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Proteolytic activity of three probiotic strains in semi-hard cheese as single and mixed cultures: *Lactobacillus acidophilus*, *Lactobacillus paracasei* and *Bifidobacterium lactis*

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ABSTRACT

The influence of three probiotic strains (*Lactobacillus acidophilus*, *Lactobacillus paracasei* and *Bifidobacterium lactis*) in semi-hard cheese proteolysis patterns was assessed. Probiotics were inoculated both as single cultures and as a three-strain mix, and added to milk either after a pre-incubation step or directly to the vat. *B. lactis* did not show any effect on proteolysis of cheeses, while *L. paracasei* showed limited impact at the end of the ripening. In contrast, *L. acidophilus* significantly influenced secondary proteolysis from the beginning of ripening, causing an increase in the levels of small nitrogen-containing compounds and free amino acids and changes in the peptide profiles. The effect of *Lactobacillus acidophilus* on peptidolysis was more noticeable when it was added to cheese–milk after pre-incubation in an enriched milk fat substrate. Similar results obtained with the three-strain mixed culture, suggesting that *L. acidophilus* played a major role in secondary proteolysis of probiotic cheeses in this trial.

1. Introduction

Dairy foods have been chosen as carriers for probiotic bacteria; of these foods, fluid products are the most commonly used (Heller, 2001). However, several recent studies have shown that cheese can be a less hostile environment for probiotic bacteria, leading to a higher survival of these microorganisms, not only in the product, but also during the gastrointestinal transit (Boylston et al., 2004; Ross et al., 2002). A special characteristic of cheeses is that, unlike other probiotic carriers, they are consumed after a ripening period that varies with cheese type and can take from a few days to several months (Ross et al., 2002). During this ripening time, biochemical reactions responsible for the change of curd into matured cheese occur, and include the transformation of carbohydrates, lipids and proteins. Lactobacilli and bifidobacteria, which are the most widely used probiotic bacteria, have been shown to possess several proteolytic and peptidolytic enzymes, and therefore have the potential to influence proteolysis (Desjardins et al., 1990a; Habibi-Najafi and Lee, 1994; Peterson et al., 1990; Shihata and Shah, 2000; Williams and Banks, 1997). Probiotic cultures are usually added as secondary or adjunct cultures into cheese–milk; however, the use of a starter entirely composed by probiotic bacteria has also been proposed and successfully achieved (Gomes et al., 1995).

Most studies on probiotic cheeses have focused on maintaining a high probiotic population during the shelf life of the food. Results have shown that several cheese varieties, e.g., Cheddar, Gouda, Canestrato Pugliese, Fresco, etc., are able to carry high numbers of different strains of probiotic bacteria for variable periods (Phillips et al., 2006; Roy et al., 1998; Vinderola et al., 2000). Technological approaches, such as immobilizations probiotics or addition of protein hydrolysate have been proposed to improve probiotic viability in cheese (Dinakar and Mistry, 1994; Gobbetti et al., 1998; Gomes and Malcata, 1998). Nevertheless, the impact of probiotics on cheese quality has been less explored and remains fairly unknown. The investigation of this aspect of probiotic addition is also very important as it can have an influence on consumer acceptability of the food. In this way, the addition of cheese-isolated lactobacilli strains have been proposed to accelerate the ripening process or enhance sensory properties of the product (Di Cagno et al., 2006; Hynes et al., 2003). If probiotic lactobacilli can be shown to have these properties, this would be an extra advantage besides the health benefits.

Studies describing the impact of the addition of probiotic bacteria on the composition and quality of specific cheese varieties are still minimal. To date, this aspect of the development of new functional cheeses has been mostly evaluated in Cheddar-type

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cheese, containing individual or mixed cultures of probiotic bacteria (Daigle et al., 1999; Gardiner et al., 1998; Ong et al., 2006, 2007). A few research studies have been carried out on other cheese varieties, such as Gouda (Gomes et al., 1995), Tallaga (El-Zayat and Osman, 2001), Canestrato Pugliese (Corbo et al., 2001), Turkish white cheese (Kasimoğlu et al., 2004) and Minas fresh cheese (Buriti et al., 2005). However, the results cannot be extrapolated to other probiotic cheeses, since environmental factors defined by cheese technology influence the growth and biochemical activities of cheese-related microoorganisms (Lane et al., 1997). Species and level of primary starter, as well as the inclusion of several probiotic strains in a mixed culture, are important factors, as is the fact that survival in the food and other technological characteristics of probiotics are strain-dependent (Corbo et al., 2001; Gardiner et al., 1998; McBrearty et al., 2001; Phillips et al., 2006). It is important to mention that information about coupling thermophilic starters with probiotic bacteria in cheeses is lacking, as most research studies have been performed in cheese models using mesophilic starters. Thermophilic lactic starters are virtually the only acidifying starters used in Argentina, the world's seventh largest cheese producer (Fox, 2003). In addition, the effect of mixed probiotic cultures on cheese quality has been studied to only a limited extent (Gobbetti et al., 1998; Ong et al., 2006).

In this study, we assessed the contribution of three probiotic bacteria to proteolysis in a semi-hard cheese, both as single and mixed cultures. For that purpose, we selected a semi-hard cheese model manufactured with a thermophilic primary starter, representative of an important share of Argentinean cheese production. We also compared two different techniques for the addition of probiotics into cheese–milk, and studied their impact on probiotics' survival and biochemical expression in the food during ripening.

2. Materials and methods

2.1. Cheese-making

Three probiotic cultures were assayed as adjunct cultures, both as single cultures and as a three-strain mixed culture. For each culture, a three-block experiment was performed; each block (cheese-making day) consisted of three cheeses: control cheeses (C, without probiotics) and two types of experimental cheeses (L and P), with the addition of the same probiotic(s) but differing in the inoculation method. In one type of experimental cheese (L), probiotic bacteria were added directly to the cheese–milk as a lyophilised culture, while in the other (P), they were pre-incubated in a substrate composed by milk and milk fat, then added to the cheese–milk (Bergamini et al., 2005). The number of cheeses (experimental units) in each experiment accounted for 9; a total of 36 cheeses were produced during the entire project. Cheeses within the same experiment were identified by subscripts according to the probiotic culture tested: *L. acidophilus* ($_{Lac}$), *L. paracasei* ($_{Lpa}$), *B. lactis* ($_{Bif}$) and the three-strain mixed culture ($_{mix}$).

Pategrás Argentino cheese was selected as a representative model of semi-hard Argentinean cheeses. The cheeses were manufactured according to the industrial technology adapted to pilot scale with pasteurised large pool milk (Bergamini et al., 2006). Fat content in milk was standardised at 3.8% (w/v) for C and L cheeses and at 3.49% (w/v) for P cheese, because the addition of the probiotic inoculums in the latter increased the fat concentration to a final content of 3.8% (w/v). Non-fat solid content was also standarised in all cheeses by adding skim milk to C and L cheeses. A lyophilised commercial culture, for direct vat inoculation, of *Streptococcus thermophilus* (Diagramma S.A., Santa Fe, Argentina) was used as primary starter. It was added after a brief activation (15 min at 37 °C, in sterile reconstituted skim milk) in a dose high enough to achieve 10^6 cfu mL^{-1} in the cheese–milk. Probiotics were added immediately after primary starter. Milk was coagulated with chymosin (Maxiren 150, Gist Brocades, Seclin Cedex, France) at 37 °C. Curd was cut in successive steps until it was the size of a corn grain (at 37 °C–approximately 20 min) and the curds were scalded (0.5 °C min^{-1} up to 45 °C and maintained at this temperature for 15–20 min). Curd was then separated from whey and moulded, moulds were piled three high and pressed. The following day, cheeses were brined for 24 h and then ripened for 2 months at 12 °C and 80% relative humidity.

