Multivariate analysis of proteolysis patterns differentiated the impact of six strains of probiotic bacteria on a semi-hard cheese

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ABSTRACT

The individual contribution of 6 strains of probiotic bacteria (3 of *Lactobacillus acidophilus* and 3 of the *Lactobacillus casei* group) to proteolysis patterns in a semi-hard cheese was assessed. Control cheeses (without probiotics) and 2 types of experimental cheeses (with the addition of probiotics either directly to milk or by a 2-step fermentation method) were manufactured. Cheeses containing *Lb. acidophilus* showed the most extensive peptidolysis, which was evidenced by changes in the peptide profiles and a noticeable increase of free amino acids compared with control cheeses. The strains of the *Lb. casei* group showed a lower contribution to cheese peptidolysis, which consisted mainly of free amino acid increase. Two-step fermentation improved peptidolytic activity for only one of the cultures of *Lb. acidophilus* tested. The addition of *Lb. acidophilus* strains into cheese may be suitable not only for their beneficial health effect but also for their influence on secondary proteolysis, consistent with acceleration of ripening and improved flavor formation.
Key words: multivariate analysis, secondary proteolysis, sensory characteristic, probiotic cheese

INTRODUCTION

An increasing number of probiotic foods have been developed in the last few years because of the interest of consumers in health-promoting foods. Fluid dairy products were the first carriers of probiotic bacteria (Heller, 2001), and they are still very popular. However, cheeses have been suggested as a more suitable food environment to hold probiotics.

Cheeses consist of a close protein matrix, which usually includes a high amount of fat; their pH ranges between 4.8 and 5.2, and they show high buffer capacity. All

these characteristics have been associated with the idea that probiotic bacteria will maintain viability better in cheese than in fermented milk (Gardiner et al., 1998; Ross et al., 2002; Boylston et al., 2004). Nevertheless, it is important to consider that cheese ripening generally covers a longer period of time than the shelf-life of fermented milks, and consequently, probiotic bacteria must remain viable for a longer time. In fact, cheese ripening can take as little as a few weeks or longer than a year, depending on the cheese type. So far, several types of cheeses (Cheddar, Gouda, Fresco, Pategrás, among others) have shown satisfactory performance in maintaining high levels of probiotic bacteria during ripening (Dinakar and Mistry, 1994; Gomes et al., 1995; Vinderola et al., 2000; Bergamini et al., 2005; Ong et al., 2006; Ong et al., 2007b).

However, probiotic bacteria in cheese are not inert ingredients. Probiotic lactobacilli, for instance, possess several peptidases, which can hydrolyze peptides to oligopeptides and free amino acids (**FAA**) and induce changes in the sensory properties of the cheese (Peterson et al., 1990; Habibi-Najafi and Lee, 1994; Williams and Banks, 1997; Shihata and Shah, 2000). Peptides and free amino acids are considered responsible for the background flavor of cheeses, and amino acids contribute principally as precursors of compounds of taste and aroma (McSweeney, 2004).

The influence of probiotic lactobacilli on cheese proteolysis has been studied on different types of cheeses: Cheddar cheese (Gardiner et al., 1998; Ong et al., 2006, 2007b), Minas fresh cheese (Buriti et al., 2005; Souza and Saad, 2009), Pecorino Foggiano cheese (Santillo and Albenzio, 2008), Gouda (Gomes et al., 1995), and Turkish white cheese (Kasimoğlu et al., 2004). However, only one strain of lactobacilli was studied in each work, with the exception of papers about Cheddar cheese, in which several strains have been tested in each case. Additionally, cheese proteolysis was studied mainly by nitrogen fractions and electrophoresis, whereas FAA and peptide profiles were determined in few works (Gardiner et al., 1998). Argentinean probiotic cheese ripening has never been characterized, although probiotic cheeses are already commercialized in our country,

the world's seventh largest cheese producer (Fox, 2003). It is important to note that, besides information about probiotic cheeses, there is much information about the effect of the addition of non-probiotic lactobacilli in cheeses (Menéndez et al., 2000; Martínez-Cuesta et al., 2001; Hynes et al., 2003; Thage et al., 2005; DiCagno et al., 2006; Morea et al., 2006; Milesi et al., 2008, among others); however, few works included *Lb. acidophilus* strains (Santillo et al., 2007).

In previous reports, we showed that Pategrás cheese was a suitable carrier for probiotic bacteria for at least 60 d of ripening. Additionally, we detected a strain of *Lactobacillus acidophilus* that strongly influenced cheese peptidolysis, and a strain of *Lactobacillus paracasei* that did not show any effect (Bergamini et al., 2005, 2006). In the present work, we compared the proteolysis and peptidolysis caused by 6 strains of probiotic lactobacilli in cheese by means of a multivariate approach. In addition, we investigated if the methodology of addition had an influence on the peptidolytic activity of each strain.

MATERIALS AND METHODS

Cheese Making

Six probiotic cultures were assayed as single adjunct cultures in 6 cheese-making trials. In each trial, 3 types of Pategrás cheeses were manufactured: control cheeses without probiotics (**C** cheeses), probiotic cheeses with the direct addition of probiotic bacteria as a lyophilized culture into cheese milk (**L** cheeses), and probiotic cheeses with the addition of probiotic bacteria by means of a 2-step fermentation procedure (**P** cheeses). At least 2 replicate cheese makings were performed on different days for each culture and using different cheese milk. Pategrás Argentino cheese was selected as a representative model of semi-hard Argentinean cheeses, whose production volume reached 152,313 t in 2006, 33% of the total cheese production in the country (Lavabre, 2006). The cheeses were manufactured according to industrial technology (adapted to pilot scale) with a large pool of pasteurized milk (Bergamini et al., 2006). Cheeses were ripened for 2 mo at 12°C and 80% relative humidity.

Cultures

Six strains of probiotic bacteria were used as adjunct cultures, 3 belonging to the species *Lb. acidophilus* (A1, A2, and A3) and 3 from the *Lactobacillus casei* group: strains of *Lb. paracasei* ssp. *paracasei* (C1), *Lb. casei* (C2), and *Lactobacillus rhamnosus* (C3). Five of the 6 strains assayed were lyophilized commercial cultures

and their suppliers claimed that they showed good survival during passage through the gastrointestinal tract and possessed probiotic properties. The companies that provided the commercial probiotic strains cannot be mentioned for confidentiality reasons. The remaining strain (*Lb. rhamnosus*) belongs to the culture collection of the Instituto de Lactología Industrial (Santa Fe, Argentina), and was selected on the basis of its technological and probiotic properties (Villarreal, 2002). This strain was cultured at 37°C in sterile de Man, Rogosa, and Sharpe broth and then centrifuged at 3,000 × *g* for 10 min. The pellet containing the bacteria was used similarly to the commercial cultures.

