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Influence of probiotic bacteria on the proteolysis profile of a semi-hard cheese

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Abstract

Two probiotic strains, *Lactobacillus acidophilus* and *Lactobacillus paracasei* subsp. *paracasei*, were used as adjunct cultures in semi-hard cheesemaking experiments, in order to study their influence on proteolysis during ripening. Cheeses with and without probiotic bacteria were manufactured. The population of probiotics remained above 10^7 cfu g^{-1} during all ripening, and they did not influence primary proteolysis. However, *L. acidophilus* produced a significant increase in the level of low molecular weight nitrogen compounds and individual free amino acids; the amino acid profiles were also different. Multivariate analysis of peptide profiles showed that samples were grouped mainly by ripening time, although the impact of probiotics was also noticeable. *L. acidophilus* showed a clear influence on secondary proteolysis, while a minor effect of *L. paracasei* was evidenced at the end of the ripening. These results showed that the tested strains influenced distinctly proteolysis of cheeses, probably as a consequence of their different proteolytic systems and their activity via the alimentary matrix (cheese).

Keywords: Probiotic bacteria; Adjunct culture; Cheese ripening; Proteolysis

1. Introduction

During recent years, consumers have become very interested about safety and quality of food products. Most consumers are concerned not only about food's safety and nutritional value, but also about its beneficial effects on health (Saarela, Lähteenmäki, Crittenden, Salminen, & Mattila-Sandholm, 2002). Precisely, these attributes describe functional foods, a new category in which probiotic products are included (Playne, Bennet, & Smithers, 2003). Guarner and Schaafsma (1998) have defined probiotic bacteria as "living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". There are different studies in humans that provide evidence about health effects of probiotics. Some of these are: relief of lactose maldigestion symptoms, shortening of rotavirus diarrhoea, immune modulation, and suppression of *Helicobacter pylori*, among others (Ouwehand et al., 2003; Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). However, in general, these effects are documented only for certain strains and more research is needed on this topic (Saxelin et al., 2005). On the other hand, there is fair agreement about a minimal concentration of probiotic of 10^7 cfu g^{-1} or mL^{-1} of food that should be present at the moment of the intake, to assure a favourable impact on consumer's health (De Vuyst, 2000).

Fermented dairy products enriched with probiotic bacteria are one of the most studied and optimised functional foods (Saxelin et al., 2005). Among these, cheese has been suggested as a better carrier product to deliver probiotic bacteria than fermented milk. The higher pH and fat content, and the solid matrix of cheese, may protect bacteria more efficiently than a fluid environment during the storage of the food and its transit through the human body (Ross, Fitzgerald, Collins, & Stanton, 2002).

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Besides, cheese is a lactose-free food, which represents an advantage over other dairy products, as many consumers are lactose-intolerant. Probiotic bacteria, such as lactobacilli, bifidobacteria and enterococci, have been incorporated into different cheese varieties: Gouda (Gomes, Malcata, Klaver, & Grande, 1995), Argentinean fresco cheese (Vinderola, Prosello, Ghiberto, & Reinheimer, 2000), white cheese (Kasimoğlu, Göncüoğlu, & Akgün, 2004), Cheddar (Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998), Cottage (Blanchette, Roy, Bélanger, & Gauthier, 1996), and Crescenza (Gobbetti, Corsetti, Smacchi, Zocchetti, & De Angelis, 1998). Most publications concerning incorporation of probiotic bacteria into cheeses have focused on their survival during manufacture and storage, but few studies have considered the effect of this incorporation on cheese composition and quality. Besides, most studies have investigated the effect of bifidobacteria strains (alone or mixed with lactobacilli strains) in cheeses manufactured with mesophilic starters (Ross et al., 2002). There is little information about probiotic cheeses manufactured with thermophilic starter cultures, which are the most widely used in Argentina. Finally, there are few studies about the addition of probiotic strains of *Lactobacillus* spp. to cheeses without the concurrent addition of bifidobacteria.

Proteolysis in cheese during ripening is an important process, as it plays a direct role on cheese flavour and texture development in most cheese varieties (Sousa, Ardö, & McSweeney, 2001). Proteinases and peptidases from different origin catalyse this process: residual coagulant, milk, starter and non-starter lactic acid bacteria, and adjunct cultures. Lactic acid bacteria possess a very comprehensive proteolytic enzymatic system, because of their complex amino acids requirements (McSweeney, 2004). Several authors have demonstrated that lactobacilli are lactic acid bacteria capable of impacting on proteolysis of different cheeses (Hynes et al., 2003; Madkor, Tong, & El Soda, 2000; Poveda, Sousa, Cabezas, & McSweeney, 2003).

During the last few years Argentina produced about 130,000 t $year^{-1}$ of semi-hard cheeses (http://www.cil.org.ar), among which Pategrás Argentino is the most important variety. Pategrás Argentino was developed by European immigrants in late 19th and early 20th centuries, inspired by similar French and Italian cheeses, but then it was modified and adapted during the last century to Argentinean raw materials and environmental conditions, to give a distinctive product. Starter cultures for Pategrás Argentino may be either commercial starters composed of *Streptococcus thermophilus* strains or "wild" cultures obtained by incubating good-quality thermised milk. Coagulant enzyme has traditionally been the extract from adult bovine stomach, but since the 1990s, fermentation-produced chymosin obtained from genetically modified organisms has almost completely replaced it. Pategrás Argentino cheese and its ripening process have been characterised in previous research studies (Tesone, Martinez, & Quevedo, 1981; Zalazar, Meinardi, Bernal, & Candioti, 1988; Zalazar, Meinardi, & Hynes, 1999; Zalazar, Meinardi, Reinheimer, Candioti, & Bernal, 1985). So far, probiotic cultures have not been added to this cheese variety.

The objective of the present study was to assess the impact of the biochemical expression of two probiotic strains of *Lactobacillus*, added by two different methodologies, on the proteolysis profile of a semi-hard Argentinean cheese.

2. Materials and methods

2.1. Cheese manufacture

Two different probiotic lactobacilli strains were tested as adjunct cultures in separate cheesemaking trials.

In each trial three types of cheeses were made: one control cheese and two experimental cheeses. Control cheeses (C) did not contain probiotic bacteria. In the first experimental cheese (EL), probiotic bacteria were added lyophilised (previously dispersed in a small amount of pasteurised milk), while in the second (EP) probiotic bacteria were added after pre-incubation in a substrate composed by milk and milk fat. The purpose of testing two different methodologies of addition of the culture was to investigate the possibility of improving probiotics' viability in cheese via pre-incubation in a substrate. As a consequence of the potential differences in viability and physiological state of the culture at the moment of the addition, changes might arise in the proteolysis patterns, so EL and EP cheeses were both compared to control cheeses and between themselves. Three cheese replicates were made on different cheesemaking days.