2.2. Probiotic cultures

Three different strains of probiotic bacteria were used as adjunct cultures: *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis*. All were lyophilised commercial cultures with a suppliers claimed that they were of human intestinal origin, have good survival in the gastrointestinal tract and probiotic properties such as protection amongst *Salmonella* infection, inhibition of *Escherichia coli* O111 and *Listeria monocytogenes*. The companies that provide them will not be mentioned for confidentiality reasons.

A milk fat-rich medium containing 14% (w/v) fat and 5.2% (w/v) proteins was prepared and heat-treated according to Bergamini et al. (2005) to pre-incubate probiotics for P cheeses. Probiotic culture was inoculated into this substrate, incubated at 37 °C for 5 h and then stored at 4 °C until the next day, when it was added into cheese–milk. The same initial amount of probiotic culture was used for the manufacture of L and P cheeses. The dose of probiotic culture was aimed to obtain high probiotic concentration in the product without increasing acidification rate or extension, because it may alter cheese composition and standard quality. Thus, when assayed individually, *B. lactis* and *L. paracasei* were added at 1.4×10^6 cfu mL^{-1} of the cheese–milk, whereas the *L. acidophilus* concentration was lower (2.6×10^5 cfu mL^{-1}), because it showed a higher acidification activity in preliminary trials (data not shown). In the mixed culture, *B. lactis* was added at same quantity as above, whereas *L. paracasei* and *L. acidophilus* were inoculated in a lower level (4×10^4 cfu mL^{-1} in the cheese–milk) because a higher acidification occurred when doses similar to those of the single starter experiments were used (data not shown).

2.3. Fat substrate analysis

During incubation and cold storage of the substrate inoculated with the probiotic(s) for P cheese, pH values and probiotic viable cell were determined at 0, 2, 5 and 20 h. When added individually in the substrate, lactobacilli were enumerated by plating sample dilutions on De Man Rogosa Sharpe (MRS) agar, whereas MRS agar with 0.15% bile was used in the trial with the mixed-strain culture. In both cases, plates were incubated 48 h at 37 °C in aerobic conditions (Vinderola and Reinheimer, 2000). Bifidobacteria were plated on propionate lithium MRS agar and incubated at 37 °C for 72 h in anaerobic conditions (Vinderola and Reinheimer, 1999).

2.4. Gross composition, pH and microbiology of cheeses

Gross composition was determined at 3 days, except for NaCl, in which case analysis was conducted at 30 days, and the pH, which was also monitored at 30 and 60 days of ripening. Moisture (oven drying at 102 ± 1 °C), fat matter (butyrometer) and protein content (Kjeldahl method) were analysed according to International Dairy Federation standard methods (IDF, 1982; IDF, 1997 and IDF, 1993, respectively). The pH was measured according to American Public Health Association (APHA) standard (Bradley et al., 1993). Sodium chloride content was analysed using a spectrophotometric method (AOAC, 1990).

Starter streptococci and probiotic bacteria were enumerated in cheese at 0 (fresh curd), 3 (curd after press), 15, 30, 45 and 60 days of ripening, according to Bergamini et al. (2005). Primary starter was enumerated on skim milk agar after 48 h of incubation at 37 °C in aerobic conditions. Probiotic bacteria in experimental cheeses were counted as described previously. Non-starter lactobacilli in C cheeses were counted in MRS agar after 48 h of incubation at 37 °C.

2.5. Proteolysis assessment

Proteolysis was determined on 3, 30 and 60 day old cheeses by nitrogen fractions, electrophoresis, soluble peptide profiles and free amino acids profiles.

2.5.1. Nitrogen fractions

Cheese samples were treated to obtain a crude citrate extract from which 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) were prepared (Bergamini et al., 2006; Gripon et al., 1975). The nitrogen content in each fraction was determined by the macro-Kjeldahl method (IDF, 1993).

2.5.2. Electrophoresis

The insoluble residue at pH 4.6 of cheeses was analysed by urea-polyacrylamide gel electrophoresis (Urea-PAGE) in a Mini-Protean II cube (BioRad Laboratories, Hercules, CA, USA) according to the method of Andrews (1983), using a 7.5% (w/v) acrylamide gel. Proteins were stained by Coomassie Blue G-250 and destained in a few changes of a solution of acetic acid/ethyl alcohol/distilled water (10:25:65) until the background became clear.

2.5.3. Peptide analysis by reverse phase-high performance liquid chromatography

The high performance liquid chromatography (HPLC) equipment consisted of a quaternary pump, an on-line degasser and UV/VIS detector, all Series 200, from Perkin Elmer (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 × 4.6 mm Aquapore OD-300 C18, 5 μm–300 A° analytical column was used (Perkin Elmer). Water-soluble extracts of the cheeses were obtained, filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil), and injected into the HPLC chromatograph. Separation was achieved under an increasing linear gradient of acetonitrile in water, over 107 min. Detection was performed at 214 nm, column temperature was 40 °C and flow rate was 1 mL min^{-1} (Bergamini et al., 2006).

2.5.4. Free amino acid analysis

A pre-column derivatisation method using 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) followed by HPLC was used for the determination of free amino acids (FAAs) in cheese samples. For this the Chemistry Package of the Waters AccQ · Tag® Amino Analysis Method (Waters Corporation, Mildford, MA, USA) was used. The package comprised reagent kit for the derivatisation reaction, column, mixture of amino acid standards, sample tubes and the eluents. The HPLC equipment was the same used for peptide profiles. A 150 × 3.9 mm Nova–Pak™ C_{18}, 4 μm column (Waters Corporation) specifically certified for use with the AccQ · Tag Method and a 15 × 3.2 mm, 7 μm guard column (Perkin Elmer) were used. The system temperature was 37 °C. Detection was performed at 248 nm and flow rate was 1 mL min^{-1}. Solvents used for the separation were: acetate-phosphate buffer pH 5.02 (solvent A), and acetonitrile/H_2O (60/40; solvent B). Gradient conditions were: initial = 100% A, 0.5 min = 98% A, 15 min = 93% A, 19 min = 87% A, 32 min = 66% A, 33 min = 66% A, 34 min = 0% A (all segments linear), followed by a wash with 100% solvent B for 3 min, a change at 100% A in 1 min and then re-equilibration for 12 min at 100% A. Sample was an aqueous extract of cheese, similar to the that used in peptide profiles analysis (Giraudo et al., 2002). L-2-aminobutyric acid (Sigma, St. Louis, MO, USA) was used as an internal standard, and the standard amino acid mixture (Waters Corporation) was used to obtain calibration curves for each amino acid. The derivatisation reaction was performed on adequately diluted cheese samples and standard solutions, according to the experimental protocol of the AccQ · Tag method, and then 20 μL of derivatives samples were injected into the HPLC chromatograph. FAAs were determined on samples at 3 and 60 days of ripening.

2.6. Sensory analysis

The sensory properties of 60 day old cheeses were assessed by a 12-member trained panel. During each session, two probiotic cheeses (L and P cheeses) and one control cheese (C), corresponding to one cheese-making day, were evaluated by each panel member. Cheeses were removed from storage, stored at room temperature for 1 h and cut into portions (25 g) for evaluation. The outer layer of the cheese (1 cm) was removed. Cheese portions were covered with glass plates for odour assessment and labelled with randomised three number codes. The order of presentation was balanced to avoid order and carry-over effects. Mineral water and bread were provided to the panellists to rinse their mouth between samples. The panel was asked to note odour intensity on a 9-point scale and to describe the olfactory, visual (aspect), and tactile (hand touching) sensations perceived during examination of the cheese portions. Samples were then rated according to three texture attributes (cohesiveness, elasticity and perceptibility of the micro-structure: from unctuous to granular), three flavour attributes (acid, creamy, salty), colour, eyes occurrence, residual bitter taste, and overall flavour intensity. A 9-point rating scale was used with 0 = undetected, 1–2 = very low, 3–4 = low, 5–6 = medium, 7–8 = strong and 9 = very strong.