A milk fat rich medium containing 14% (wt/vol) fat and 5.2% (wt/vol) proteins was prepared and heat-treated according to Bergamini et al. (2005) for the first step of probiotic fermentation for P cheeses. Probiotic culture was inoculated into 1 L of 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 amount of probiotic culture was used for the manufacture of L and P cheeses, independently of the potential variation of population during incubation of the substrate. Thus, probiotic cultures were added to reach approximately 10^6 cfu/mL of cheese milk (45 L) for L cheeses and 5×10^7 cfu/mL of the substrate (1 L) for the manufacture of P cheeses.

Gross Composition and pH of Cheeses

Gross composition was determined in 3-d-old cheeses, except for salt-in-moisture, for which 30-d-old cheeses were analyzed, and for pH, which was also monitored at 30 and 60 d of ripening. Moisture (oven drying at 102 ± 1°C), fat matter (butyrometer), and protein content (Kjeldahl method) were analyzed according to International Dairy Federation standard methods (IDF, 1982, 1997, 1993, respectively). The pH was measured according to American Public Health Association standard (Bradley et al., 1993). Sodium chloride content was analyzed following a spectrophotometric method (AOAC, 1990; method 985.35).

Microbiology of Cheeses

Lactobacilli probiotic counts were determined on cheese samples during ripening. They were plated onto de Man, Rogosa, and Sharpe agar and were enumerated after 48 h of incubation at 37°C in aerobiosis (Bergamini et al., 2005).

Assessment of Proteolysis

Peptide Profiles Analysis by Reverse Phase HPLC. The HPLC equipment consisted of a qua-

ternary pump, an on-line degasser, and UV visible detector, all Series 200, purchased from Perkin Elmer (Norwalk, CT). An interface module connected to a computer was used for acquisition of chromatographic data with the software Turbochrom (Perkin Elmer). A 220 mm × 4.6 mm Aquapore OD-300 C18, 5 µm – 300 A° analytical column (Perkin Elmer) was used. Water-soluble extracts of the cheeses at 60 d of ripening 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 obtained at 214 nm, column temperature was 40°C, and flow rate was 1 mL/min (Bergamini et al., 2006).

FAA Assessment. A precolumn derivatization method using 6-aminoquinolyl-N-hydroxi-succinimidyl carbamate (AQC) followed by HPLC was used for the determination of FAA in cheese samples, employing the Chemistry Package of the Waters AccQ·Tag Amino Analysis Method (Waters Corporation, Milford, MA). This package comprises the reagent kit for the derivatization reaction, the column, a standard mixture of AA, sample tubes, and the eluents. The HPLC equipment was the same that was used for determination of the peptide profiles. A 3.9 × 150 mm Nova-Pak C_{18}, 4-µm column (Waters) specifically certified for use with the AccQ·Tag Method and a 15 × 3.2 mm, 7-µm guard column (Perkin Elmer) were used, and the system temperature was set at 37°C. Detection was achieved at 248 nm and flow rate was 1 mL/min. Mobile phases used for the separation were: acetate-phosphate buffer pH 5.02 (A), and acetonitrile/H_2O 60/40 (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% 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). An internal standard was included (L-2-aminobutyric acid, Sigma, St. Louis, MO), and calibration curves for each amino acid were built using a standard mixture (Waters). The derivatization reaction was carried out on adequately diluted cheese samples and standard solutions according to the method, and then 20 µL of derivatized samples were injected into the HPLC chromatograph. Free amino acids were determined on samples at 3 and 60 d of ripening.

Sensory Analysis

Cheeses at 60 d of ripening were analyzed by the difference-from-control test (Meilgaard et al., 2006). The panel was composed of 20 assessors, familiar with cheese science and technology, although untrained in sensory analysis, which is allowed by the selected method. During each session, 2 probiotic cheeses (L and P cheeses) and one blinded control cheese (C, without probiotics) were simultaneously compared by each panelist with a control cheese. The panel assessed 3 attributes: flavor intensity, acid taste, and global texture; assessors were asked to quantify differences between each sample and the control. A 9-point rating scale was used with 0 = no difference and 9 = extremely different. Cheeses were removed from cold storage, kept at room temperature for 1 h and cut into portions (~25 g) before sensory evaluation. The outer layer of the cheese (1 cm) was removed. Cheese portions were covered with glasses and labeled with randomized 3-number codes. Mineral water and bread were provided to the panelists to rinse their mouth between samples.

Statistical Analysis

Data analysis was carried out with SPSS 10.0 (SPSS Inc., Chicago, IL). Results of cheese composition, FAA, and sensory analysis were compared by ANOVA. For significant differences ($P < 0.05$), the Duncan's test was applied to identify groups of homogeneous means.

Peptide profiles and FAA were analyzed by principal component analysis (**PCA**) with standardization to a mean of zero and to a standard deviation of one (correlation matrix). For PCA, the peaks of peptide profiles that showed the highest variation among samples were selected, and their areas considered as independent variables (Pripp et al., 2000). Selected peaks were identified with the letters a to q, in alphabetical order (Figure 1). The concentrations of FAA, expressed as milligrams per 100 grams of cheese, were considered as entry variables for PCA. In all cases, principal components with an eigenvalue greater than 1 were retained.

Peptide profiles and FAA were also analyzed by hierarchical cluster analysis (**HCA**), where linkage between groups was chosen as the amalgamation rule. The linkage distance was calculated as the squared Euclidean distance between cheese samples in the space defined by the area of selected peaks or the concentration of each FAA.