Cheesemaking was performed at pilot plant scale, by adapting the industrial technology for the semi-hard cheese Pategrás Argentino (Zalazar et al., 1999). Raw milk, obtained from a nearby dairy factory (Milkaut S.A., Franck, Santa Fe, Argentina), was batch pasteurised at 65 °C for 20 min, and cooled to 37 °C. Calcium chloride 1.6 M (Merck, Darmstadt, Germany) was added to a final concentration of 0.02% (w/v). After that, milk was divided in three aliquots of 45 L each. Fat concentration was standardised at 3.8% (w/v) for control and EL cheeses and at 3.49% (w/v) for EP cheese, because the addition of the substrate increased the fat concentration to a final content of 3.8% (w/v). In addition, the same amount of milk powder used in the preparation of the substrate was added to cheesemilk for C and EL cheeses, in order to obtain the same non-fat solid content in all cheeses. A lyophilised culture of *S. thermophilus* (Diagramma, Santa Fe, Argentina) was used as primary starter; it was dispersed in approximately 100 mL of pasteurised milk and maintained for 5–10 min at 37 °C before addition to cheesemilk. After that, adjunct probiotic starter was added in both types of experimental cheeses, according to the addition methodology explained above. All the lactic bacteria (starter and

adjunct) were added in a dose high enough to achieve 10^6 cfu mL^{-1} in cheesemilk. After 15 min, 1 g of chymosin produced by fermentation of genetically modified *Kluyveromyces lactis* (Maxiren 150, Gist Brocades, France) was dispersed in 25 mL of distilled water and added to the vat. When the curd reached the appropriated strength, which was assessed empirically by testing its hardness with a spatula, it was cut in successive steps (with manual stirring between steps) until it reached the size of a corn grain (at 37 °C—approx. 20 min). The mixture of curd particles and whey was gently stirred and heated at the rate of 0.5 °C min^{-1} until 45 °C for 15–20 min approximately, in order to reduce the moisture content of curd grains.

After that, the curd was separated from whey and moulded. The three moulds were piled and pressed during 24 h (0.2–0.3 kg cm^{-2}). Young cheeses were brined in 20% (w/v) pH 5.4 brine for 24 h and ripened for 2 months at 12 °C and 80% relative humidity.

2.2. Probiotic cultures

Lyophilised commercial cultures of two *Lactobacillus* species were used. In trial 1, a strain of *Lactobacillus acidophilus* was studied, whereas in trial 2 a strain of *Lactobacillus paracasei* subsp. *paracasei* was tested. The cultures were available in the Argentinean market; the companies that provide them will not be mentioned for confidentiality reasons. However, we are able to state that suppliers claimed both strains to be probiotic, from human intestinal origin and showing survival in conditions of the gastrointestinal tract.

The substrate used for addition of probiotic bacteria to EP cheeses was prepared according to Bergamini, Hynes, Quiberoni, Suárez, and Zalazar (2005). Water was added to reach a final volume of 1 L to a mixture of 147.3 g of skim milk powder (Molico, Nestlé Argentina, Buenos Aires, Argentina) and 350 g of raw, freshly obtained cream, 40% fat (w/w) (Milkaut S.A., Franck, Argentina) to reach a final concentration of 14% (w/v) for fat and 5.2% (w/v) for proteins. The substrate was treated at 80 °C during 5 min and then cooled at 37 °C. Probiotic culture was added to this substrate to attain approximately 5×10^7 cfu mL^{-1}. The inoculated substrate was incubated at 37 °C for 5 h and then stored at 4 °C until the next day, when it was used to make EP cheese.

2.3. Fat substrate analysis

During the incubation and cold storage of the substrate, pH values and probiotic bacteria plate counts were determined at 0, 2, 5 and 20 h.

The population of lactobacilli present in the fat substrate was determined by plating sample dilutions on MRS agar and counting plate colonies after 48 h of incubation at 37 °C (Bergamini et al., 2005).

2.4. Gross composition and microbiology of cheeses

Gross composition was determined in 3-day old cheeses, except for NaCl, in which case 30-day old cheeses were analysed, and for pH, which was also monitored at 30 and 60 days of ripening. Dry matter was analysed by drying the sample at 105 °C until constant weight according to IDF standards (IDF, 1982). Fat matter (by Gerber's method) and pH were determined according to standard of American Public Health Association (APHA) (Bradley et al., 1993). Protein content was determined by the Kjeldahl method according to IDF standards (IDF, 1993). Sodium chloride content was analysed following a standard spectrophotometric method (AOAC, 1990).

The population of lactobacilli present in cheeses after 0 (fresh curd), 3 (curd after press), 15, 30, 45 and 60 days of ripening was determined by plating sample dilutions on MRS agar and counting plate colonies after 48 h of incubation at 37 °C, according to Bergamini et al. (2005). Similarly, the population of streptococci was determined on Skim Milk Agar (SMA). Even though the selected culture media were not specific (both streptococci and lactobacilli were able to grow on them), we tested that they were selective as lactobacilli and streptococci yielded different colonies (Bergamini et al., 2005).

2.5. Proteolysis assessment

Proteolysis was assessed on 3-, 30- and 60-day old cheeses by techniques detailed below.

2.5.1. Soluble nitrogen (SN)

Cheese samples were treated to obtain crude citrate extract and soluble fractions at pH 4.6 (SN—pH 4.6), in 12% (w/v) trichloroacetic acid (SN-TCA) and in 2.5% (w/v) phosphotungstic acid (SN-PTA). The extract was obtained by adding 20 mL of sodium citrate 0.5 M to 10 g of cheese and grounding to homogeneity using a pestle. Deionised water was added to ~90 mL and the pH was adjusted to 4.6 with HCl 3 N. After centrifugation ($3000 \times g$, 15 min), the soluble fraction volume was adjusted to 100 mL. SN-TCA and SN-PTA were obtained from SN—pH 4.6 according to Gripon, Desmazeaud, Le Bars, and Bergère (1975). The nitrogen content in each fraction was determined by the macro-Kjeldahl method.

2.5.2. Electrophoresis

Cheese N fraction insoluble at pH 4.6 was analysed by Urea-Polyacrylamide Gel Electrophoresis (Urea-PAGE) in a Mini-Protean II cube (BioRad Laboratories, California, USA) according to the Andrews (1983) method, onto a 7.5% (w/v) acrylamide gel. Proteins were stained with Coomassie Blue G-250.