In addition, a consumer test was performed using 9-point hedonic scale ranging from "dislike extremely" to "like extremely" with all incremental categories labelled appropriately. For that, 114 (non-trained) consumers were asked to classify cheeses according to their overall liking: points 6–9 meant that the subject liked the sample; points 1–4 represented a dislike of the sample, while point 5 was taken as neither liking nor disliking of the sample.

2.7. Statistical analysis

Data analysis was carried out with SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Results of fat substrate analysis, cheese composition, microbiological counts and nitrogen fractions were compared by one-way analysis of variance. When differences were found ($\alpha < 0.05$), the least significant difference test (LSD) was applied to identify differing means groups.

Peptide profiles were analysed by principal component analysis (PCA). PCA is a non-supervised multivariate technique that allows easier interpretation of multivariate data sets, as it reduces the number of original variables into a fewer number of unobservable variables, called principal components, that are linear combinations of the original ones (Hair et al., 1999). The areas of peaks that showed the highest variation among samples were considered as independent variables for PCA, with standardization to a mean of zero and their original variances (covariance matrix) (Pripp et al., 2000). As for PCA samples, they consisted of all the cheeses in each experiment: C, L and P cheeses (three replicates of each) at 3, 30 and 60 days of ripening. In each analysis, principal components with an eigenvalue higher that the mean of eigenvalues were reserved (Hair et al., 1999).

3. Results

3.1. Fat substrate analysis

An increase of probiotic population, which paralleled a decrease of pH, was detected for all the probiotic cultures assayed during incubation in the fat substrate (Table 1). The decrease in pH caused by the mixed culture was most pronounced. As for microbial counts, the increase in bifidobacteria was always somewhat lower than that of lactobacilli, even when included in the mixed culture, where they showed their maximum increase. Lactobacilli also showed a higher increase when pre-incubated in the mixed culture: they reached similar final levels as those of the individual trials, even when the initial dose was lower (approximately 3×10^6 cfu mL^{-1} in the mixed culture versus 6×10^7 cfu mL^{-1} in the individual assays).

3.2. Gross composition and pH of cheeses

Fat matter content, total protein, dry extract and sodium chloride concentration in control and probiotic cheeses did not significantly differ. Similarly, significant differences in pH were not found, except for cheeses containing the probiotic mixed culture: in this case, pH of cheeses containing the pre-incubated culture was 4.84 ± 0.14 at 3 days of ripening, which is significantly lower than the pH of the other two types of cheese, control and probiotic cheeses with lyophilised culture (5.20 ± 0.03 and 5.09 ± 0.12, respectively). However, pH values in all the cheeses evened out after 30 days of ripening (data not shown).

3.3. Microbiological analysis of cheeses

Primary starter populations showed a similar evolution in all cheeses during ripening. An initial increase of approximately 1 log was observed between 0 and 3 days, then counts remained more or less constant at 10^9 cfu g^{-1} (data not shown).

Probiotic bacteria also increased significantly during the first days of ripening (between 1.2 and 2.5 log after 3 days), except for *B. lactis* in all cheeses and *L. acidophilus* in P_{mix} cheeses, in which case the increase was less than 0.8 log. Overall, the concentration of probiotic bacteria in P and L cheeses did not significantly differ. Only in cheeses made with the mixed starter *L. acidophilus* and *L. paracasei* counts were significantly higher in P cheeses than in L, and this was just observed at the beginning of the ripening (Fig. 1). At the end of the ripening, the counts of each probiotic strain assayed were similar in all cheeses, regardless their addition as single or mixed cultures, lyophilised or after pre-incubation. *L. paracasei* strain showed the highest levels, approximately 10^9 cfu g^{-1}, while *L. acidophilus* and *B. lactis* were at a lower cell concentration, approximately 10^8 cfu g^{-1} and 10^7 cfu g^{-1}, respectively.

Non-starter lactobacilli counts in control cheeses were below 10^6 cfu g^{-1} at 3 days of ripening, then increased up to approximately 5×10^7 cfu g^{-1} at the end of the ripening.

3.4. Proteolysis assessment

3.4.1. Soluble nitrogen and electrophoresis

Nitrogen content in all soluble fractions assayed significantly increased during ripening as a consequence of proteolysis in the cheese. Probiotic cultures addition did not influence on SN-pH 4.6 and SN-TCA, but some of the cultures significantly ($\alpha < 0.05$) increased SN-PTA level: these were *L. acidophilus* and the three-strain mixed culture (Fig. 2). Two homogenous groups of means, which differed along ripening time, were detected by the LSD test for SN-PTA of cheeses made with *L. acidophilus*: C_{Lac}/L_{Lac} and P_{Lac} at 3 days of ripening, and C_{Lac}/L_{Lac} and L_{Lac}/P_{Lac} at 30 and 60 days. In cheeses made with the mixed probiotic culture, SN-PTA of C_{mix}/L_{mix} differed from P_{mix} at 3 and 30 days of ripening, while C_{mix}, L_{mix} and P_{mix} had significantly different SN-PTA levels at the end of the ripening (60 days). Whether or not significant differences were found, control cheeses had always the lowest values of SN-PTA, and cheeses with the probiotic culture added after pre-incubation, the highest.

Primary proteolysis evidenced by urea-PAGE was similar for all cheeses at equivalent ripening times. Extensive hydrolysis of α_{s1}-casein and the concomitant increase of the α_{s1}(f24–199)-casein was observed during ripening, as a result of residual chymosin. On the contrary, β-casein remained almost intact during ripening and only a light, barely visible, band of γ-casein was observed, which indicated a low activity of plasmin (data not shown).

3.4.2. Peptide profiles

In general, all the peaks in the chromatograms increased during ripening. Some qualitative and quantitative differences among peptide profiles of cheeses with and without probiotics were detected by visual comparison, however, objective comparison of all the profiles obtained in each trial for a given probiotic culture was achieved by PCA analysis.

For *L. acidophilus* trial, PCA was applied to 14 peaks selected by visually matching the chromatograms. The two first components described about 89.8% of the total variation of sample: PC1 73.7% and PC2 16.1%. Grouping of samples according to type of cheese and

Table 1
pH values and probiotic cell counts during incubation and cold storage within a fat substrate (Mean and standard deviation of three replicates are reported).

Strain	Parameter	Incubation time (h)			
		0	2	5	20
Lactobacillus acidophilus	pH	6.55 ± 0.07^a	6.45 ± 0.07^a	6.23 ± 0.07^b	6.17 ± 0.07^b
	Log_{10} cfu mL^{-1}	7.61 ± 0.25^a	7.48 ± 0.12^a	7.78 ± 0.21^a	8.18 ± 0.16^b
Lactobacillus paracasei	pH	6.45 ± 0.03^a	6.30 ± 0.03^b	6.25 ± 0.03^c	6.25 ± 0.02^c
	Log_{10} cfu mL^{-1}	7.89 ± 0.30^a	8.32 ± 0.18^{ab}	8.21 ± 0.22^a	8.70 ± 0.28^b
Bifidobacterium lactis	pH	6.53 ± 0.03^a	6.20 ± 0.03^b	5.88 ± 0.16^c	5.88 ± 0.20^c
	Log_{10} cfu mL^{-1}	8.01 ± 0.21^a	8.06 ± 0.14^a	8.21 ± 0.18^a	8.15 ± 0.13^a
Mixed culture					
L. acidophilus+	pH	6.72 ± 0.04^a	6.15 ± 0.07^b	5.80 ± 0.10^c	5.65 ± 0.07^d
	Log_{10} cfu mL^{-1}	6.08 ± 0.25^a	6.75 ± 0.12^b	7.48 ± 0.15^c	7.98 ± 0.13^d
L. paracasei+		6.68 ± 0.14^a	6.88 ± 0.13^a	7.51 ± 0.17^b	8.15 ± 0.09^c
B. lactis		7.71 ± 0.14^a	8.26 ± 0.23^b	8.18 ± 0.16^b	8.26 ± 0.16^b

[a,b,c,d] Means in the same row with different superscript differ ($\alpha < 0.05$).