RESULTS AND DISCUSSION

Gross Composition and pH

Fat matter content, total protein, moisture, and salt-in-moisture in control and probiotic cheeses in each trial were not significantly different (Table 1). Similarly, significant differences in pH between control and probiotic
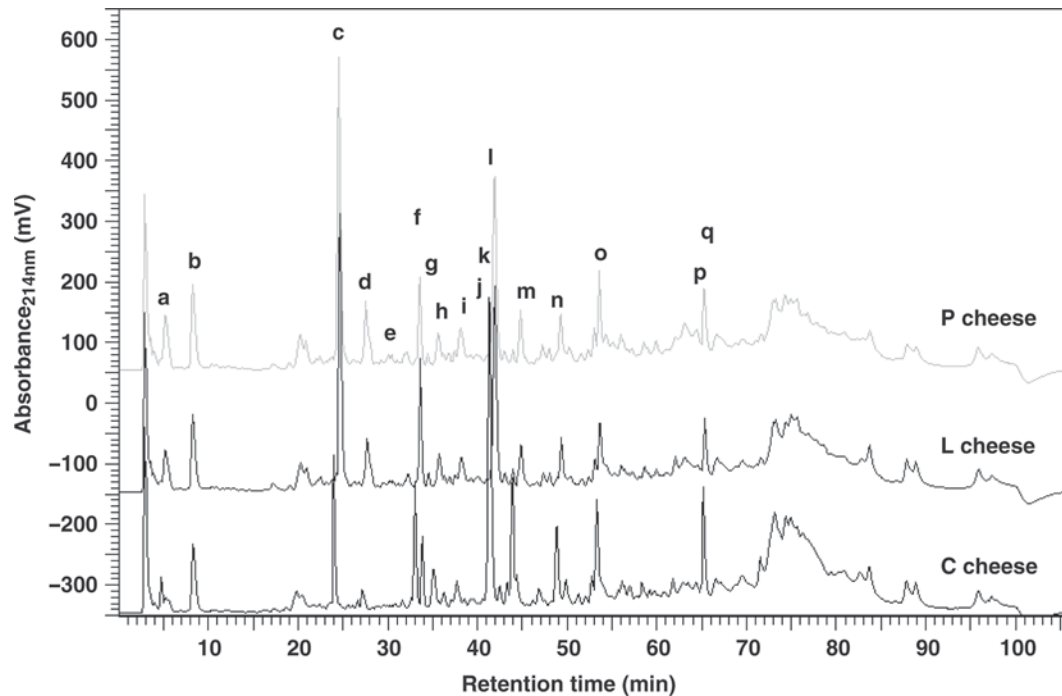
Figure 1. Reverse phase liquid chromatography profiles of water-soluble extract of control (C cheese) and probiotic cheeses with the direct addition of *Lactobacillus casei* C2 (L cheese) and by a 2-step fermentation method (P cheese) at 60 d of ripening. Letters a to q indicate the peaks selected for principal component analysis.

cheeses were not found, except for cheeses containing *Lb. acidophilus* A1 or *Lb. acidophilus* A2, whose pH differed ($P < 0.05$) from that of C cheeses at 3 and 60 d of ripening, respectively (Table 1). For *Lb. acidophilus* A1, only the pH of P cheeses had a significantly lower pH than C cheeses, whereas L cheeses showed an intermediate value. For *Lb. acidophilus* A2, pH of both types of probiotic cheeses (L and P) differed from control cheeses.

These results reveal the contribution to acidification of *Lb. acidophilus* A1 and *Lb. acidophilus* A2, which is an important characteristic of the cultures, as it can affect not only cheese composition, but also the activity of proteolytic enzymes during ripening and the final texture of the product (Gobbetti et al., 1999; Watkinson et al., 2001). The influence of both *Lb. acidophilus* strains on cheese pH was detected only at one sampling point, and therefore the possible effect on enzymatic activities during ripening should be low. As for cheese texture, changes in pH are implicated in calcium equilibrium and this, in turn, strongly affects cheese texture (O'Mahony et al., 2005). Large changes in pH may impair the correct development of the cheese body (Hynes et al., 1999), but information about slight or temporary pH changes during cheese ripening and their relation with cheese texture are lacking. The decrease of pH in cheeses with probiotic bacteria has been reported

previously (Gobbetti et al., 1998; El-Zayat and Osman, 2001; Mc Brearty et al., 2001; Ong et al., 2006).

Microbiology Analysis

Counts of all the strains of probiotic bacteria assayed remained above the minimum required for a probiotic food (Table 2). No significant differences were found in lactobacilli populations between both types of probiotic cheeses in all trials. Consequently, the 2-step fermentation procedure did not increase the concentration of probiotics in cheeses.

Peptide Profiles

In a previous work (Bergamini et al., 2006), we detected grouping of samples (control and probiotic cheeses) principally by ripening time through PCA of peptide profiles at 3, 30, and 60 d of ripening. In that work, we found low variation among samples at the beginning of the ripening, whereas 30- and 60-d-old cheeses were more variable according to the type of cheese or cheese making day. In the present work, we applied PCA and HCA to peptide profiles of 60-d-old cheeses, merging samples from all trials, to distinctively assess the influence of each probiotic strain on proteolysis patterns. In this way, we compared control cheeses

Table 1. Gross composition and pH of control and probiotic cheeses (means ± SD)

Probiotic strain	Cheese type[1]	pH 3 d	pH 30 d	pH 60 d	Fat matter (% wt/wt)	Dry extract (% wt/wt)	Total protein (% wt/wt)	NaCl in moisture (% wt/wt)
Lactobacillus	C	5.25[a] ± 0.05	5.15 ± 0.14	5.15 ± 0.14	28.7 ± 2.2	55.7 ± 1.4	22.01 ± 1.14	3.34 ± 0.46
acidophilus A1	L	5.08[ab] ± 0.08	4.98 ± 0.18	5.00 ± 0.14	29.0 ± 3.0	55.0 ± 2.4	22.25 ± 0.49	3.85 ± 0.13
	P	4.92[b] ± 0.16	4.85 ± 0.01	4.90 ± 0.07	29.5 ± 2.2	55.2 ± 0.8	21.71 ± 0.61	3.61 ± 0.19
Lactobacillus	C	5.35 ± 0.01	5.31 ± 0.01	5.29[a] ± 0.19	29.0 ± 0.1	54.6 ± 2.1	21.62 ± 0.86	3.64 ± 0.16
acidophilus A2	L	5.30 ± 0.01	5.08 ± 0.32	4.93[b] ± 0.04	28.3 ± 0.4	54.2 ± 0.2	21.37 ± 0.29	3.76 ± 0.22
	P	5.17 ± 0.21	5.03 ± 0.20	4.92[b] ± 0.05	27.3 ± 1.0	54.3 ± 0.9	21.48 ± 0.13	3.13 ± 0.30
Lactobacillus	C	5.27 ± 0.24	5.33 ± 0.19	5.09 ± 0.15	30.0 ± 1.0	55.8 ± 1.5	21.31 ± 0.80	3.28 ± 0.21
acidophilus A3	L	5.33 ± 0.24	5.14 ± 0.21	5.02 ± 0.05	30.3 ± 0.6	56.8 ± 0.4	22.17 ± 0.62	2.73 ± 0.24
	P	5.28 ± 0.31	5.03 ± 0.03	4.99 ± 0.04	30.3 ± 0.6	57.0 ± 0.8	21.58 ± 0.32	3.18 ± 0.29
Lactobacillus	C	5.20 ± 0.07	4.98 ± 0.04	5.15 ± 0.07	27.0 ± 1.4	54.6 ± 1.1	21.63 ± 1.89	3.69 ± 0.17
paracasei C1	L	5.15 ± 0.18	4.98 ± 0.06	5.17 ± 0.08	28.0 ± 1.1	54.6 ± 0.4	21.50 ± 1.08	3.42 ± 0.53
	P	5.05 ± 0.10	4.98 ± 0.06	5.07 ± 0.03	28.5 ± 1.3	55.2 ± 1.4	21.47 ± 0.50	3.16 ± 0.28
Lactobacillus	C	5.37 ± 0.08	5.14 ± 0.13	5.08 ± 0.07	29.5 ± 1.3	56.0 ± 0.7	21.77 ± 0.35	3.15 ± 0.57
casei C2	L	5.31 ± 0.02	4.93 ± 0.05	5.09 ± 0.11	28.3 ± 0.6	55.2 ± 1.1	21.95 ± 0.10	3.03 ± 0.32
	P	5.24 ± 0.23	4.90 ± 0.11	5.05 ± 0.02	28.5 ± 1.3	55.2 ± 0.5	21.08 ± 0.23	2.75 ± 0.13
Lactobacillus	C	5.19 ± 0.07	5.28 ± 0.05	5.03 ± 0.04	29.0 ± 0.1	55.4 ± 0.1	21.25 ± 0.42	3.05 ± 0.78
rhamnosus C3	L	5.16 ± 0.01	4.95 ± 0.04	4.93 ± 0.03	28.8 ± 0.4	55.8 ± 0.4	22.05 ± 0.61	3.05 ± 0.38
	P	5.05 ± 0.01	4.95 ± 0.14	4.94 ± 0.04	29.8 ± 0.4	56.1 ± 0.3	21.18 ± 0.10	2.79 ± 0.01

