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Mini soft cheese as a simple model for biochemical studies on cheese-making and ripening

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Abstract

A new miniature cheese model obtained under controlled microbiological conditions was proposed, characterized and tested for reproducibility. Optimal heat treatment of cheesemilk was defined, as well as maximal ripening time. Miniature cheeses were obtained with batch pasteurized milk (65 °C, 30 min) and ripened at 5 °C. Lactic and nonlactic microbial populations were monitored by plate counts. Proteolysis was assessed by nitrogen fractions, electrophoresis and liquid chromatography, and a sniffing test was applied to evaluate aroma. Coliform bacteria decreased during ripening but moulds and yeasts increased up to 10^4 cfu/g after 60 d, which defined the end of ripening period. Starter population remained constant during all ripening (10^9 cfu/g), while nonstarter lactic acid bacteria increased from $\sim 10^2$ to 10^4 cfu/g. Soluble nitrogen levels at pH 4.6, in trichloracetic acid (0.73 mol/l) and in phosphotungtic acid (0.009 mol/l) were 151, 67, and 10 g/1000 g of the total nitrogen, respectively, after 60 d of ripening, which are usual values for soft cheeses. Proteolytic patterns as measured by electrophoresis were also similar to those of standard cheeses, as well as the aroma of the products. Peptide profiles revealed that the areas of most peaks increased with ripening time. The proposed model showed to be suitable for the production of mini cheese specimens for laboratory testing of cultures and enzymes in similar conditions to their real environment in the food matrix.

Keywords: Soft cheeses; Miniature cheese model; Cheese composition; Starter and nonstarter bacteria; Proteolysis

1. Introduction

Many aspects of the biochemical events involved in cheese production and ageing are not yet completely known. The impact of processing variables such as cheesemilk, temperature profile during cheese-making and starter or adjunct cultures on the quality of the resulting cheese are usually assessed by means of cheese-making experiments (Hunter, McNulty, & Banks, 1997). However, cheese-making trials are both expensive and time consuming. Reproducibility may also be difficult to obtain at pilot plant scale, and contamination with nonstarter microorganisms as well as sub-lethal phage infections can change ripening patterns leading to misinterpretation of the results (Chapot-Chartier, Deniel, Rousseau, Vassal, & Gripon, 1994; Martley & Crow, 1993). In this context, miniature cheese models have been proposed as an alternative to pilot plant experiments, as they can be prepared under controlled microbiological conditions and are more economical, reproducible and easier to obtain (Shakeel-Ur-Rehman, Fox, McSweeney, Madkor, & Farkye, 2001). Miniature cheese models have been reported for Cheddar cheese and 'Saint-Paulin' washed-curd cheese, but no soft cheese variety has been proposed yet (Hynes, Ogier, & Delacroix-Buchet, 2000; Shakeel-Ur-Rehman, McSweeney, & Fox, 1998).

Cremoso Argentino cheese consists of a relatively simple ecosystem which only includes a thermophilic starter of *Streptococcus thermophilus* strains, and contrary to many soft cheeses, does not comprise a surface flora

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(Choisy et al., 1997; Gripon, 1993; Zalazar, Meinardi, & Hynes, 1999). In addition, the cheese-making does not involve cooking or washing of the curd, or cheddaring and stretching steps, which makes it relatively easy to miniaturize and contributes to the reproducibility of the model (Hynes, Zalazar, & Delacroix Buchet, 1999; Zalazar et al., 1999).

Cremoso cheese is a protein matrix with high fat and moisture content: minimum value for fat content in the dry matter is 500 g/1000 g, according to Argentinean legislation (Código Alimentario Argentino). The product is also rich in calcium (8–12 g/1000 g), as coagulation is basically enzymatic, and contains relatively high initial content of galactose (8–10 g/1000 g) as most *S. thermophilus* strains are unable to metabolize this carbohydrate (Zalazar et al., 1999). Cremoso's texture is crispy and hard at the beginning of the ripening, but afterwards the cheese softens and can even liquefy if the ripening period is too long. Softening occurs throughout the whole body of the cheese, on the contrary than Camembert and other surface mould-ripened cheeses, which soften from the inside to the outer part, due to a pH gradient verified in the cheese body. Cremoso cheese softening has been traditionally related to proteolysis as α_{s1} casein was long considered as a structuring protein and it is extensively degraded during ripening (de Jong, 1976; Guinee, 2003; Hynes et al., 2001). However, other studies suggest that pH may have as much importance in cheese texture as proteolytic changes (Alonso, Candioti, & Hynes, 2005; Hynes, Delacroix-Buchet, Meinardi, & Zalazar, 1999; O'Mahony, Lucey, & McSweeney, 2005).