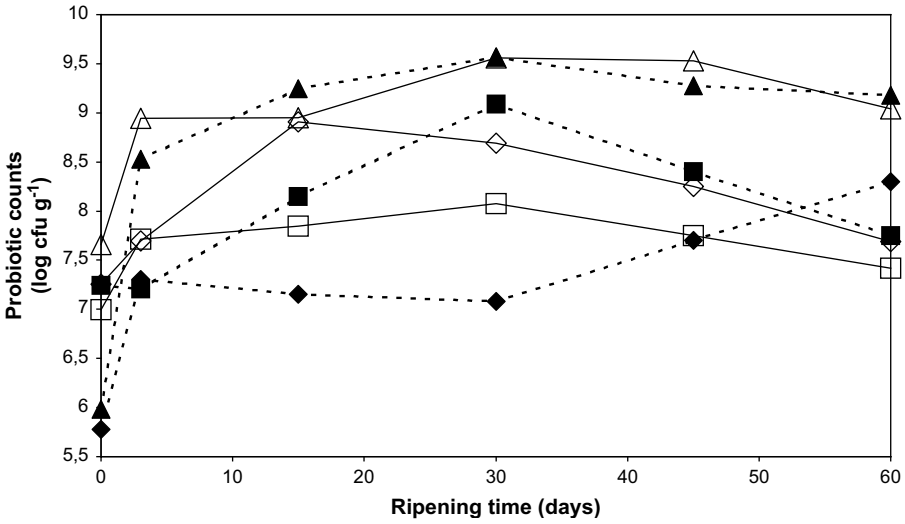

Fig. 1. Population of probiotic bacteria in cheeses: ◆, ◇, *L. acidophilus*; ▲, △, *L. paracasei*; ■, □, *B. lactis*. Mixed culture-added cheeses are shown; results from other assays were similar. Filled symbols: cheeses with probiotic bacteria added as a lyophilised culture; open symbols: cheeses with probiotic bacteria added after pre-incubation.

ripening time was observed in the score plot. Control cheeses (C_{Lac}) and cheeses with *L. acidophilus* added directly (L_{Lac}) were clearly differentiated from cheeses with pre-incubated probiotics (P_{Lac}) along PC1 and PC2 at 30 and 60 days of ripening; this trend was already evident for young cheeses (3 days). In addition, C_{Lac} was distinguished from L_{Lac} cheeses along PC2 in the score plot, above all at 30 and 60 days of ripening. Grouping by ripening time could also be based on the two first principal components; P_{Lac} cheeses of different age were separated on PC2, while C_{Lac} and L_{Lac} cheeses were principally differentiated by PC1. The 3 day old samples were characterised by low values for all variables and had lower inter-replicate variation than 30 and 60 day old samples. On the other hand, C_{Lac} and L_{Lac} cheeses were positively correlated with the variables with a high impact on PC1. In addition, peaks with a high impact on PC2 characterized the P_{Lac} cheeses. Finally, all cheeses were characterized at the end of the ripening by high values of the variables that showed high impact on both first principal components. Summarizing, *L. acidophilus* had a marked influence on cheese peptide profile, which was more evident when the probiotic culture was added after a pre-incubation step.

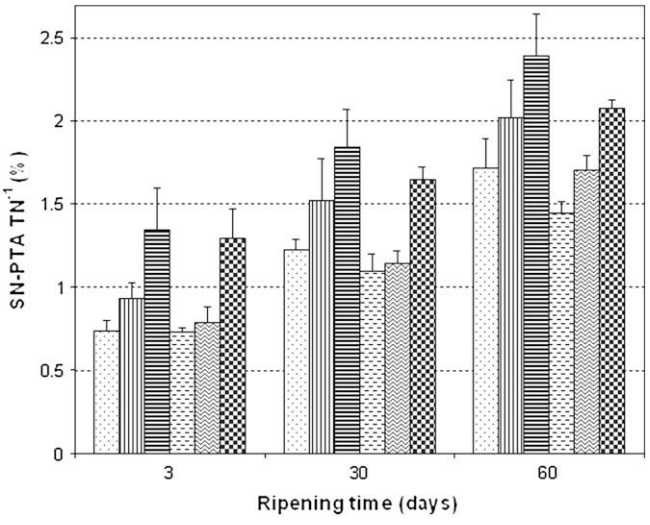

Fig. 2. Nitrogen content in cheese soluble fraction in 2.5% phosphotungstic acid (SN-PTA), expressed as the percentage of total nitrogen (TN), at 3, 30 and 60 days of ripening in trials with *L. acidophilus* (□, C_{Lac}; ▥, L_{Lac}; ▤, P_{Lac},) and the mixed culture (⬚, C_{mix}; ▨, L_{mix}; ▩, P_{mix}). C, control cheese without probiotics; L, cheese with probiotic bacteria added as a lyophilised culture; P, cheese with probiotic bacteria added after pre-incubation.

As for *Lactobacillus paracasei* trial, 12 peaks were selected from chromatograms for the PCA analysis. Two components explained about 93.7% of the total variance: PC1 83.8% and PC2 9.9%. As previously noted for *L. acidophilus*, grouping of samples according to ripening time was detected, in this case along PC1, and 3 day old samples showed low dispersion. On the contrary, samples of 30 and 60 days of ripening showed higher variability due both to cheese-making day and type of cheese. Nonetheless, differentiation between control and probiotic cheeses by PC2 was found, especially at the end of ripening. Low levels of all variables characterized the samples at 3 days of ripening. One peak exhibiting high positive impact on PC2 was characteristic of control cheeses (C_{Lpa}), while probiotic cheeses at the end of the ripening were positively correlated with variables with a high negative impact on PC2. In this trial, no differentiation was observed between cheeses with *L. paracasei* added directly or after a pre-incubation step (L_{Lpa} and P_{Lpa}, respectively).

Some of the trends found for probiotic lactobacilli were also observed in the experiment with *Bifidobacterium lactis*: samples were grouped according to the ripening time and inter-replicate variation increased with cheese age. However, unlike *L. acidophilus* and *L. paracasei*, Bifidobacteria did not influence cheese peptide profiles.

Sixteen peaks were chosen from peptide profiles of cheeses made with the mixed probiotic culture, then used as variables for PCA. Two components were identified, which described 91.3% of the total variance: PC1 68.9% and PC2 22.4%. The samples were clearly clustered in the score plot according to ripening time and type of cheese, both by PC1 and PC2. Probiotic cheeses in which the inocula were added after pre-incubation (P_{mix}) were grouped according to ripening time by PC2, while control (C_{mix}) cheeses and cheeses with the addition of a lyophilised probiotic culture (L_{mix}) were separated by age mainly based on PC1. However, P_{mix} cheeses were clustered separately from C_{mix} and L_{mix} cheeses, especially at 30 and 60 days of ripening, but the trend was already detected at 3 days. In addition, separate grouping of C_{mix} and L_{mix} cheeses was found for 30 and 60 day old cheeses. As before, 3 day old samples were characterized by low values of all variables. Peaks with a high impact on PC2 were typical of P_{mix} cheeses, while peaks that

exhibited high impact on PC1, described C_{mix} and L_{mix} cheeses. The mixed probiotic culture demonstrated a greater influence on the peptide profiles when it was added after pre-incubation step than when it was added directly as a lyophilised culture.