[a,b]For each strain, means with a different superscript within a column significantly differ ($P < 0.05$).

[1]C = control cheeses without probiotic bacteria; L = probiotic cheeses with the direct addition of probiotic bacteria as a lyophilized culture; P = probiotic cheeses with the addition of probiotic bacteria by a two-step fermentation method.

with probiotic cheeses, and probiotic cheeses containing different strains among themselves. Additionally, we examined whether the methodology of probiotics addition had an influence on peptide profiles. Five principal components were extracted, which explained 77.2% of the total variance. Score plots for PC1 versus PC2 and PC3 versus PC4 are presented in Figures 2 and 3. Additionally, similar samples clustered by HCA are shown enclosed by rectangles in the score plots.

Principal component analysis and HCA showed that peptide profiles of most probiotic cheeses were different from their respective control cheeses. Cheeses with different probiotic strains also grouped separately, and addition methodology showed some influence as well (Figures 2 and 3). Hierarchical cluster analysis clustered the samples in 5 groups: 1) cheeses with *Lb. acidophilus* A1 (L and P) and *Lb. acidophilus* A3 (only P cheeses); 2) cheeses with *Lb. acidophilus* A2; 3) cheeses with *Lb. paracasei* C1; 4) cheeses with *Lb. casei* C2; and 5) control cheeses and probiotic cheeses with *Lb. rhamnosus* C3 (L and P) and *Lb. acidophilus* A3 (L cheeses). The effect of *Lb. acidophilus* strains on cheese peptide profiles was the strongest, as the samples containing these cultures (except L cheeses with *Lb. acidophilus* A3) were grouped separately from the others on the 2 first principal components, which explained most of the total variation of data (Figure 2). Peaks that eluted early during the chromatographic separation characterized these samples; they are considered hydrophilic peptides (data not shown). In contrast, control cheeses and probiotic cheeses with strains of the *Lb. casei* group (identified as groups 3–5), were characterized by peaks

that eluted only after the gradient has reached a high acetonitrile concentration (retention times longer than 35 min) and therefore are considered as hydrophobic peptides (data not shown).

Decrease in the ratio of hydrophobic/hydrophilic peptides during ripening has been reported for several types of cheeses (Saldo et al., 2002; Picon et al., 2005; Morea et al., 2006; Bergamini, 2007). This is generally considered a favorable biochemical event, because hydrophobic peptides are usually bitter and therefore impact negatively on sensory characteristics of the product (Monnet and Gripon, 1997). In this way, the influence of the *Lb. acidophilus* strains assayed in this work on the ratio of hydrophobic/hydrophilic peptides can be evaluated as beneficial, as it is consistent with an acceleration of ripening and a potential debittering effect. Several strains of lactobacilli have been shown

Table 2. Probiotic counts in 60-d-old L and P probiotic cheeses (means ± standard deviation)

Probiotic strain	Cheese type[1]	
	L	P
Lactobacillus acidophilus A1	7.78 ± 0.74	8.09 ± 0.33
Lactobacillus acidophilus A2	7.91 ± 0.29	8.50 ± 0.91
Lactobacillus acidophilus A3	8.08 ± 0.53	7.98 ± 0.71
Lactobacillus paracasei C1	9.11 ± 0.36	9.23 ± 0.20
Lactobacillus casei C2	9.13 ± 0.13	8.83 ± 0.12
Lactobacillus rhamnosus C3	8.59 ± 0.90	9.15 ± 0.35

[1]L = probiotic cheeses with the direct addition of probiotic bacteria as a lyophilized culture; P = probiotic cheeses with the addition of probiotic bacteria by a two-step fermentation method.
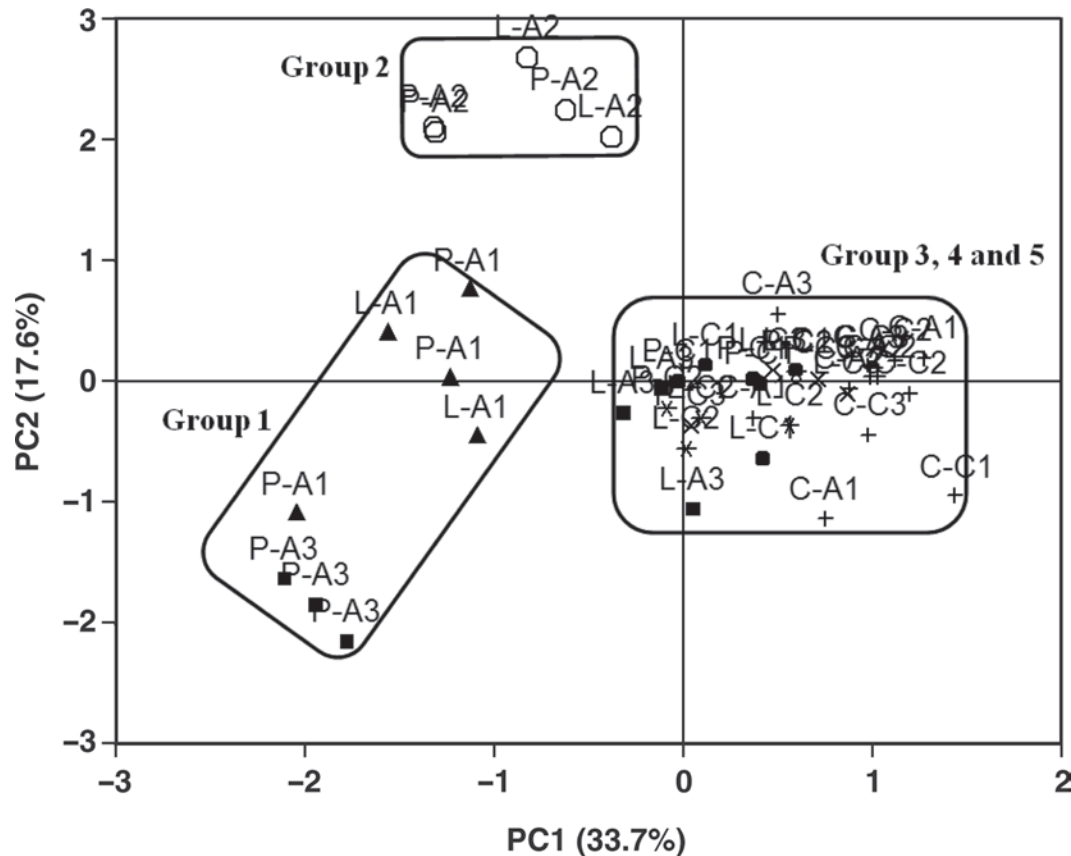
Figure 2. Principal component (PC) analysis of peptide profiles: score plot (PC1 vs. PC2) of 60-d-old cheese samples: control cheeses (+) and probiotic cheeses with *Lactobacillus acidophilus* A1 (▲), *Lb. acidophilus* A2 (○), *Lb. acidophilus* A3 (■), *Lactobacillus paracasei* C1 (●), *Lactobacillus casei* C2 (*), and *Lactobacillus rhamnosus* C3 (×). C = control cheeses without probiotics; L = cheeses with probiotic bacteria added directly as a lyophilized culture; P = cheeses with probiotic bacteria added by a 2-step fermentation method. Rectangles enclose cheese samples in a cluster by hierarchical cluster analysis.