2.5.3. Free amino acid (FAA) analysis

Free amino acid analysis was applied only when significant differences in SN-PTA were found, as the latter is an

index of total FAA (Ardö, 1999). Five grams of cheese was homogenised with 5 mL of distilled water at room temperature by blending with morter and pestle. After that, 10 mL of 20% TCA (w/v) was added and the mixture was homogenised and centrifuged at $5000 \times g$ for 10 min. The supernatant was filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil) and then the filtrate was diluted (1:2) using borate buffer pH 10. Amino acids were derivatised with *o*-phthalaldehyde-3-mercaptopropionic acid (OPA) and 25 μL of derivative sample was injected into the HPLC chromatograph. Liquid chromatography was performed in a Gilson system provided with a fluorimeter model 121 (Gilson Medical Electronics, Inc., Middleton, WI, USA). Chromatographic separation was achieved by linear gradient elution from 0% to 85% B solvent in 70 min, on a reverse phase Spherisorb ODS-2, 5 μm C_{18} column (250×4.6 mm), with a flow of 1.0 mL min^{-1}. Solvents used for the separation were: A: methanol, 10 mM sodium phosphate buffer, pH 7.3, and tetrahydrofuran (19:80:1), and B: methanol and 10 mM sodium phosphate buffer, pH 7.3 (80:20). For detection, wavelength was set by means of filters: 305–395 nm was used for excitation and 430–470 nm for emission.

2.5.4. Reverse phase-high performance liquid chromatography (RP-HPLC)

The HPLC equipment consisted of a quaternary pump, an on-line degasser and UV/VIS detector, all Series 200, purchased from 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 x 4.6 mm Aquapore OD-300 C18, 7 μm – 300 Å analytical column was used (Perkin Elmer). Water-soluble extracts of the cheeses were obtained by blending 5 g of cheese and 15 mL of distilled water with mortar and pestle, then warmed up to 40 °C and maintained for 1 h. The suspension was centrifuged at $3000 \times g$, 30 min, 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 membranes (Millex, Millipore, São Paulo, Brazil), and 60 μL was injected into the HPLC chromatograph. Detection was performed at 214 nm, and column temperature was 40 °C. The gradient starting from 100% of solvent A (H_2O:trifluoroacetic acid (TFA) 1000:1.1, v/v) and 0% of solvent B (acetonitrile:H_2O:TFA 600:400:1, v/v), was generated 10 min after injection. The proportion of solvent B was increased by 1% min^{-1} (80 min), 20% min^{-1} (1 min), 0% min^{-1} (4 min), and then returned to starting conditions, which took 1 min. These last setting conditions were maintained for 10 min (Hynes, Bergamini, Suárez, & Zalazar, 2003).

2.6. Statistics

Data from microbiological and compositional analysis were processed by one-way analysis of variance (ANOVA) using SPSS 10.0 (SPSS Inc., Chicago, Estados Unidos). When differences were found, means were compared by the least significant difference test (LSD) using the same tool.

Principal component analysis (PCA) and non-hierarchical cluster analysis (CA) (*K*-means) were applied to peptide profiles, in order to reduce dimensionality, compare chromatograms objectively, and detect subjacent structures in the data ensemble. The areas of peaks expressed on arbitrary units were considered as independent variables for PCA, with standardisation to a mean of zero and their original variances (covariance matrix) (Pripp, Stepaniak, & Sørhaug, 2000). One way-ANOVA analysis was also performed on the principal component scores in order to detect any variation attributable to the type of cheese or age. All multivariate techniques were performed with SPSS 10.0 (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Fat substrate analysis

A significant ($p < 0.05$) increase in lactobacilli population was observed during incubation of the substrate inoculated with *L. acidophilus* or *L. paracasei*, which went along with a significant decrease in pH value ($p < 0.05$) (Table 1). The decrease of pH value in the substrate with *L. acidophilus* was more pronounced. The two studied strains of probiotics remained viable throughout the incubation and storage of the substrate (Bergamini et al., 2005).

3.2. Gross composition and microbiology of cheeses

Gross composition of control and experimental cheeses was similar (Table 2), with the only exception of pH value, which showed some significant differences ($p < 0.05$) among cheeses of trial 1 (*L. acidophilus*). In fact, the pH values of EP1 cheeses were significantly lower than those of C1 cheeses at 3 days of ripening, then pH values levelled out at 30 and 60 days of ripening.

Gross composition results demonstrated that the proposed Pategrás cheese was a repetitive model, which is a pre-requisite to detect differences on the proteolysis attributable to the biochemical expression of probiotic bacteria, and not to different environmental conditions.

Primary starter was about 10^9 cfu g^{-1} in cheese samples after pressing and brining. This number remained more or less constant during ripening and no significant differences were detected in primary starter number between control and experimental cheeses (results not shown). On the other hand, the tested probiotic strains in both trials were viable during all ripening at levels of 10^8 cfu g^{-1} (Table 3). These population numbers are higher than the required to meet probiotic food standards. Lactobacilli population in EP cheeses was always slightly higher than in EL cheeses. However, differences were significant ($p < 0.05$) only for 0 and 3 days in *L. acidophilus* trial, probably as a consequence of the increase in lactobacilli population

Table 1
pH values and probiotic cell counts during incubation and cold storage within a fat substrate[a]

Time (h)[b]		0	2	5	20
Trial 1[c]	pH	6.50 ± 0.06^{e}	6.42 ± 0.08^{e}	6.35 ± 0.05^{f}	6.00 ± 0.10^{g}
	Cell counts ($\log_{10}$ cfu mL^{-1})	6.97 ± 0.31^{e}	6.82 ± 0.10^{e}	7.18 ± 0.23^{e}	7.88 ± 0.33^{f}
Trial 2[d]	pH	6.45 ± 0.08^{e}	6.30 ± 0.05^{f}	6.25 ± 0.10^{f}	6.25 ± 0.08^{f}
	Cell counts ($\log_{10}$ cfu mL^{-1})	7.89 ± 0.21^{e}	8.32 ± 0.16^{f}	8.21 ± 0.07^{e}	8.70 ± 0.38^{f}

[a]Means and standard deviation of three replicate substrates are reported. Means in the same row with different superscripts differ ($p<0.05$).
[b]Time: total time (incubation + storage).
[c]Trial 1: fat substrate with *L. acidophilus*.
[d]Trial 2: fat substrate with *L. paracasei* subsp. *paracasei*.