3.4.3. Free amino acid analysis

Most free amino acids increased significantly ($\alpha < 0.05$) in probiotic cheeses made with *L. acidophilus*, compared with their control cheeses, both at 3 and 60 days of ripening. In general, the largest differences were detected between P_{Lac} and C_{Lac} cheeses, while L_{Lac} cheeses showed intermediate values. The total amount of FAAs at 60 days of ripening was approximately 1.4 and 2.0 times higher in probiotic cheeses, L_{Lac} and P_{Lac} respectively, than in C_{Lac} cheeses. Similar results were obtained with the mixed culture, where the content of most FAAs in P_{mix} cheeses was at significantly higher ($\alpha < 0.05$) concentrations than in C_{mix} and L_{mix} cheeses. The total amount of FAAs at the end of the ripening was 1.3 and 2.2 times higher in L_{mix} and P_{mix} cheeses, respectively, than in C_{mix} cheeses (Table 2).

For probiotic cheeses with *L. paracasei*, significant ($\alpha < 0.05$) differences were observed for few amino acids at the end of the ripening, and the total amount of FAAs did not significantly increase, although the absolute amount of FAAs was slightly higher in probiotic cheeses than in control cheeses (Table 2). *B. lactis* did not show any influence on the release of FAAs (results not shown).

3.5. Sensory analysis

Overall, sensory analysis did not reveal significant differences among probiotic and control cheeses. All the attributes had low variation, mean values were always around the middle of the scale, and ranges were as follows: cohesiveness: 4.4–6.7, elasticity: 4.0–6.6, perceptibility of the micro-structure: 3.5–5.9, acid taste: 4.4–7.0, creamy flavour: 3.9–5.7, salty taste: 4.5–6.5, colour: 5.1–6.3. Although acid taste of probiotic cheeses manufactured with *L. acidophilus* and the mixed probiotic culture was somewhat higher than acid taste of control cheeses, the differences were not significant. Cheeses did not show any cracks or eyes. As for flavour intensity and residual flavour, all cheeses were mild-flavoured with very low residual bitterness (4.8–6.7 and 0.9–2.0, respectively).

Results of the consumer test are presented in Table 3. Degrees of liking did not differ among control and experimental cheeses for each probiotic strain tested, and about 80% of consumers tested liked all cheeses.

4. Discussion

4.1. Pre-incubation of probiotics

Previously, the addition of probiotic bacteria after pre-incubation on a fat substrate was evaluated as an approach to increase inoculum numbers and improve probiotic viability in the product (Shah, 2000). In this research growth and acidification rate and extent varied from one probiotic culture to another but all were viable and active when added to cheese–milk.

Lactobacilli, as do other lactic bacteria, possess a more complete proteolytic system than bifidobacteria, which enables them to hydrolyse casein and grow in a dairy medium (Boylston et al., 2004). These differences in proteolytic abilities can explain the fact that *L. acidophilus* and *L. paracasei* grew better than bifidobacteria during the pre-incubation step. Bifidobacteria, on the other hand, caused a significant decrease in pH, which probably led to growth inhibition; high acidifying activity by bifidobacteria has been attributed to the metabolic production of different acids in relatively high concentrations (Desjardins et al., 1990b). The strain of *B. lactis* used here has been shown to possess good viability during aerobic incubation in a high fat substrate, which suggested good tolerance to oxidative stress (Boylston et al., 2004).

Both positive and negative interactions can take place in a culture containing a high concentration of different bacteria (Boylston et al., 2004). In the present work, the results suggest that a synergist interaction occurred among the strains assayed, as higher increase was observed in their populations when they were incubated together as a mixed culture in the substrate.

4.2. Cheese gross composition, probiotic counts and primary proteolysis

A cheese model which is reproducible is required to detect differences among cheeses attributable to the addition of an adjunct culture. In our work, gross composition was similar in all cheeses, which provided the cultures with similar environmental conditions.

The microbial counts of all the strains used were above the minimum required for probiotic foods. During the ripening period in both experimental cheeses, *L. paracasei* was the most resistant to cheese environment, followed by *L. acidophilus* and *B. lactis*. The higher probiotic population in P cheeses at the beginning of ripening probably occurred as a consequence of higher inoculums, probiotic counts then remained more or less constant. However, probiotics in L cheeses increased markedly during the first days of ripening and rapidly attained similar levels to those in P cheeses. As a result, the direct addition of probiotics as a lyophilised culture was considered more efficient, as direct addition was easier, more rapid and less vulnerable to contaminations (Bergamini et al., 2005).

None of the tested probiotic cultures showed a significant contribution to primary proteolysis. Similar results were reported for other cheeses: Cheddar (Dinakar and Mistry, 1994; Gardiner et al., 1998), Gouda (Gomes et al., 1995), Minas fresh cheese (Buriti et al., 2005), and goat cheese (Gomes and Malcata, 1998). However, other authors have found that some probiotic cultures do influence primary proteolysis in other types of cheeses (Corbo et al., 2001; Gobbetti et al., 1998; Kasimoğlu et al., 2004; Ong et al., 2007).

4.3. Secondary proteolysis

Secondary proteolysis contributes to cheese flavour development principally by release of amino acids, the most important precursors of taste and aroma compounds (Yvon, 2006). In our work, the addition of probiotic cultures to Pategrás cheese did not increase the nitrogen content in TCA soluble fraction, which is mainly composed of peptides produced by the primary starter, although some are derived from chymosin activity (Rank et al., 1985). As for the other indexes of secondary proteolysis studied, i.e., SN-PTA, peptide profiles and FAAs, results varied from one probiotic culture to another.

B. lactis did not produce any detectable change when inoculated as a single culture. This is not surprising as low proteolytic activity has been reported for several bifidobacteria strains (Boylston et al., 2004; Shihata and Shah, 2000). Corbo et al. (2001) verified in Canestrato Pugliese cheese that neither the amino acid profile nor total amount was modified by the addition of two strains of bifidobacteria. Nevertheless, other bifidobacteria strains possess high peptidase activity (Desjardins et al., 1990a; Shihata and Shah, 2000).

L. paracasei strain used showed little influence on secondary proteolysis, as only at the end of ripening peptide profiles and a few FAA concentrations showed some changes. This is in contrast to several *L. casei* and *L. paracasei* strains that showed significant

Table 2
Levels of individual free amino acids (FAAs) and total FAAs in cheese (mg 100^{-1} g^{-1}) at 3 and 60 days of ripening in trials with *L. acidophilus* (*Lac*), *L. paracasei* (*Lpa*) and the mixed culture (mix)[a].