to possess peptidolytic enzymes that are able to hydro-lyze bitter/hydrophobic peptides (Peterson et al., 1990; Macedo et al., 2000; Martínez-Cuesta et al., 2001).

Principal component analysis of peptide profiles also showed that samples containing different strains of *Lb. acidophilus* were grouped separately, A3 and A1 were distinct from A2, which we identified as groups 1 and 2 (Figure 2), respectively. These results indicate that each *Lb. acidophilus* strain tested in this work showed a distinctive effect on cheese peptidolysis, which can be explained by their specific and strain-dependent enzymatic pool. On the other hand, an interesting finding was that *Lb. acidophilus* A3 showed a distinguishable effect on peptidolysis only when 2-step fermentation was carried out: P cheeses (group 1) were clustered separately from L cheeses, which were grouped together with control cheeses (group 5). As was mentioned, the addition methodology did not influence probiotic viability for any of the strains assayed. Thus, the different effect of *Lb. acidophilus* A3 on peptidolysis of P cheeses

most probably depends on the addition methodology and not in probiotics count. Either the differences in the cell physiological state at the moment of their addition to cheese milk or the composition of the growth medium may be the cause of the differences observed (Habibi-Najafi and Lee, 1994; Gilbert et al., 1997; Williams et al., 2002; Savijoki et al., 2006). Therefore, 2-step fermentation may be a suitable approach to obtain an increased peptidolysis by this particular strain on semi-hard cheeses.

The influence of *Lb. paracasei* C1 and *Lb. casei* C2 on peptide profiles was less than that of the *Lb. acidophilus* strains, as they were differentiated from control cheeses and the rest of probiotic cheeses on PC3, which described 13.3% of total variance (Figure 3). In addition, these 2 strains were grouped separately on PC4, which explained only 6.4% of variance, and they are identified as groups 3 and 4 in the score plot. All probiotic cheeses containing *Lb. rhamnosus* C3 and one-step fermented cheeses with *Lb. acidophilus* A3 were the
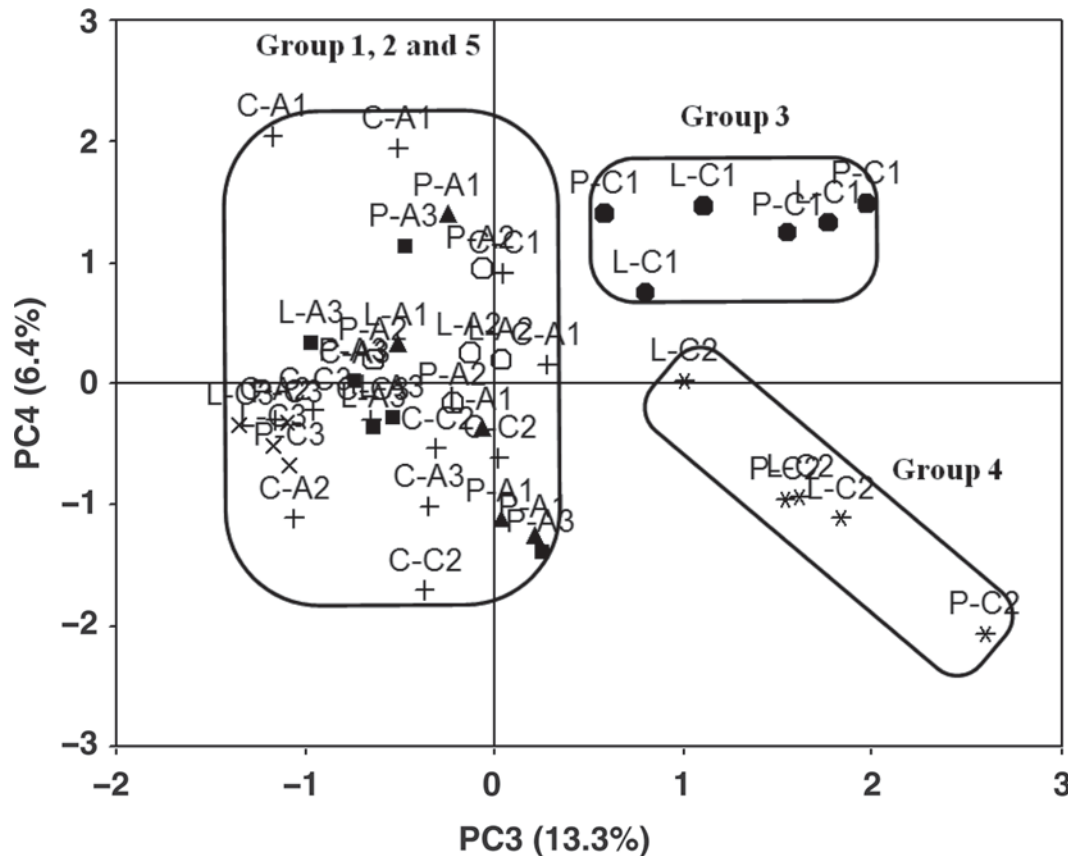
Figure 3. Principal component (PC) analysis of peptide profiles: score plot (PC3 vs. PC4) of 60-d-old cheese samples: control cheeses (+) and probiotic cheeses with *Lactobacillus acidophilus* A1 (▲), *Lb. acidophilus* A2 (○), *Lb. acidophilus* A3 (■), *Lactobacillus paracasei* C1 (●), *Lactobacillus casei* C2 (*), and *Lactobacillus rhamnosus* C3 (×). C = control cheeses without probiotics; L = cheeses with probiotic bacteria added directly as a lyophilized culture; P = cheeses with probiotic bacteria added by a 2-step fermentation method. Rectangles enclose cheese samples in a cluster by hierarchical cluster analysis.

exceptions—their sample scores were grouped together with the control cheeses.