Table 2
Gross composition of cheeses[a]

	Trial 1 (*L. acidophilus*)			Trial 2 (*L.paracasei*)		
	C1[b]	EL1[c]	EP1[d]	C2[b]	EL2[c]	EP2[d]
pH-3	5.25 ± 0.05^{e}	$5.08 \pm 0.08^{e,f}$	4.92 ± 0.16^{f}	5.20 ± 0.07	5.15 ± 0.18	5.05 ± 0.10
pH-30	5.15 ± 0.14	4.98 ± 0.18	4.85 ± 0.15	4.98 ± 0.04	4.98 ± 0.06	4.98 ± 0.06
pH-60	5.15 ± 0.14	5.00 ± 0.14	4.90 ± 0.07	5.15 ± 0.07	5.17 ± 0.08	5.07 ± 0.03
Fat matter (%)	28.70 ± 2.23	29.03 ± 3.02	29.47 ± 2.24	27.00 ± 1.41	27.97 ± 1.05	28.50 ± 1.32
Total protein (%)	22.01 ± 1.14	22.25 ± 0.49	21.71 ± 0.61	21.63 ± 1.89	21.50 ± 1.08	21.47 ± 0.50
Dry matter (%)	55.71 ± 1.35	54.99 ± 2.41	55.23 ± 0.80	54.57 ± 1.12	54.63 ± 0.36	55.24 ± 1.39
Salt in moisture (%)	3.34 ± 0.46	3.85 ± 0.13	3.61 ± 0.19	3.69 ± 0.17	3.52 ± 0.25	3.24 ± 0.20

[a]Means and standard deviation of three replicate cheeses are reported. All the analyses were performed in 3-day old cheeses, except Salt in Moisture (carried out on samples of 30-day old cheeses) and pH, which was analysed after 3 (pH-3), 30 (pH-30) and 60 (pH-60) days of ripening. Means in a row, and within each trial, with different superscripts differ ($p<0.05$).
[b]C1 and C2: control cheeses without probiotic bacteria, in trials 1 and 2, respectively.
[c]EL1 and EL2: experimental cheeses with the addition of probiotic bacteria as a lyophilised culture, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively.
[d]EP1 and EP2: experimental cheeses with the addition of probiotic bacteria pre-incubated in a substrate, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively.

Table 3
Population of probiotic lactobacilli in cheeses during ripening[a]

Ripening time (days)	Trial 1 (*L. acidophilus*)		Trial 2 (*L. paracasei*)	
	EL1[b] ($\log_{10}$ cfu g^{-1})	EP1[c] ($\log_{10}$ cfu g^{-1})	EL2[b] ($\log_{10}$ cfu g^{-1})	EP2[c] ($\log_{10}$ cfu g^{-1})
0	6.86 ± 0.21^{d}	7.69 ± 0.16^{e}	7.39 ± 0.12	7.95 ± 0.34
3	8.29 ± 0.18^{d}	8.73 ± 0.04^{e}	8.78 ± 0.09	9.13 ± 0.38
15	8.38 ± 0.38	8.61 ± 0.07	8.98 ± 0.23	9.13 ± 0.14
30	8.43 ± 0.49	8.82 ± 0.31	9.09 ± 0.35	9.21 ± 0.12
45	8.00 ± 1.08	7.83 ± 0.72	8.95 ± 0.41	9.28 ± 0.04
60	7.78 ± 0.74	8.09 ± 0.33	9.11 ± 0.36	9.23 ± 0.20

[a]Means and standard deviation of three replicate cheeses are reported. Means in a row, and within each trial, with different superscripts differ ($p<0.05$).
[b]EL1 and EL2: experimental cheeses with the addition of probiotic bacteria as a lyophilised culture, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively.
[c]EP1 and EP2: experimental cheeses with the addition of probiotic bacteria pre-incubated in a substrate, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively.

during incubation in the fat substrate (Bergamini et al., 2005). The results of plate counting on MRS agar for C cheeses represent the non-starter lactobacilli population, as the morphology of the colonies differed from the characteristic shape and colour found for lactobacilli colonies in the experimental cheeses, the fat substrate and the pure

Lactobacillus culture. In addition, the microscopic characteristics of the bacteria recovered from MRS plates of control and experimental cheeses were also different. Non-starter lactobacilli in C cheeses remained always lower than 10^6 cfu g^{-1}.

3.3. Proteolysis assessment

3.3.1. Soluble nitrogen

Levels of SN-pH 4.6, SN-TCA and SN-PTA increased during ripening as a consequence of proteolysis, in both trials (Table 4). The levels of SN-pH 4.6 at 3, 30 and 60 days, did not differ significantly ($p>0.05$) between cheeses with and without added probiotic bacteria, in both trials. These results show that no influence of probiotic bacteria was detected on primary proteolysis, which is not surprising taking into account that other enzymatic agents are most likely responsible for primary proteolysis in semi-hard cheeses. In fact, it has been shown that this process is mainly catalysed by residual chymosin and, in a lower degree, by other proteinases present in the curd such as plasmin or cell envelope proteases from the starter (Sousa et al., 2001). Our observations agreed with those obtained by Gardiner et al. (1998), who did not find any differences in SN-pH 4.6 between cheeses with and without a probiotic culture of *L. paracasei*. In contrast, it was reported that *L. acidophilus* produced an increase in this fraction during the ripening of a probiotic white cheese (Kasimoğlu et al., 2004). Even though the pH value of EP1 cheeses was significantly lower than that of C1 cheeses at 3 days of ripening, we did not detect changes in primary proteolysis (SN-pH 4.6 and electrophoresis, see below) attributable to this environmental difference.

In the same way, the level of SN-TCA did not show significant differences ($p>0.05$) between control and experimental EL and EP cheeses, in both trials. These results suggest that probiotic bacteria added to cheeses did not influence the production of medium and small-sized peptides. On the contrary, the level of SN-PTA was significantly different ($p<0.05$) between cheeses with and without probiotic bacteria either at 3, 30 or 60 days of ripening, in trial 1 (Table 4). The two types of probiotic cheeses showed a higher level of SN-PTA than control cheeses. EL1 and EP1 cheeses did not significantly differ in SN-PTA amount, although this index of secondary proteolysis was always slightly higher in EP1 cheeses. In contrast, in trial 2 where the strain of *L. paracasei* was tested, no differences were detected in SN-PTA level.