	Cheese[b]	Time	Asp	Ser	Glu	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Lys	Ile	Leu	Phe	Total FAA
L. acidophilus	C_{Lac}	3	0.27	1.50	5.59[a]	0.42	2.60[a]	4.33[a]	0.61	2.75[a]	4.97[a]	2.53	1.44	0.36[a]	4.84	0.74[a]	4.39[a]	3.94	45.20[a]
			(0.10)	(0.05)	(1.39)	(0.08)	(0.31)	(0.65)	(0.12)	(0.34)	(0.35)	(0.31)	(0.14)	(0.10)	(2.37)	(0.05)	(0.75)	(0.39)	(5.27)
	L_{Lac}		1.36	4.17	13.67[a]	0.73	7.13[a]	9.79[ab]	2.24	3.78[a]	9.50[ab]	4.61	4.21	0.90[ab]	8.68	2.12[ab]	12.73[ab]	7.29	96.45[ab]
			(0.08)	(2.06)	(7.15)	(0.31)	(2.92)	(4.75)	(1.55)	(0.72)	(2.91)	(1.65)	(2.06)	(0.44)	(4.11)	(1.08)	(6.57)	(2.83)	(40.81)
	P_{Lac}		2.46	4.78	33.08[b]	1.22	12.59[b]	16.78[b]	3.68	5.52[b]	16.24[b]	3.96	7.29	1.93[b]	13.28	4.63[b]	23.46[b]	7.95	162.99[b]
			(2.01)	(1.04)	(14.87)	(0.46)	(2.97)	(5.19)	(1.45)	(0.83)	(6.20)	(1.07)	(3.39)	(0.88)	(6.46)	(2.16)	(9.01)	(2.15)	(58.80)
	C_{Lac}	60	2.28	9.90	20.67	2.23	6.67[a]	19.22	3.97[a]	5.52[a]	7.97	6.26	9.74	2.92	13.27[a]	3.09[a]	29.11[a]	29.90	177.90
			(1.06)	(4.57)	(7.28)	(1.17)	(3.01)	(1.57)	(1.78)	(0.66)	(2.79)	(1.81)	(3.83)	(1.68)	(1.68)	(2.47)	(7.28)	(9.85)	(48.62)
	L_{Lac}		3.46	16.09	30.16	1.94	8.98[a]	24.73	6.31[ab]	7.59[ab]	17.33	8.26	12.32	3.26	22.74[ab]	5.08[a]	43.50[b]	32.64	250.30
			(1.43)	(1.97)	(18.59)	(0.19)	(1.89)	(5.17)	(0.60)	(1.41)	(10.67)	(2.07)	(0.72)	(0.46)	(6.12)	(0.90)	(3.36)	(2.87)	(31.01)
	P_{Lac}		6.17	15.96	44.45	3.94	19.56[b]	31.65	7.33[b]	14.14[b]	27.82	9.73	17.17	4.64	46.29[b]	12.82[b]	48.63[b]	30.33	346.69
			(3.91)	(2.12)	(17.32)	(2.48)	(6.62)	(7.35)	(1.01)	(5.62)	(21.65)	(1.16)	(4.07)	(0.98)	(19.67)	(2.31)	(5.26)	(2.85)	(64.55)
L. paracasei	C_{Lpa}	3	0.12	1.22	1.69	0.37	2.88	2.80	0.84	2.07	5.28	2.08	1.22	0.31	6.03	0.67	4.17	4.26	36.02
			(0.10)	(0.12)	(0.10)	(0.05)	(0.28)	(0.30)	(0.09)	(0.25)	(0.50)	(0.26)	(0.20)	(0.08)	(2.02)	(0.09)	(0.88)	(0.52)	(6.90)
	L_{Lpa}		0.55	1.30	3.07	0.57	2.64	5.47	0.47	1.77	6.55	2.84	1.44	0.37	7.13	0.66	4.10	3.61	42.53
			(0.04)	(0.45)	(2.15)	(0.13)	(0.59)	(1.46)	(0.18)	(0.36)	(3.30)	(0.69)	(0.01)	(0.09)	(0.37)	(0.08)	(1.79)	(1.26)	(7.02)
	P_{Lpa}		0.76	1.13	4.24	0.50	2.80	6.78	0.40	2.04	8.96	3.43	1.93	0.57	8.38	0.76	5.85	4.35	52.89
			(0.02)	(0.12)	(4.35)	(0.01)	(0.09)	(1.52)	(0.06)	(0.06)	(5.54)	(0.23)	(0.41)	(0.01)	(0.67)	(0.15)	(2.59)	(1.21)	(6.81)
	C_{Lpa}	60	3.95[a]	6.16	3.28	1.69[a]	4.67	13.22	1.89	2.74[a]	26.04	2.90[a]	6.77	1.59	13.68	1.38	27.41	24.45	141.81
			(0.60)	(0.47)	(0.79)	(0.03)	(0.16)	(12.16)	(0.08)	(0.56)	(3.54)	(1.61)	(1.16)	(0.11)	(2.67)	(0.42)	(2.20)	(5.19)	(22.33)
	L_{Lpa}		3.09[a]	7.35	9.47	2.10[a]	5.40	13.87	2.28	3.26[a]	19.20	5.83[b]	8.99	2.17	14.33	1.80	28.58	24.13	151.86
			(0.71)	(1.38)	(10.75)	(0.39)	(0.85)	(8.20)	(0.55)	(0.35)	(12.59)	(0.61)	(2.20)	(0.77)	(1.36)	(0.37)	(4.06)	(3.34)	(25.32)
	P_{Lpa}		1.27[b]	9.27	8.85	2.45[b]	6.82	15.13	2.30	6.16[b]	22.29	7.11[b]	10.38	2.83	15.96	2.16	34.12	25.19	172.30
			(0.35)	(1.32)	(9.56)	(0.31)	(0.23)	(4.67)	(0.12)	(0.13)	(8.35)	(0.76)	(0.75)	(0.43)	(0.42)	(0.41)	(1.98)	(1.91)	(3.26)
Mixed culture	C_{mix}	3	0.31[a]	1.50[a]	5.30	0.46	2.10[a]	4.51[a]	0.65[a]	2.30	4.71[a]	2.84	1.48[a]	0.40[a]	5.16[a]	0.73[a]	4.20[a]	3.91[a]	40.56[a]
			(0.26)	(0.24)	(0.97)	(0.06)	(0.74)	(0.39)	(0.08)	(0.49)	(0.27)	(0.48)	(0.18)	(0.13)	(0.26)	(0.08)	(0.32)	(0.50)	(2.21)
	L_{mix}		0.54[a]	2.42[a]	7.65	0.49	3.40[a]	7.27[a]	1.01[ab]	2.68	5.49[a]	3.88	2.01[a]	0.48[a]	7.49[a]	0.90[a]	7.52[a]	6.20[a]	59.42[a]
			(0.20)	(0.91)	(4.37)	(0.15)	(0.81)	(1.67)	(0.38)	(0.80)	(0.80)	(0.65)	(0.69)	(0.07)	(1.27)	(0.35)	(2.57)	(1.16)	(15.31)
	P_{mix}		4.34[b]	4.58[b]	29.24	1.43	10.32[b]	15.44[b]	2.89[b]	3.69	14.90[b]	5.09	6.50[b]	1.58[b]	16.24[b]	3.79[b]	20.85[b]	9.27[b]	150.16[b]
			(2.68)	(1.56)	(20.17)	(0.76)	(4.39)	(4.55)	(1.73)	(1.28)	(0.83)	(1.35)	(3.39)	(0.83)	(5.28)	(1.94)	(8.18)	(2.25)	(60.76)
	C_{mix}	60	2.82[a]	6.31[a]	8.69	1.38	3.97[a]	14.95[a]	2.50	4.16	13.80	6.13	7.74[a]	2.07[a]	9.45[a]	1.42[a]	26.82[a]	26.65	138.85[a]
			(0.24)	(0.51)	(8.52)	(0.09)	(1.39)	(6.94)	(0.24)	(0.99)	(5.20)	(1.21)	(0.34)	(0.15)	(1.93)	(0.03)	(1.94)	(3.52)	(18.71)
	L_{mix}		3.82[a]	10.34[b]	16.67	1.63	5.94[a]	22.44[ab]	2.51	4.91	14.19	7.84	10.44[a]	2.54[a]	16.18[a]	1.83[a]	33.66[a]	31.00	185.94[a]
			(0.30)	(2.60)	(15.78)	(0.51)	(1.70)	(5.14)	(1.02)	(1.41)	(6.26)	(2.49)	(2.66)	(0.24)	(4.99)	(0.98)	(5.96)	(5.65)	(45.27)
	P_{mix}		6.37[b]	13.15[b]	42.44	2.69	14.47[b]	29.48[b]	4.37	7.85	20.58	11.01	19.19[b]	4.49[b]	41.77[b]	6.56[b]	46.39[b]	34.01	304.81[b]
			(0.97)	(1.55)	(28.95)	(0.93)	(4.74)	(3.04)	(1.28)	(2.00)	(8.39)	(1.94)	(4.01)	(0.81)	(8.19)	(3.29)	(7.16)	(1.52)	(60.49)

[a] Mean and standard deviation of three cheeses (replicates) are reported. Different superscripts indicate significant differences ($\alpha < 0.05$) among means in a column, within each trial and for the same ripening time.
[b] C, control cheeses; L, cheeses with addition of probiotic bacteria directly as a lyophilised culture; P, cheeses with addition of probiotic bacteria after a pre-incubation. Subscripts identify the probiotic bacteria used in the different cheesemakes.

