiles were analysed by a multivariate method. In the present work the chromatographic profiles were divided into 20 classes of retention time, which were identified with letters a–t in alphabetical order (Fig. 5). The areas of these classes were considered as entry variables for PCA, with standardisation to a mean of zero and their original variances (covariance matrix). Three PCs with an eigenvalue higher than the mean of eigenvalues were extracted, which explained 84.1% of the total variance. Scores and loading plots for PC1 *vs* PC2 are presented in Fig. 6.

In the score plot it was observed that all samples were very similar at the beginning of ripening (2 days). In contrast, 45-, 90- and 180-day-old cheese samples showed a higher variability according to the type of cheese and ripening time. Among these samples, S cheeses were grouped separately from L cheeses, principally along PC1, which extracted more than half of the total variance (53.3%). In effect, L cheeses presented positive scores for this PC, while S cheeses in general showed negative scores (Fig. 6A). Classes with the lowest retention time characterised L cheeses, while those with a higher retention time typified S cheeses (Fig. 6B). These results are in agreement with the visual analysis. Peptides that elute in the initial region of chromatograms are considered hydrophilic because of their elution at low acetonitrile concentration. In addition, aromatic free amino acids (Tyr, Phe and Trp) elute in this initial region. Conversely, peptides with higher retention time are considered hydrophobic owing to their elution at high acetonitrile concentration. Thus hydrophilic and hydrophobic peptides characterised L and S cheeses respectively. A decrease in the hydrophobic/hydrophilic peptide ratio has been reported as a characteristic change during ripening for several types of cheeses.[33–35] This fact is considered favourable, because hydrophobic peptides have been associated with bitter taste.[36] Thus the results obtained for L cheeses are consistent with an acceleration of ripening and a potential debittering effect.

In addition to the differentiation by type of cheese, a separation according to the ripening time was also observed along PC1 (Fig. 6A). In effect, sample scores of both types of cheeses were higher as the ripening time increased, with the exception of 180-day-old L cheese samples. These samples were differentiated from 45- and 90-day-old L cheese samples along PC2 and showed negative scores on this axis, as did 2-day-old cheese samples. These results coincide with those observed by

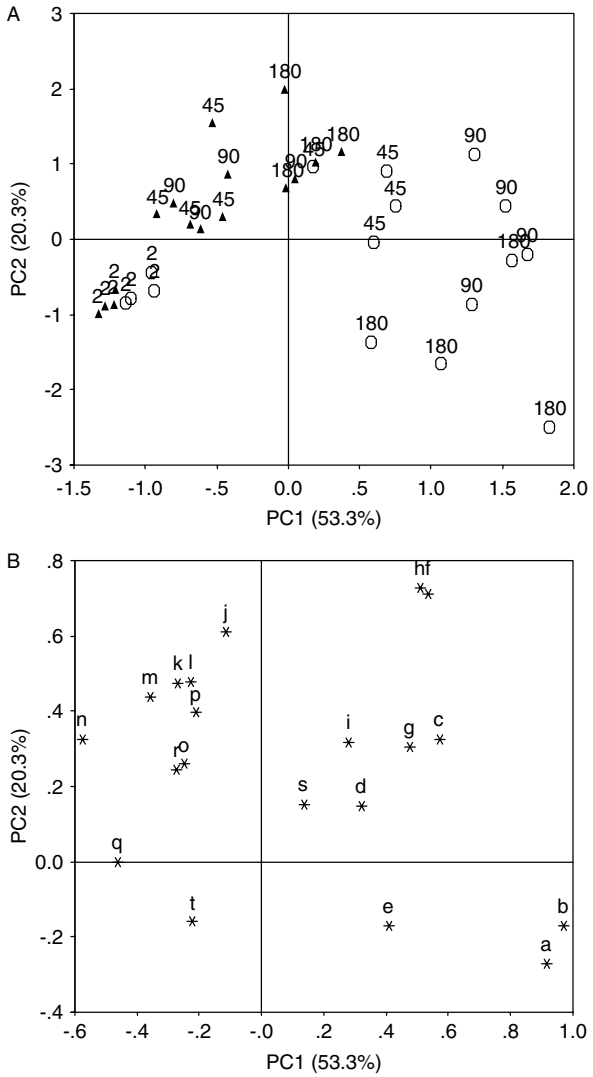

Figure 6. Principal component analysis (PCA) of soluble peptide profiles of S (▲) and L (○) cheeses. (A) Score plot for PC1 *vs* PC2. The labels of sample scores indicate their ripening time (days). (B) Loading plot for PC1 *vs* PC2. The labels of loading indicate the identification of classes of retention time of chromatograms used for PCA.

visual analysis of the chromatograms, indicating that soluble peptides of these cheeses reach a maximum level and then decrease.

Finally, S cheese samples at the end of ripening were close to 45-day-old L cheese samples in the score plot (Fig. 6A). These results also showed a higher proteolysis and acceleration of ripening in L cheeses compared with S cheeses, probably produced by the activity of proteolytic enzymes released by *L. helveticus* and *L. bulgaricus*. Similarly, other authors have demonstrated an increase in proteolysis and acceleration of ripening in cheeses with the addition of different strains of *L. helveticus*.[23,37]

Sensory evaluation

The triangle test performed at 45 days of ripening with an untrained panel of 12 subjects showed significant differences between S and L cheeses ($P < 0.01$). Eleven subjects correctly identified the different sample and described the flavour charac-

teristics of L cheeses as more intense than those of S cheeses. These sensory appreciations agree very well with the differences reported previously in microbiological and physicochemical characteristics.

CONCLUSIONS

This work has provided information on the ripening process of sheep cheeses made by two different production methods, currently lacking in Argentina. Differences in the starters used in cheesemaking were noticeable in the production of SN-TCA and SN-PTA fractions. Values for both fractions were higher in L cheeses, bacterial lysis of the lactobacilli population being the reason for this situation. Electrophoretic profiles showed higher hydrolysis of α_{s1} casein in L cheeses, because S cheeses retained a lower level of coagulant as a consequence of washing and their higher pH. PCA of peptide profiles also showed evident differences between S and L cheeses; higher peptidolysis was verified in L cheeses. Results of the sensory test demonstrated more intense flavour development in L cheeses than in S cheeses.

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