The SN-TCA fraction contains medium and small-sized peptides, amino acids and smaller N compounds such as amines, urea and ammonium. On the other hand, the SN-PTA fraction is composed of very small peptides, amino acids and smaller N compounds other than dibasic amino acids and ammonia, which makes it a fair index of FAA content (Ardö, 1999). The results of the increased levels of SN-PTA in cheeses manufactured using *L. acidophilus* suggest that this strain in particular has a more active peptidolytic system than the *L. paracasei* strain tested. This enzymatic ability significantly influenced the production of small peptides and FAA during ripening, even as early as at 3 days. Similarly, others authors (Hynes et al., 2003; Madkor et al., 2000) have determined that adjunct cultures of lactobacilli can produce an increase on the level of SN-PTA or total FAAs. On the contrary, Poveda et al. (2003) found no differences in the SN-PTA fraction between Manchego cheeses with and without an adjunct culture of *Lactobacillus plantarum*, even at 150 days of ripening.

Environmental conditions in EP1 cheeses (initial lower pH) seem not to be the cause of increased secondary proteolysis, as EL1 cheeses, whose pH did not differ from

Table 4
Nitrogen content in cheese soluble fractions at pH 4.6 (SN-pH 4.6), in 12% trichloroacetic acid (SN-TCA) and in 2.5% phosphotungstic acid (SN-PTA), expressed as the percentage of total N, and total amount of free amino acids (FAA), expressed as mg 100^{-1} g^{-1} of cheese, at 3, 30 and 60 days of ripening[a]

Cheeses		SN-pH 4.6			SN-TCA			SN-PTA			FAA		
		Days of ripening			Days of ripening			Days of ripening			Days of ripening		
		3	30	60	3	30	60	3	30	60	3	30	60
Trial 1	C1[b]	5.2±0.6	10.9±1.4	14.2±1.6	2.5±0.3	6.0±0.3	7.5±1.1	0.7±0.1[e]	1.2±0.2[e]	1.4±0.1[e]	12±1[e]	25±2[e]	48±4[e]
	EL1[c]	5.7±0.3	10.7±1.1	13.9±0.7	2.6±0.2	6.1±0.2	7.7±0.7	1.1±0.2[f]	1.7±0.2[f]	2.0±0.2[f]	73±5[f]	102±6[f]	125±6[f]
	EP1[d]	6.1±0.9	11.4±1.8	13.9±1.2	3.0±0.4	6.3±0.5	7.9±0.3	1.2±0.1[f]	1.9±0.1[f]	2.1±0.1[f]	73±6[f]	93±5[f]	124±7[f]
Trial 2	C2[b]	5.8±0.1	12.4±1.3	15.5±1.0	2.0±0.1	5.8±0.4	8.1±0.7	0.6±0.2	1.0±0.2	1.5±0.3	ND	ND	ND
	EL2[c]	5.5±0.1	10.8±0.4	13.9±0.3	2.1±0.1	5.5±05	7.8±0.3	0.6±0.2	1.2±0.2	1.6±0.1	ND	ND	ND
	EP2[d]	5.4±0.3	11.0±0.4	13.4±1.4	2.1±0.3	5.8±0.2	7.7±0.6	0.6±0.2	1.2±0.1	1.6±0.1	ND	ND	ND

[a]Means and standard deviations of three replicate cheeses are reported. Means in a column, and within each trial, with different superscripts differ ($p<0.05$).

[b]C1 and C2: control cheeses in trials 1 and 2, respectively. ND: not determined.

[c]EL1 and EL2: experimental cheeses with the addition of probiotic bacteria as a lyophilised culture, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively.

[d]EP1 and EP2: experimental cheeses with the addition of probiotic bacteria pre-incubated in a substrate, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively.

C1 cheeses, also showed SN-PTA augmented values. In addition, cheese environments were all similar after 30 and 60 days of ripening.

As for probiotic viability, *L. acidophilus* counts declined about 0.5 log during ripening, while the *L. paracasei* subsp. *paracasei* population did not show any reduction (Table 3). This change in cell viability could explain the observed differences in peptidolytic activity, being due to cell lysis and release of intracellular peptidases into the cheese matrix (Hynes, Bergamini et al., 2003). However, differences in secondary proteolysis were found as early as 3 days of ripening, when *L. acidophilus* viability had not yet declined. On the other hand, Gardiner et al. (1998) found that adjunct lactobacilli, whether they survived to high levels or not, increased formation of FAA in Cheddar cheese, but this ability is shown to be species- and strain dependent (Hynes et al., 2003).

3.3.2. Electrophoresis

Electrophoretic profiles of the pH 4.6-insoluble N fraction of control and experimental cheeses showed no differences, in both trials. These results agreed with the similarity observed in the levels of SN-pH 4.6 fraction, which is not surprising taking into account that both are indices of primary proteolysis in cheese. Some examples of electrophoretic patterns of cheese samples after 3, 30 and 60 days of age from trial 1 are presented in Fig. 1. Degradation of α_{s1}-casein was extensive and only a light band was present at the end of ripening (60 days). Disappearance of α_{s1}-casein showed a concomitant increase of α_{s1}(f24-199) fraction, a peptide resulting from α_{s1}-casein breakdown by residual chymosin. On the other hand, β-casein remained almost intact during ripening and only a light band of γ-casein, barely visible, was observed, which indicated a poor activity of plasmin. These electrophoretograms were as could be expected for a cheese variety where a mild cooking step is performed (McSweeney, 2004).

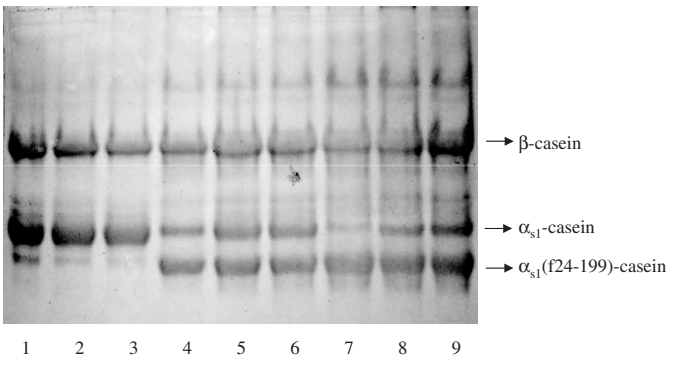

Fig. 1. Urea-polyacrylamide gel electrophoresis at pH 8.4 of 3-, 30- and 60-day old Pategrás cheeses. Lanes 1–3: C1, EL1 and EP1 cheeses at 3 days of ripening. Lanes 4–6: C1, EL1 and EP1 cheeses at 30 days of ripening. Lanes 7–9: C1, EL1 and EP1 cheeses at 60 days of ripening. C1: control cheese in trial 1. EL1: experimental cheese with addition of probiotic bacteria as a lyophilised culture in trial 1 (*L. acidophilus*). EP1: experimental cheese with addition of probiotic bacteria pre-incubated in a substrate in trial 1 (*L. acidophilus*).