Table 3
Consumer test of probiotic and control cheeses ($n = 114$): degree of liking (mean and standard deviations) for the cheese samples.

Probiotic strain	Cheeses[a]		
	C	L	P
L. acidophilus	6.7 ± 1.3	6.7 ± 1.2	6.7 ± 1.4
L. paracasei	6.9 ± 1.4	6.7 ± 1.5	6.9 ± 1.4
B. lactis	6.8 ± 1.4	6.9 ± 1.4	6.7 ± 1.5
Mixed culture	6.6 ± 1.5	6.1 ± 1.8	6.4 ± 1.6

[a] C, control cheeses (without probiotics); L, cheeses with the addition of probiotic bacteria directly as a lyophilised culture; P, cheeses with the addition of probiotic bacteria after a pre-incubation step.

increases in FAA when used as adjunct cultures in other cheese varieties (Di Cagno et al., 2006; Gardiner et al., 1998; Hynes et al., 2001, 2003).

L. acidophilus had the most influence on secondary proteolysis. From the beginning of the ripening, its addition modified the peptide profiles, increased SN-PTA and the concentration of most FAAs. This impact was more evident when the culture was added after a pre-incubation step.

A remarkable finding of the present study is that secondary proteolysis patterns of probiotic cheeses made either with the single culture of *L. acidophilus* or the mixed three-strain probiotic culture were very similar. These results support the hypothesis that *L. acidophilus* increases secondary proteolysis. While a great number of peptidases, amino, di and tripeptidases, as well as proline-specific peptidases have been reported for several strains of *L. acidophilus* (Khalid and Marth, 1990; Shihata and Shah, 2000; Upadhyay et al., 2004). No previous reports about FAA production in cheese by *L. acidophilus* are available.

The greater influence of probiotic bacteria in P_{Lac} and P_{mix} cheeses is most probably due to probiotics inoculation method. Our results suggest that production of peptidases by *L. acidophilus* can be modified by growth conditions. Protein content in the rich-fat substrate was 5.2% versus 22.0% in the cheeses. Although the nitrogen compounds of the substrate were not fractionated, it is well known that the FAA and small peptide concentrations in the milk are low. However, these compounds increase in cheeses during ripening as a result of proteolysis. Therefore, the fat substrate supplied a poorer medium in small nitrogen compounds, possibly leading to a higher production of proteolytic and peptidolytic enzymes by *L. acidophilus* and, eventually, greater secondary proteolysis in P_{Lac} and P_{mix} cheeses.

Several authors have detected an influence of probiotics on the sensory characteristics of cheeses (Gomes et al., 1995; Gomes and Malcata, 1998; Kasimoğlu et al., 2004; McBrearty et al., 2001). In our work, sensory profiles of probiotic cheeses did not differ from those of control cheeses, indicating that the addition of the probiotic cultures and the differences in proteolysis did not impact texture and flavour during the 60 days of ripening. Thus, incorporation of probiotics to Pategrás cheeses had no adverse impact on their acceptability by the consumers.

5. Conclusions

Pategrás Argentino cheese showed excellent performance as a carrier for the probiotic cultures assayed: they could be easily added in cheese-making and maintained high counts during ripening.

Each culture influenced proteolysis differently; these results highlight the importance of testing a probiotic strain in a food when developing a new functional food. For Pategrás cheese, *B. lactis* did not impact proteolysis, *L. paracasei* showed a minor influence, and *L. acidophilus* increased the level of small nitrogen compounds and free amino acids, a desirable change that may lead to cheese ripening acceleration.

When a three-strain mixed culture was used, synergistic effects were observed, as *L. paracasei* and *L. acidophilus* reached higher levels in the substrate and showed improved growth in the cheeses. *L. acidophilus* seemed to increase secondary proteolysis when added as a constituent in the mixed culture.

Pre-incubation of probiotics did not enhance their viability in the food; however, for *L. acidophilus*, it modified biochemical activities. *L. acidophilus* caused a greater change in the proteolysis patterns of cheeses when added after a pre-incubation step, either alone or in the three-strain mixed culture.

Finally, the probiotic strains produced acceptable functional foods, with similar acceptability scores to regular Pategrás cheeses.

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References

Andrews, A.T., 1983. Proteinases in normal bovine milk and their action on caseins. Journal of Dairy Research 50, 45–55.
AOAC, 1990. Official method 985.35. Minerals in ready-to-feed milk-based infant formula. Atomic absorption spectrophotometric method. In: Heilrich, K. (Ed.)Official Methods of Analysis of Association of Official Analytical Chemists, fifteenth ed., Vol. II. AOAC, Arlington, Virginia, USA, p. 1110.
Bergamini, C., Hynes, E., Quiberoni, A., Suárez, V., Zalazar, C., 2005. Probiotic bacteria as adjunct starters: influence of the addition methodology on their survival in a semi-hard Argentinean cheese. Food Research International 38, 597–604.
Bergamini, C., Hynes, E., Zalazar, C., 2006. Influence of probiotic bacteria on the proteolysis profile of a semi-hard cheese. International Dairy Journal 16, 856–866.
Boylston, T.D., Vinderola, C.G., Ghoddusi, H.B., Reinheimer, J.A., 2004. Incorporation of bifidobacteria into cheeses: challenges and rewards. International Dairy Journal 14, 375–387.
Bradley, R.L., Arnold, E., Barbano, D.M., Semerad, R.G., Smith, D.E., Vines, B.K., 1993. Chemical and physical methods. In: Marshall, R. (Ed.), Standard Methods for the Examination of Dairy Products. American Public Health Association, Washington, DC, USA, pp. 433–531.
Buriti, F.C.A., da Rocha, J.S., Saad, S.M.I., 2005. Incorporation of *Lactobacillus acidophilus* in Minas fresh cheese and its implications for textural and sensorial properties during storage. International Dairy Journal 15, 1279–1288.
Corbo, M.R., Albenzio, M., De Angelis, M., Sevi, A., Gobbetti, M., 2001. Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. Journal of Dairy Science 84, 551–561.
Daigle, A., Roy, D., Bélanger, G., Vuillermard, J.C., 1999. Production of probiotic cheese (Cheddar-like cheese) using enriched cream fermented by *Bifidobacterium infantis*. Journal of Dairy Science 82, 1081–1091.
Desjardins, M.-L., Roy, D., Goulet, J., 1990a. Growth of bifidobacteria and their enzyme profiles. Journal of Dairy Science 73, 299–307.
Desjardins, M.-L., Roy, D., Toupin, C., 1990b. Uncoupling of growth and acids production in *Bifidobacterium* ssp. Journal of Dairy Science 73, 1478–1484.
Di Cagno, R., Quinto, M., Corsetti, A., 2006. Assessing the proteolytic and lipolytic activities of single strains of mesophilic lactobacilli as adjunct cultures using a Caciotta cheese model system. International Dairy Journal 16 (2), 119–130.
Dinakar, P., Mistry, V.V., 1994. Growth and viability of *Bifidobacterium bifidum* in Cheddar cheese. Journal of Dairy Science 77, 2854–2864.
El-Zayat, A.I., Osman, M.M., 2001. The use of probiotics in Tallaga cheese. Egyptian Journal of Dairy Science 29, 99–106.
Fox, P.F., 2003. In: Roginsky, H., Fuquay, J., Fox, P. (Eds.), Encyclopedia of Dairy Sciences. Cheese – Biochemistry of Cheese Ripening, Vol. 1. Academic Press, Reino Unido, Argentina, pp. 320–326.
Gardiner, G.E., Ross, R.P., Collins, J.K., Fitzgerald, G., Stanton, C., 1998. Development of a probiotic cheddar cheese containing human-derived *Lactobacillus paracasei* strains. Applied and Environmental Microbiology 64, 2192–2199.
Giraudo, M., Sánchez, H., Muset, G., Pavesi, R., Castañeda, R., Fernández, M., Nocedal, D., Markowski, I., Guirin, G., 2002. Determinación cuantitativa de aminoácidos libres en quesos Reggianito Argentino por derivatización con 6-AQC y RP-HPLC. Alimentaria 337, 121–126.
Gobbetti, M., Corsetti, A., Smacchi, E., Zocchetti, A., De Angelis, M., 1998. Production of Crescenza cheese by incorporation of bifidobacteria. Journal of Dairy Science 81, 37–47.