In this work, a new miniature cheese model based on Cremoso Argentino cheese and obtained under controlled microbiological conditions was proposed, characterized and tested for reproducibility. The model is aimed to study the impact of processing variables on biochemical events during soft cheeses cheese-making and ripening.

2. Material and methods

2.1. Cheese-making

Bulk raw milk was obtained from a nearby dairy plant. Cheeses were prepared with pasteurized milk, as cheese cannot be obtained with autoclaved or sterile milk (heat damage on milk proteins impairs coagulation).

Prior to cheese-making experiments, we assayed increasing slightly the pasteurization temperature, in order to identify the highest temperature that did not significantly decrease coagulation properties of the milk. This approach was aimed to diminish as much as possible the initial microbial charge of the cheesemilk, especially nonstarter lactic acid bacteria (NSLAB), which constitute an uncontrolled factor (Martley & Crow, 1993). Three heat treatments were assayed: the standard low-temperature long-time batch pasteurization (63 °C, 30 min), and two higher temperatures: 65 and 67 °C, both for 30 min. After pasteurization, milk was cooled up to 37 °C, and 30 ml were poured in 50 ml-centrifuge tubes, which were placed in a water-bath at the same temperature. Rennet was added: 700 μl of a solution of 1 g/l chymosin (9 g active chymosin/1000 g, Maxiren®, Gist-Brocades, Seclin, France) in acetic-acetate buffer (pH 5.50). Tubes were vortexed and quickly placed again in the water-bath. Coagulation time was determined subjectively by rocking the tubes gently and visually detecting the casein flocculation on their walls. After that, the tubes were kept in the water-bath for a period equivalent to the coagulation time, and then coagulum was cut with a stainless-steel spatula. The ability of the milk to coagulate was evaluated subjectively by observation of: cleanness of the cut, gel strength, consistency of the curd grain, fine particles occurrence and whey colour. This experiment was performed twice, with different milk on different days, and its results defined cheesemilk pasteurization for cheese-making trials.

Miniature Cremoso cheeses were manufactured in large-neck glass containers of 2.5 l of capacity, provided with glass covers and previously sterilized in an oven (140 °C, 2 h). The containers were filled with 2 l of bulk pasteurized milk, and placed in a water-bath at 37 °C. Calcium chloride (0.2 g/l) was added into the milk to compensate thermal damage caused by pasteurization. A lyophilized direct-to-vat starter culture composed of selected strains of *S. thermophilus* (Diagramma, Santa Fe, Argentina) was inoculated into the milk after being hydrated for 30 min at 37 °C in sterile reconstituted skim milk. Rennet was the same as described above; it was suspended into sterile water and added into the milk 15 min later than the starter culture (0.0225 g/l). The coagulation time was controlled as described; it generally was about 18–20 min. The coagulum was kept still for a similar period of time for gel strengthening, and then cut with miniature tools in large cubes (0.5 cm side). After healing of the curd for 3 min, the mixture whey–curd particles was gently stirred for 5 min. This operation was repeated thrice. Whey was drained and the curd was moulded; moulds were stored in an oven at 45 °C until pH of the cheeses was near 5.20–5.30. In the oven, cheeses were kept in a sterile stainless-steel box and inverted every 30 min. After that, they were salted in the same containers used as cheese vats, by pouring 1 l of sterile brine (200 g/l NaCl, pH 5.40) at 5 °C. After 10 min, the brine was discarded and the cheeses were placed in sterile boxes fitted with a grid to facilitate whey drainage. Boxes were stored overnight at 5 °C for salt and moisture balance in the cheeses. The next day, cheeses were dried with sterile tissue paper (sterilized in an oven at 120 °C for 4 h), vacuum packed and ripened at 5 °C, which is the common ripening temperature for Cremoso cheese.