3.3.3. Free amino acid (FAA) analysis

FAA analysis was performed only for trial 1 (*L. acidophilus*). Significant differences ($p<0.05$) between total amount of FAAs in experimental and control cheeses were detected, after 3, 30 and 60 days of ripening. Probiotic cheeses had a higher content of FAA than control cheeses, but no difference was found between EP1 and EL1. These results agree with the trend detected by SN-PTA analysis, and indicated a higher peptidolytic activity in cheeses with *L. acidophilus* (Table 4).

Not only was each individual FAA higher in probiotic cheeses than in control ones, but also profiles varied after 3, 30 and 60 days of ripening. The major FAAs in experimental cheeses were Lys, Ile, Tyr and Ala, in decreasing order of concentration, throughout the whole ripening period. In contrast, Lys, Tyr and Arg/Trp/Ile represented the majority of FAAs in control cheeses, in decreasing order of concentration (the third main FAA changed with ripening time) in samples from 3 and 30 days of ripening. Main FAAs in 60-day old C1 cheeses were Trp, Tyr, Lys and Ile, in decreasing order of concentration. The individual FAA profiles at 3 and 60 days of ripening are shown in Figs. 2A and B. These results are in agreement with those of Madkor et al. (2000), who found that the greatest contribution of lactobacilli to cheese ripening was to increase FAA levels in adjunct-treated cheeses.

Increased levels of FAAs in probiotic cheese may be favourable for developing cheese flavour, since amino acids can contribute, mainly as precursors, to the taste and aroma of cheeses (McSweeney, 2004).

3.3.4. Reverse phase-high performance liquid chromatography (RP-HPLC)

In general, all the peaks in the chromatograms of cheeses increased throughout ripening. A visual analysis of the peptide profiles obtained by HPLC allowed detecting some qualitative and quantitative differences among cheeses. Twelve peaks were chosen by visually matching all the chromatograms and selecting the peaks whose areas varied most evidently (Pripp et al., 2000). Selected peaks were identified with characters from "a" to "l", in alphabetical order. An example of peptide profiles for C1, EL1 and EP1 cheeses at 60 days, and the position of selected peaks on the chromatograms are shown in Fig. 3.

In trial 1, during all ripening period, peaks a, b, d, g and k were higher in experimental cheeses than in control cheeses, while c, e, h, i and j showed an opposite behaviour. On the other hand, peaks f and l were similar between cheeses with and without added probiotic bacteria.

In trial 2, a, b, c and l were higher in experimental cheeses than in control cheeses, while h and principally j were larger in control cheeses. Peaks d and g were similar for control and experimental cheeses, whereas e, f, i and k showed a variable behaviour.

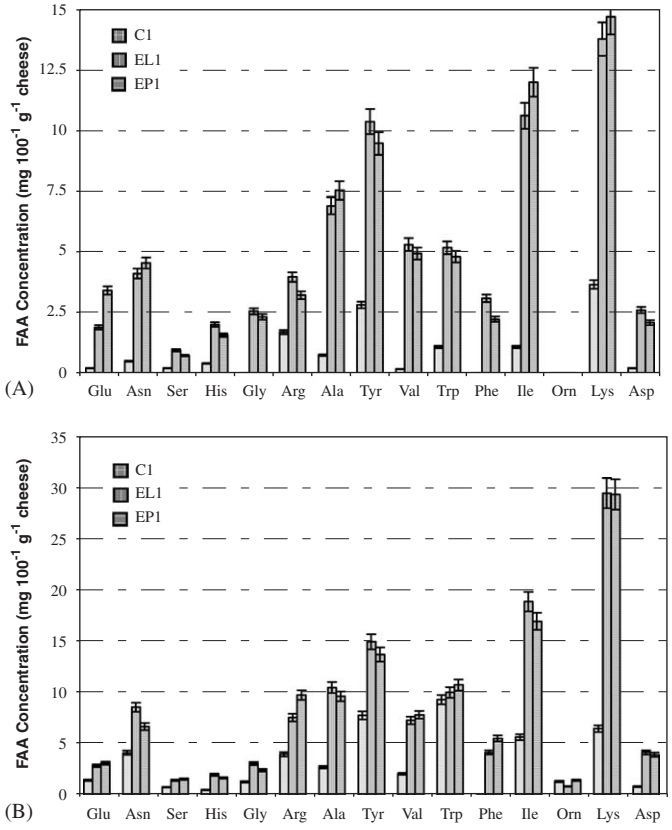

Fig. 2. Profiles of individual free amino acids in cheeses at 3 (A) and 60 (B) days of ripening. C1 (▨): control cheese in trial 1. EL1 (▥): experimental cheese with addition of probiotic bacteria as a lyophilised culture in trial 1 (*L. acidophilus*). EP1 (▤): experimental cheese with addition of probiotic bacteria pre-incubated in a substrate in trial 1 (*L. acidophilus*).

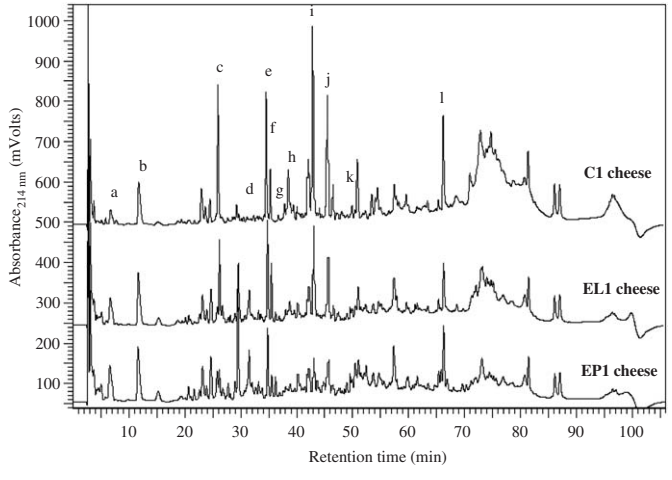

Fig. 3. Reverse phase liquid chromatography profiles of water-soluble extract of C1, EL1 and EP1 cheeses at 60 days of ripening. C1: control cheese in trial 1. EL1: experimental cheese with addition of probiotic bacteria as a lyophilised culture in trial 1 (*L. acidophilus*). EP1: experimental cheese with addition of probiotic bacteria pre-incubated in a substrate in trial 1 (*L. acidophilus*). Characters a to l indicate the peaks selected for principal component analysis.