Gomes, A.M.P., Malcata, F.X., 1998. Development of probiotic cheese manufactured from goat milk: response surface analysis via technological manipulation. Journal of Dairy Science 81, 1492–1507.
Gomes, A.M.P., Malcata, F.X., Klaver, F.A.M., Grande, H.J., 1995. Incorporation and survival of *Bifidobacterium* sp. Strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. Netherlands Milk and Dairy Journal 49, 71–95.
Gripon, J.-C., Dezmazeaud, M.J., Le Bars, D., Bergere, J.L., 1975. Etude du role des micro-organismes et des enzymes au cours de la maturation des fromages. II. – Influence de la presure commerciale. Lait 55, 502–516.
Habibi-Najafi, M.B., Lee, B.H., 1994. Proline-specific peptidases of *Lactobacillus casei* subspecies. Journal of Dairy Science 77, 385–392.
Hair, J.F., Anderson, R.E., Tatham, R.L., Black, W.C., 1999. Análisis Multivariante; Chap. 3: Análisis Factorial. Prentice Hall Iberia, Madrid, Spain, pp. 79-123.
Heller, K.J., 2001. Probiotic bacteria in fermented foods: product characteristics and starter organisms. American Journal of Clinical Nutrition (supplement) 73, 374S–379S.
Hynes, E., Ogier, J.-C., Delacroix-Buchet, A., 2001. Proteolysis during ripening of miniature washed-curd cheeses manufactured with different strains of starter bacteria and a *Lactobacillus plantarum* adjunct culture. International Dairy Journal 11, 587–597.
Hynes, E., Ogier, J.-C., Son, O., Delacroix-Buchet, A., 2003. Influence of starter and adjunct lactobacilli culture on ripening of miniature washed-curd cheeses. Lait 83, 31–43.
IDF, 1982. Formaggio e formaggio fuso. Determinazione della materia secca. Metodo di riferimento N° 4 A. International Dairy Federation, Brussels, Belgium.
IDF, 1993. Latte. Determinazione del tenore in azoto. Metodo di riferimento N° 20 B. International Dairy Federation, Brussels, Belgium.
IDF, 1997. Lait produits laitiers. Determination de la teneur en matiere grasse. Guide de directives generales appliquees aux methodes butyrometriques. International Dairy Federation, Brussels, Belgium.
Kasimoğlu, A., Göncüoğlu, M., Akgün, S., 2004. Probiotic white cheese with *Lactobacillus acidophilus*. International Dairy Journal 14, 1067–1073.
Khalid, N.M., Marth, E.H., 1990. Lactobacilli – their enzymes and role in ripening and spoilage of cheese: a review. Journal of Dairy Science 73, 2669–2684.
Lane, C.N., Fox, P.F., Walsh, E.M., Folkertsma, B., McSweeney, P.L.H., 1997. Effect of compositional and environmental factors on the growth of indigenous non-starter lactic acid bacteria in Cheddar cheese. Lait 77, 561–573.
McBrearty, S., Ross, R., Fitzgerald, G., Collins, J., Wallace, J., Stanton, C., 2001. Influence of two commercially available bifidobacteria cultures on Cheddar cheese quality. International Dairy Journal 11, 599–610.
Ong, L., Henriksson, A., Shah, N.P., 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus, Lb. casei, Lb. paracasei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal 16, 446–456.
Ong, L., Henriksson, A., Shah, N.P., 2007. Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus, Lb. paracasei, Lb. casei* or *Bifidobacterium* sp. International Dairy Journal 17, 67–78.
Peterson, S.D., Marshall, R.T., Heymann, H., 1990. Peptidase profiling of lactobacilli associated with cheddar cheese and its application to identification and selection of strains for cheese-ripening studies. Journal of Dairy Science 73, 1454–1464.
Phillips, M., Kailasapathy, K., Tran, L., 2006. Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp.; *L. casei*, *L. paracasei* and *L. rhamnosus*) in Cheddar cheese. International Journal of Food Microbiology 108, 276–280.
Pripp, A.H., Stepaniak, L., Sørhaug, T., 2000. Chemometrical analysis of proteolytic profiles during cheese ripening. International Dairy Journal 10, 249–253.
Rank, T.C., Grappin, R., Olson, N.F., 1985. Secondary proteolysis of cheese during ripening: a review. Journal of Dairy Science 68, 801–805.
Ross, R.P., Fitzgerald, G., Collins, K., Stanton, C., 2002. Cheese delivering biocultures – probiotic cheese. Australian Journal of Dairy Technology 57, 71–78.
Roy, D., Mainville, I., Mondou, F., 1998. Selective enumeration and survival of bifidobacteria in fresh cheese. International Dairy Journal 7, 785–793.
Shah, N.P., 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. Journal of Dairy Science 83, 894–907.
Shihata, A., Shah, N.P., 2000. Proteolytic profiles of yoghurt and probiotic bacteria. International Dairy Journal 10, 401–408.
Upadhyay, V.K., McSweeney, P.L.H., Magboul, A.A.A., Fox, P.F., 2004. Proteolysis in cheese during ripening. In: Fox, P., McSweeney, P., Cogan, T., Guinee, T. (Eds.), Cheese: Chemistry, Physics and Microbiology. General Aspects, Vol. 1. Academic Press, Estados Unidos, Argentina, pp. 391–433.
Vinderola, C.G., Reinheimer, J.A., 1999. Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria. International Dairy Journal 9, 497–505.
Vinderola, C.G., Reinheimer, J.A., 2000. Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products. International Dairy Journal 10, 271–275.
Vinderola, C.G., Prosello, W., Ghiberto, D., Reinheimer, J.A., 2000. Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in Argentinian Fresco cheese. Journal of Dairy Science 83, 1905–1911.
Williams, A.G., Banks, J.M., 1997. Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. International Dairy Journal 7, 763–774.
Yvon, M., 2006. Key enzymes for flavour formation by lactic acid bacteria. Australian Journal of Dairy Technology 61, 16–24.