All the operations that required the opening of the containers were performed after taking them out of the water-bath, in controlled microbiological conditions (adjacent to a flame). Miniature stainless-steel tools, boxes and grids were sterilized in an oven before use. Germicide UV light was directed to the work surface

before cheese-making and during milk transfer to the miniature vats to diminish contamination from the environment.

Four cheeses of 200 g were obtained by the cheese-making day. One experiment of four replicated cheese-makings (4 miniature cheeses × 4 d) was performed in order to test the reproducibility of the model.

2.2. Ripening period

Standard ripening period for Cremoso Argentino cheese is 15–30 d (Código Alimentario Argentino). However, it is probable that this period of time is not long enough to detect some of the biochemical transformations such as the secondary proteolysis or the increase in the free amino acids level. Hence, ripening was prolonged and microbiological and chemical changes were monitored in order to fix the maximum ripening time.

2.3. Analysis of cheeses

The pH of the mini cheeses and in the final products was determined during cheese-making using a Horiba pH metre (Horiba, Kyoto, Japan).

Microbiological counts were performed in the products after 0, 3, 15, 30, 45 and 60 d of ripening. Population of starter streptococci was determined by counting samples on Skim Milk Agar (SMA) after 48 h of incubation at 37 °C (Frank, Christen, & Bullerman, 1993). NSLAB were determined on MRS agar (Biokar, Beauvais, France) by incubation at 34 °C for 48 h under anaerobic conditions. Coliforms were enumerated on Bile Red Violet Agar (BRVA); the plates were incubated for 24 h at 32 °C (Christen, Davidson, McAllister, & Roth, 1993). Moulds and yeasts were developed on yeast extract–dextrose (glucose)–cloramphenicol agar (YGCA) at 25 °C for 5 d (Frank et al., 1993).

Gross composition was determined in 3-d-old cheeses. Dry matter, protein content and fat matter were analysed according to the International Dairy Federation (standard methods IDF, 1962:4A; IDF, 1993:20B; IDF, 1997:152A, respectively).

Proteolysis was monitored on 3-, 30-, and 60-d-old cheeses by nitrogen fractions, urea-polyacrylamide gel electrophoresis (urea-PAGE) and reverse phase high-performance liquid chromatography (RP-HPLC).

Cheese samples were treated to obtain crude citrate extract and soluble fractions at pH 4.6 (pH 4.6-SN), in 0.73 mol/l trichloroacetic acid (TCA-SN) and in 0.009 mol/l phosphotungstic acid (PTA-SN). The extract was obtained by adding 20 ml of sodium citrate (0.5 mol/l) to 10 g of cheese and grounding to homogeneity using a pestle. Deionized water was added up to ~90 ml and the pH was adjusted to 4.6 with HCl (3 mol/l). After centrifugation (3000*g*, 15 min), the soluble fraction volume was adjusted to 100 ml with deionized water (Bergamini, Hynes, & Zalazar, 2006). TCA-SN and PTA-SN were obtained from pH 4.6-SN according to Gripon, Desmazeaud, Le Bars, & Bergère (1975). The nitrogen content in the fractions was assessed by the Kjeldahl method.

Urea-PAGE of the pH 4.6-insoluble fraction of the cheeses was performed using a Mini-Protean II cube (BioRad Laboratories, CA) according to the method of Andrews (1983). The gels were stained directly with Coomassie Brillant Blue G250, as described by Blakesley and Boezi (1977).

Water-soluble nitrogen (WSN) fraction of the cheeses was obtained and analysed by RP-HPLC. Cheese samples (5 g) were homogenized with 15 ml of distilled water by using mortar and pestle and incubated in a water-bath at 40 °C during 1 h. The suspension was centrifuged (3000*g*, 10 min) and filtered through fast flow filter paper. The filtered solution was adjusted to a final volume of 25 ml. Samples were filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil), and 60 μl were injected into the HPLC chromatograph. Detection was performed at 214 nm, and column temperature was 40 °C. The gradient, starting from 100% of solvent A (H_2O–trifluoroacetic acid (TFA), 1000:1.1, vol/vol) and 0% of solvent B (acetonitrile–H_2O–TFA 600:400:1, vol/vol/vol), was generated 10 min after injection. The proportion of solvent B was increased by 1%/min (80 min), 20%/min (1 min), 0%/min (4 min), and then returned to starting conditions, which took 1 min. These last setting conditions were maintained for 10 min (Bergamini et al., 2006). The HPLC equipment consisted of a quaternary pump, an on-line degasser and UV/VIS detector, all Series 200, purchased from Perkin Elmer (Norwalk, CT, USA). An interface module connected to a computer was used for acquisition of chromatographic data with the software Turbochrom (Perkin Elmer, Norwalk, CT, USA). A 220 × 4.6 mm Aquapore OD-300 C18, 7 μm 300 Å analytical column was used (Perkin Elmer, Norwalk, CT, USA).