In both trials, areas of peaks b, c, e, and i showed the greatest variation during ripening, while a, f, h, j, k and l had the lowest increase. On the other hand, c, d and g were the peaks that varied the most from one trial to another.

Changes on the peptide profiles reflect the dynamic between peptide production and degradation to FAAs and their metabolic products. Differences of area for a given peak, between control and experimental cheeses, could be explained as a consequence of the peptidolytic activity of lactobacilli that increased the production of the peptide or peptides eluting at a particular retention time. At the same time, decreasing the area of certain peaks in experimental cheeses may have originated by the demolition of peptides that act as substrates for lactobacilli peptidases (Hynes, Bergamini et al., 2003).

Multivariate methods of analysis have been increasingly applied to study peptide profiles obtained by RP-HPLC (Hynes, Bergamini et al., 2003; Poveda et al., 2003; Pripp, Shakeel-Ur-Rehman, McSweeney, & Fox, 1999). Multivariate analysis allows reducing system dimensionality and helps to better understanding and interpretation of data (Pripp et al., 2000). In this work, PCA and non-hierarchical CA were applied to HPLC data. Chromatograms of the samples in each trial at all the studied ripening times were processed together in the same analysis. The first two PCs retained most information existing in the original data (based on the criterion Eigenvalue >1), and represented 91.1% and 93.5% of the total variance in trials 1 and 2, respectively.

Loadings for variables (peak areas) in trials 1 and 2 are presented in Figs. 4A and B, respectively. In trial 1, peaks that were higher in control cheeses than in experimental cheeses, mostly influenced PC1 (Fig. 4A). On the contrary, peaks that were higher in experimental cheeses than in control ones, showed the highest loadings on PC2. In trial 2, the variables that influenced PC1 the most were the peaks that showed the highest variation during ripening. On the contrary, the peak that mostly influenced PC2 was characteristic of control cheeses peptide profile.

Score plots of trials 1 and 2 are presented in Figs. 5A and B, respectively. These plots clearly showed grouping of samples according to ripening time primarily along PC1 axis. Samples of 3 days of ripening showed very low variability, while 30- and 60-dayold cheeses were more variable. On the other hand, a tendency to group by cheese type (control and experimental) was observed along PC2 axis in both trials. An ANOVA allowed confirming our interpretation of PCs significance, as it showed significant differences in the first two PCs for ripening time, and only in PC2 for cheese type ($p<0.05$) (Table 5).

Changes in peptide profiles in cheeses with and without lactobacilli were not localised in one particular region of the chromatograms, which evidenced a non-specific contribution to peptide production and degradation. These results differ from other peptide mapping studies, which report the appearance of specific peptides in cheeses with

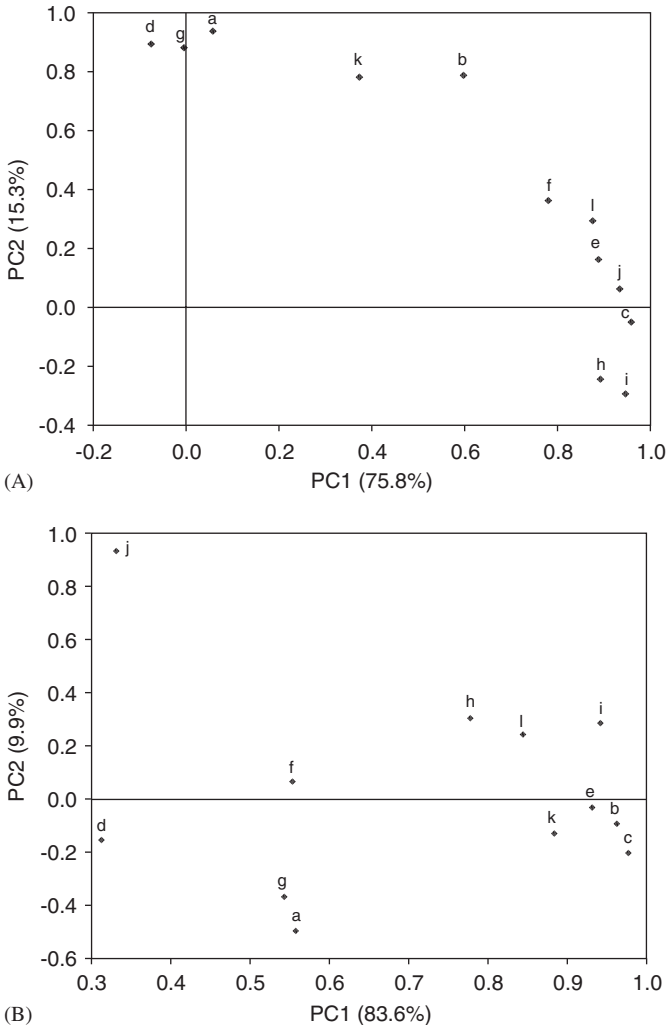

Fig. 4. Principal component (PC) analysis of chromatographic profiles: loading plot of the independent variables (areas of peaks a to l on the chromatograms) on PC1 and PC2, in trial 1 (A) and trial 2 (B).