Secondary proteolysis has been assessed by specific methods only in a small proportion of the existent studies on probiotic cheeses. Gardiner et al. (1998) and McBrearty et al. (2001) did not detect any influence of probiotic cultures of lactobacilli and bifidobacteria on chromatograms obtained by size exclusion HPLC of water-soluble peptides (pH 4.6-soluble N) of Cheddar cheese. In contrast, Corbo et al. (2001) verified that peptide profiles obtained by reversed-phase fast protein liquid chromatography of the soluble fraction of probiotic Canestrato Pugliese cheeses (sheep cheeses) containing bifidobacteria were more complex than those of control cheese. In our work, we obtained peptide profiles by HPLC and applied multivariate techniques on the obtained data. Principal component analysis and HCA showed that probiotic and control cheeses were clustered separately, with a few exceptions.

On the other hand, other authors have found an increase of proteolysis in cheeses caused by the addition of probiotic lactobacilli, but they described proteolysis by other indexes, such soluble-nitrogen fraction or electrophoresis (El-Zayat and Osman, 2001; Ong et al., 2006, 2007b; Santillo and Albenzio, 2008).

FAA

The total amount of FAA (expressed as the sum of the individual contents of all the FAA assessed) increased by 2 to 4 times during the ripening of the cheeses (Figure 4). The total amount of FAA in cheeses with *Lb. acidophilus* was double that of control cheeses, both at 3 and 60 d of ripening, except for the samples with *Lb. acidophilus* A3 added directly as a lyophilized culture (L cheeses). These cheeses, as well as those containing *Lb. casei* C2, also showed an increase of FAA at 3 and 60 d of ripening compared with control cheeses, but
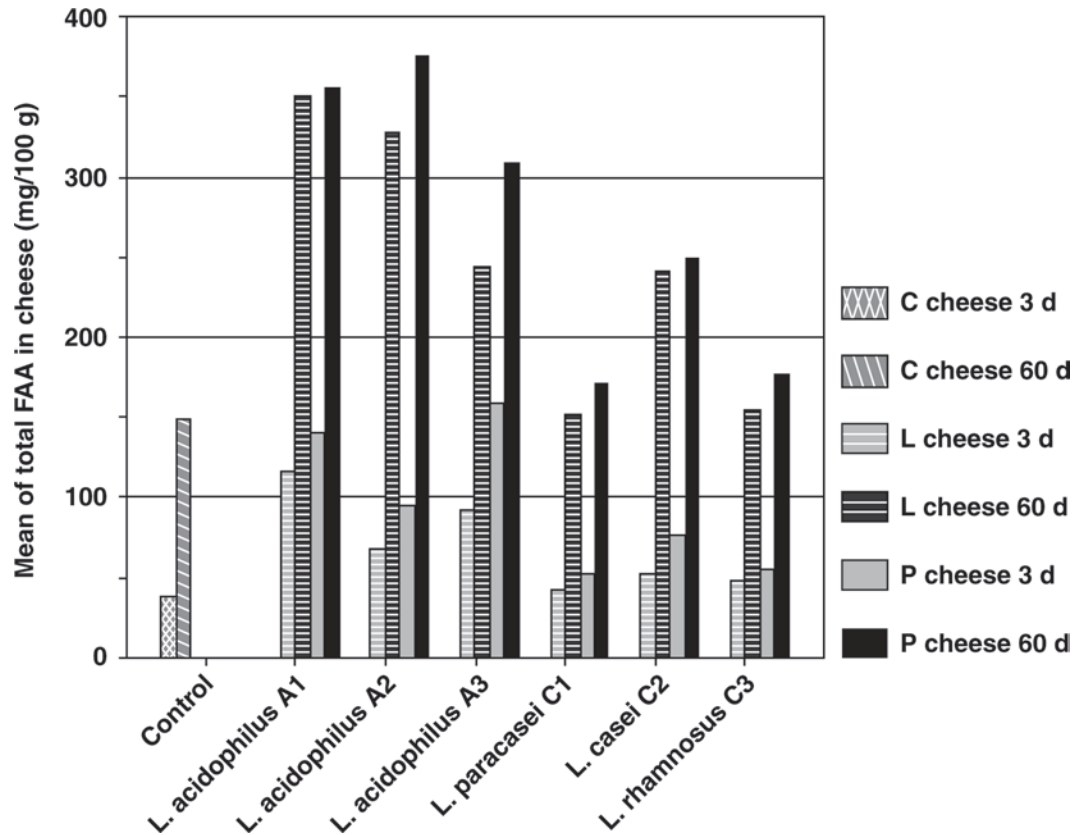
Figure 4. Total amount of free amino acids (mg/100 g of cheese) in control and experimental (L and P) cheeses at 3 and 60 d of ripening. C = control cheeses without probiotics; L = cheeses with probiotic bacteria added directly as a lyophilized culture; P = cheeses with probiotic bacteria added by a 2-step fermentation method.

the increase was less than that achieved by the other *Lb. acidophilus* strains (1.7 times was the maximum increase). Finally, cheeses with *Lb. paracasei* C1 and *Lb. rhamnosus* C3 only showed a slight increase of total FAA compared with control cheeses; this increase was slightly greater when 2-step fermentation was used (Figure 4).

Results of ANOVA and post-hoc Duncan's test on FAA in cheeses of each trial are shown in Table 3. The 3 strains of *Lb. acidophilus* produced a significant increase in the concentration of several FAA in probiotic cheeses compared with those in control cheeses; this effect was more marked in P cheeses than in L cheeses for A2 and A3 strains. As for the strains of the *Lb. casei* group, *Lb. casei* C2 produced the more evident influence, as several FAA increased in cheeses containing this strain, whereas cheeses with *Lb. paracasei* C1 and *Lb. rhamnosus* C3 only increased the level of a few AA.