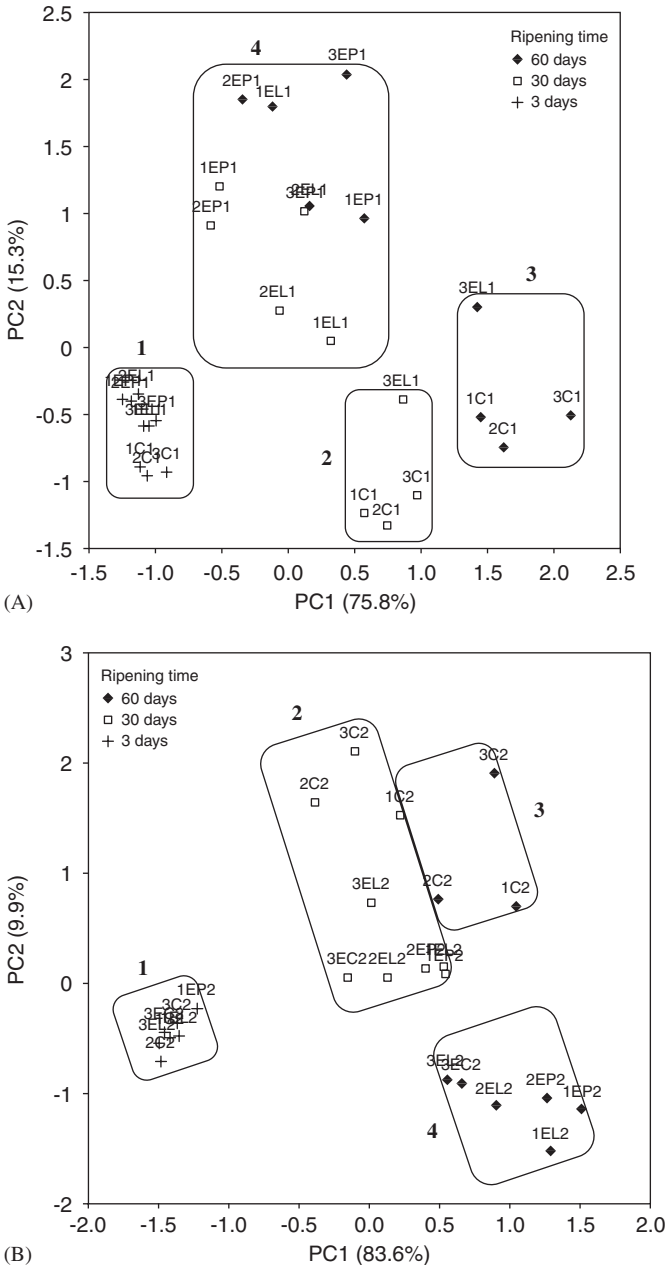

Fig. 5. Principal component (PC) analysis of chromatographic profiles: score plot of 3—(+), 30—(□), and 60—(◆) dayold-cheese samples from trial 1 (A) and trial 2 (B) on PC1 and PC2. C1 and C2: control cheeses in trials 1 and 2, respectively. EL1 and EL2: experimental cheeses with addition of probiotic bacteria as a lyophilised culture, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively. EP1 and EP2: experimental cheeses with addition of probiotic bacteria pre-incubated in a substrate, in trials 1 (*L. acidophilus*) and 2 (*L. paracasei*), respectively. The first number in the sample label indicates the cheesemaking replicate. Rectangles enclose cheese samples in a cluster.

different starter or from diverse farms (Fallico et al., 2004; Pripp et al., 1999).

CA confirmed results from PCA, as can be observed in score plot, where samples in the same cluster are surrounded by rectangles (Figs. 5A and B). In trial 1, CA grouped samples in four different groups. Cluster 1 consisted of all the 3-day old cheeses, regardless of cheese type. Cluster 2 was composed of all 30-day old C1 cheeses, and one of the 30-day old EL1 cheeses. The same cheeses were grouped together at 60 days in cluster 3. Finally, cluster 4 grouped EL1 and EP1 cheeses of 30 and 60 days of ripening, with the only exception of the EL1 cheese in clusters 2 and 3. In trial 2, again four clusters were defined by CA. As in trial 1, 3-day old cheeses were grouped in cluster 1. Cluster 2 consisted of all 30-day old cheeses. Cluster 3 grouped all 60-day old C2 cheeses, and cluster 4 all EL2 and EP2 at 60 days of ripening.

Multivariate analysis did not detect sample grouping according to the methodology of probiotic addition in both trials. EL and EP cheeses were grouped together (CA), and only a slight tendency to separation along PC2 axis was evidenced in score plot for EL1 and EP1 30-dayold cheeses.

The results of chromatographic studies and multivariate analysis showed that the two tested strains influenced the proteolytic pattern of the cheese in a different way.

Table 5
Effect of the ripening time and the addition of probiotic cultures on the first two principal components (PCs) obtained by principal component analysis of peptide profiles, in trials 1 and 2

	PC	Ripening time[a]	Addition of probiotic culture
Trial 1 (*L. acidophilus*)	1	$<0.001^{***}$	NS
	2	0.013^{*}	$<0.001^{***}$
Trial 2 (*L. paracasei*)	1	$<0.001^{***}$	NS
	2	0.018^{*}	0.007^{**}

NS: not significant at the 5% level, $p>0.05$.
[a]Asterisks indicate level of significance.
*significant at the 5% level or better, $0.01<p\leqslant0.05$; **significant at the 1% level or better, $0.001<p\leqslant0.01$; ***significant at the 0.1% level or better, $p\leqslant0.001$.

The proteolysis of cheeses with probiotic bacteria was different from the proteolysis of control cheeses without probiotics, both for trials 1 and 2. However, differences in cheeses made with *L. acidophilus* appeared earlier during ripening. This may be due to a higher peptidolytic activity, which was evidenced by several proteolysis indexes: FAA, HPLC profiles and SN-PTA. On the contrary, HPLC profiles of cheeses made with and without *L. paracasei* differed basically at the end of the ripening, and this did not go along with significant differences in SN-PTA.

In previous works, differences among peptide profiles of cheeses with and without lactobacilli were not significant (Antonsson, Molin, & Ardö, 2003; Gardiner et al., 1998; Hynes et al., 2003). In our study, the activity of the tested strains was high enough as to be detected even by comparing samples of 3, 30 and 60 days of ripening together.

4. Conclusions

The influence of probiotic lactobacilli on Pategrás cheese proteolysis was assessed. The impact of the probiotic culture varied from one probiotic strain to another and, in one case, significantly modified the proteolytic pattern of the standard cheese. Methodology of culture addition, on the contrary, did not affect cheese chemical composition.

The two strains of probiotic bacteria studied in the present work distinctly influenced the proteolysis pattern of semi-hard cheeses, probably as a consequence of their different proteolytic systems and their activity via the alimentary matrix. The observed effects were an increase in the production of short peptides and free amino acids (FAAs), and modification of peptide profiles.

The tested strain of *L. paracasei* presented the advantage of not markedly influencing proteolysis pattern of Pategrás cheese. As a consequence, the obtained probiotic food was not significantly different from the traditional cheese. Taking into account this similarity in chemical composition of the products, it is very likely that the addition of this particular probiotic culture would not impair the acceptability of the cheese by the consumers. However, sensory analysis is needed to confirm this hypothesis; such experiments are currently in course in our laboratory.

On the other hand, the tested strain of *L. acidophilus*, besides of its probiotic potential, could have the extra advantage of producing acceleration of ripening or promoting flavour enhancement, via an increased production of FAAs. In fact, the addition of lactobacilli as adjunct cultures has been proposed as an approach to accelerate ripening or improve flavour of cheeses, because of their contribution to FAA production and their catabolism. As before, this possibility will be explored in our institute using sensory analysis tools.

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