Finally, the individual concentrations of each AA at 60 d of ripening were used as entry variables in a PCA aimed to detect eventual grouping of samples by probiotic strain, and to evaluate the differences of

probiotic samples with their respective controls and the interreplicate variation. Two principal components were retained, which explained 78.0% of the variance. In the score plot of PC1 versus PC2 it was possible to observe distinctive sample grouping; HCA confirmed this sample grouping (Figure 5A). The principal source of data variation was the concentration of all FAA; this variance was extracted by PC1. One group of samples (group 4 in Figure 5A), which included, in general, control cheeses and probiotic cheeses with *Lb. paracasei* C1 and *Lb. rhamnosus* C3, had negative scores on PC1 and accordingly they were characterized by the lowest concentration of each FAA (Figure 5A and 5B). The rest of the samples, that is, cheeses with any of the *Lb. acidophilus* strains tested or *Lb. casei* C2, showed positive scores on PC1, and were characterized by the highest levels of FAA (Figure 5A and 5B). The last group of samples was, in turn, divided into 3 subgroups along PC2 because of the selective increase of certain amino acids (Figure 5B). Cheeses with *Lb. acidophilus* A2 strain (designed as group 3) were characterized by a higher content of glutamic acid, whereas those with

Table 3. Results[1] of one-way ANOVA and post-hoc Duncan's test on free amino acids contents in C, L, and P cheeses of each trial, on d 3 and 60 of ripening

Free amino acids	*Lactobacillus acidophilus* A1		*Lactobacillus acidophilus* A2		*Lactobacillus acidophilus* A3		*Lactobacillus paracasei* C1		*Lactobacillus casei* C2		*Lactobacillus rhamnosus* C3	
	3 d	60 d	3 d	60 d	3 d	60 d	3 d	60 d	3 d	60 d	3 d	60 d
Asp	*	—	—	—	—	—	—	†	§	*	§	‡
Ser	*	*	—	*	—	—	—	—	—	*	—	—
Glu	*	*	—	—	†	—	—	—	†	*	—	†
Gly	‡	—	—	—	—	—	—	—	—	*	—	—
Hys	§	*	—	—	†	†	—	†	—	—	—	—
Arg	*	*	—	‡	‡	—	—	—	—	—	—	—
Thr	*	—	—	—	—	‡	—	—	*	—	—	—
Ala	*	—	—	*	†	‡	—	†	*	‡	—	—
Pro	*	*	—	—	‡	—	—	—	†	—	—	—
Tyr	—	*	—	*	—	—	—	*	†	‡	—	—
Val	*	*	—	‡	—	—	—	—	†	‡	—	—
Met	*	—	—	—	‡	—	—	—	†	#	—	—
Lys	*	*	—	*	—	‡	—	—	—	—	—	—
Ile	*	*	—	—	‡	†	—	—	—	‡	†	—
Leu	*	*	—	*	‡	*	—	—	†	—	—	—
Phe	*	—	—	—	—	—	—	—	‡	—	—	—
Total FAA	*	*	—	*	‡	‡	—	—	†	*	—	—

[1]Significant differences were found in amino acid concentration in control cheeses without probiotic bacteria (C), probiotic cheeses with the direct addition of probiotic bacteria as a lyophilized culture (L), and probiotic cheeses with the addition of probiotic bacteria by a two-step fermentation procedure (P), mean grouping: * = C ≠ L/P; † = C/L ≠ P; ‡ = C/L ≠ L/P; § = C ≠ L ≠ P; # = C/P ≠ L/P; — = amino acid concentration in C, L, and P cheeses did not differ ($P > 0.05$).

Lb. acidophilus A1 (designed as group 1) showed an increased level of proline. The high level of proline suggests the presence of proline-specific peptidases such as proline iminopeptidase, prolidase, or prolinase in *Lb. acidophilus* A1. In addition, as proline-containing peptides have been associated with bitter taste in cheeses (Habibi-Najafi and Lee, 1996), the peptidolysis caused by *Lb. acidophilus* A1 suggests a potential debittering activity.

A great variety of peptidolytic enzymes, aminopeptidases, di- and tripeptidases, and proline-specific peptidases, was observed in *Lb. acidophilus* as well as in several strains of the *Lb. casei* group; these enzymatic activities were shown to be largely strain-dependent (Khalid and Marth, 1990; Peterson et al., 1990; Habibi-Najafi and Lee, 1994; Macedo et al., 2000; Shihata and Shah, 2000; DiCagno et al., 2006). The increase of FAA observed during ripening of Pategrás cheeses with *Lb. acidophilus* or *Lb. casei* C2 strain suggests that these probiotic strains possess peptidolytic enzymes, which expressed their activity in the environment of cheese. Similarly, Gardiner et al. (1998) and Stanton et al. (1998) found an increase of the total amount of FAA and the concentration of some specific AA in Cheddar cheese caused by the addition of probiotic strains of *Lb. paracasei*, but no previous report about probiotic cheeses with strains of *Lb. acidophilus* is available. Santillo et al. (2007) investigated the effect of one probiotic strain of *Lb. acidophilus* delivered in lamb-paste rennet on FAA production in Pecorino cheeses; they did not detect any changes in total concentration of FAA attributable to the peptidolytic activity of the strain. On the other hand, Ong et al. (2006, 2007b) assessed FAA production in cheeses by means of an unspecific index (soluble nitrogen in phosphotungstic acid) and verified an increase of this fraction after 4 mo of ripening in Cheddar cheese with probiotic strains of *Lb. acidophilus*, *Lb. casei*, *Lb. paracasei*, and bifidobacteria. In our work, a significant increase in FAA was verified both at 3 and 60 d of ripening for *Lb. acidophilus* A1 and A3 and *Lb. casei* C2, whereas for *Lb. acidophilus* A2, a significant enhance was verified only after 60 d of ripening.

Sensory Analysis

Probiotic cheeses and blind control differed equally from control sample for the attribute flavor intensity (Table 4). This result implies that the flavor of probiotic and control cheeses was perceived as similarly strong for the panel. As for acid taste, the test applied showed that probiotic cheeses with *Lb. acidophilus* A1 and A2 were significantly different from the control (Table 4), which is in agreement with the changes in pH caused by these strains. Results for global texture showed the same trend as acid taste: cheeses with *Lb. acidophilus* A1 and A2 were different from the control (Table 4). Assessors were not asked to describe differences, but
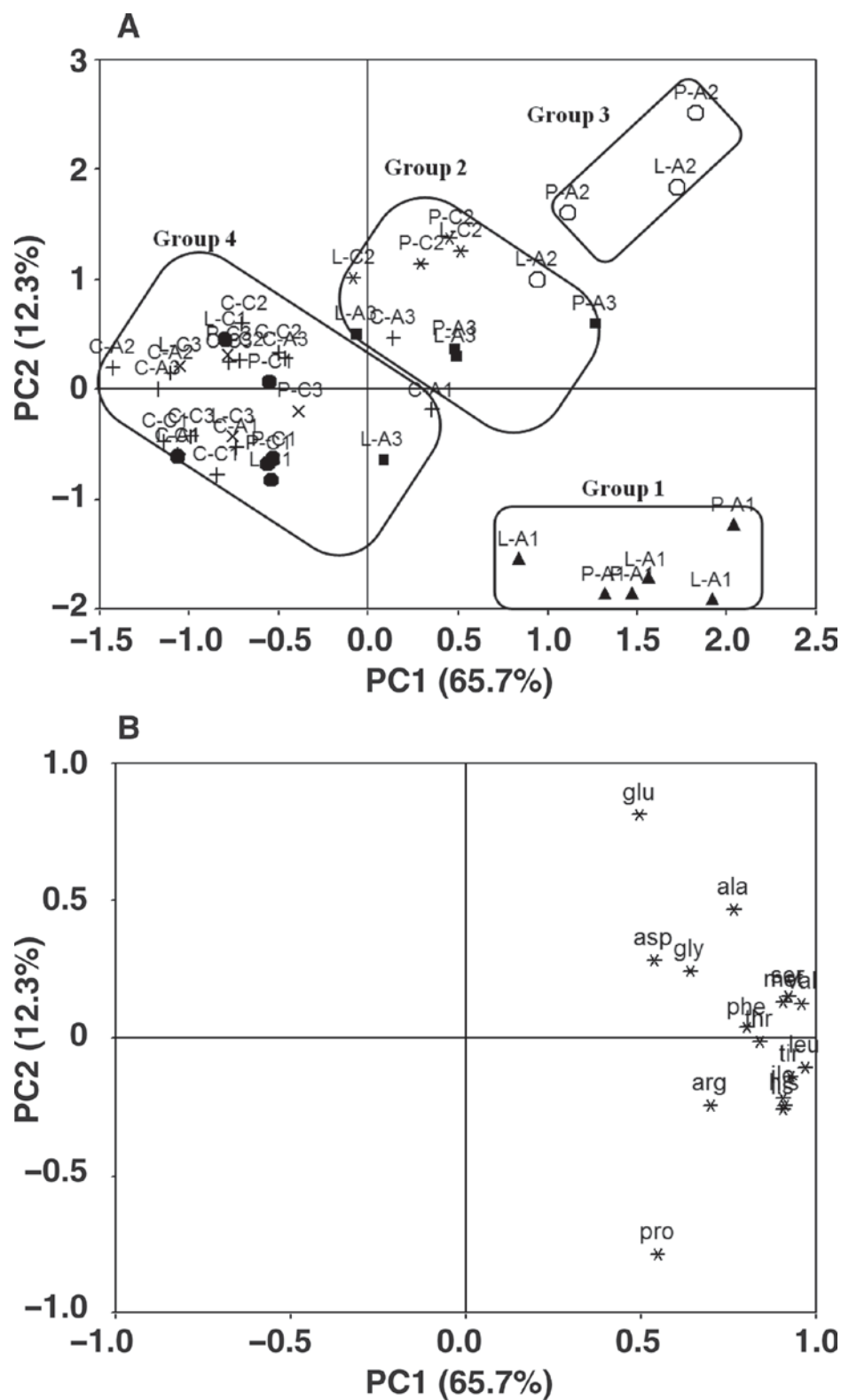
Figure 5. Principal component (PC) analysis of individual amount of free amino acids. (A) Score plot (PC1 vs. PC2) of 60-d-old cheese samples: control cheeses (+) and probiotic cheeses with *Lactobacillus acidophilus* A1 (▲), *Lb. acidophilus* A2 (○), *Lb. acidophilus* A3 (■), *Lactobacillus paracasei* C1 (●), *Lactobacillus casei* C2 (*), and *Lactobacillus rhamnosus* C3 (×). C = control cheeses without probiotic bacteria; L = cheeses with probiotic bacteria added directly as a lyophilized culture; P = cheeses with probiotic bacteria added by a 2-step fermentation method. Rectangles enclose cheese samples in a cluster by hierarchical cluster analysis. (B) Loading plot of amino acids (PC1 vs. PC2).

Table 4. Sensory characteristics of cheeses assessed by the difference-from-control test[1] (means ± standard deviation)

Probiotic strain	Cheese type[2]	Flavor intensity	Acid taste	Global texture
Lactobacillus acidophilus A1	C	1.8	1.4[a]	1.6[a]
	L	2.1	2.7[b]	2.9[b]
	P	1.4	2.9[b]	2.9[b]
Lactobacillus acidophilus A2	C	1.6	1.5[a]	1.4[a]
	L	2.0	3.0[b]	2.8[b]
	P	1.7	3.1[b]	3.1[b]
Lactobacillus acidophilus A3	C	1.9	2.0	1.7
	L	1.8	1.5	2.3
	P	2.1	2.3	2.2
Lactobacillus paracasei C1	C	2.1	1.9	1.8
	L	2.1	1.6	2.2
	P	1.7	2.0	2.3
Lactobacillus casei C2	C	2.2	2.1	1.6
	L	1.7	2.1	2.0
	P	2.2	1.7	2.2
Lactobacillus rhamnosus C3	C	2.1	2.0	1.9
	L	2.1	1.6	2.3
	P	1.9	2.0	2.3

[a,b]For each strain, means with a different superscript within a column significantly differ ($P < 0.05$).

[1]Nine-point rating scale (0 = no difference to 9 = extremely different).

[2]C = control cheeses without probiotic bacteria; L = probiotic cheeses with the direct addition of probiotic bacteria as a lyophilized culture; P = probiotic cheeses with the addition of probiotic bacteria by a two-step fermentation procedure.

they observed that probiotic cheeses with *Lb. acidophilus* A1 and A2 were crumblier than the controls, and their texture was somewhat "shorter."

Other authors have studied sensory properties of different types of probiotic cheeses. Most of them recorded that probiotics did not interfere in the development of the typical sensory profile of the cheese (Dinakar and Mistry, 1994; Gardiner et al., 1998; Gobbetti et al., 1998; Santillo and Albenzio, 2008). However, both detrimental and positive effects have also been shown, depending on species and strains (Gomes et al., 1995; Kasimoğlu et al., 2004; Buriti et al., 2005; Ong et al., 2007a).

CONCLUSIONS

Each probiotic strain assayed showed a different impact on secondary proteolysis of probiotic cheeses, which can be attributed to their heterogeneity in peptidolytic potential and actual activity of the enzymes in the cheese matrix. In this way, the 3 strains of *Lb. acidophilus* tested expressed a significant peptidolytic activity in the food, which was higher than the peptidolysis caused by the strains of the *Lb. casei* group. Additionally, we found that 2-step fermentation was an effective approach to increase the peptidolytic action of *Lb. acidophilus* A3; however, it had little or no effect on the other cultures studied. Further studies are needed to verify if this methodology of addition can increase peptidolytic activity for other strains.

Sensory characteristics of probiotic cheeses were similar to cheeses without probiotics, with a few exceptions linked to overproduction of acid by 2 of the tested strains. Flavor enhancement via increased peptidolysis was not observed.

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