A sniffing test was performed to evaluate the aroma of the miniature cheeses by comparing them with standard Cremoso cheese obtained in our pilot plant. Samples were placed in sealed glass containers and maintained in an oven at 30 °C for 30 min, after which they were provided to the panel, that was composed of 12 trained members. Three samples were supplied to each panellist; two of the samples had been taken from the same cheese while the other was different. The samples were identified with randomly fixed three-figure codes. The test consisted of a triangular analysis, aimed to detect differences between the two types of samples. No description was required (AFNOR, NF V09-013, 1995).

2.4. Statistical analyses

Cheese composition and nitrogen fractions data were subjected to one-way ANOVA. Principal components analysis (PCA) was applied on peptide profiles, using the covariance matrix (Bergamini et al., 2006). Both types of analyses were run on SPSS 10.0 (SPSS Inc., Chicago,

USA). As for sniffing test, the numbers of total and correct answers were interpreted using the binomial distribution (parameter $P = 1/3$), in order to assess if the samples were significantly different and, in such case, provide the signification level (AFNOR, NF V09-013, 1995).

3. Results and discussion

Cheesemilk was treated at 65 °C for 30 min as higher temperature damaged the ability of milk to coagulate by rennet. Strong heat treatments impair milk coagulation probably as a consequence of whey protein binding with κ-casein. Changes on soluble calcium concentration are also likely involved, as the secondary phase of coagulation seems more affected than the primary (enzymatic) step (Hyslop, 2003). In this study, milk treated at 63 and 65 °C coagulated after a similar period of time (30–32 min), and gave comparable, good quality gels. Coagulation time was longer for the milk pasteurized at 67 °C (38 min), and the obtained coagulum resulted to be weaker and more difficult to cut. The supernatant whey contained fine curd particles only in this last case.

The proximate composition of miniature cheeses did not show significant differences in dry matter and fat and protein content; the values were within the normal range for standard Cremoso Argentino cheese (Table 1). The evolution of pH during cheese-making was also similar in all cheeses; a typical pH curve is shown in Fig. 1.

Ripening time of the mini cheeses was fixed taking into account the evolution of the studied groups of micro-organisms (Fig. 2).

The number of streptococci remained constant up to 60 d, in the order of 10^9 cfu/g, while NSLAB were always below 10^4 cfu/g in the same period of time. NSLAB final number in the model cheese can be considered as appropriate, as Cremoso cheeses obtained from the local market exhibited 10^7 and 10^8 cfu/g after 10 and 70 d of ripening, respectively (Bude-Ugarte, Guglielmotti, Giraffa, Reinheimer, & Hynes, 2006).

Coliform bacteria counts were surprisingly high after cheese-making ($\sim 10^4$ cfu/g) but then diminished rapidly during ripening, reaching numbers of about 10^2 cfu/g after 60 d. As cheeses were obtained under controlled micro-biological conditions, the source of coliform bacteria is probably in highly contaminated raw-milk; however, no microbiological counts in the cheesemilk were performed.

Table 1
Dry-matter, protein content, fat content and pH for four cheese-making days (average values and standard deviation of four replicate cheeses by cheese-making day)

Cheese-making day	1	2	3	4
Dry matter (g/1000 g)	464.6±11.3	465.4±20.3	471.7±25.6	480.6±22.5
Fat content (g/1000 g)	230.0±0.6	227.0±15.3	241.0±21.2	239.0±23.3
Protein content (g/1000 g)	182.0±3.0	182.0±9.0	194.0±10.0	192.0±7.0
PH	5.25±0.04	5.24±0.03	5.23±0.07	5.21±0.05

Significant differences were not detected ($P > 0.05$).

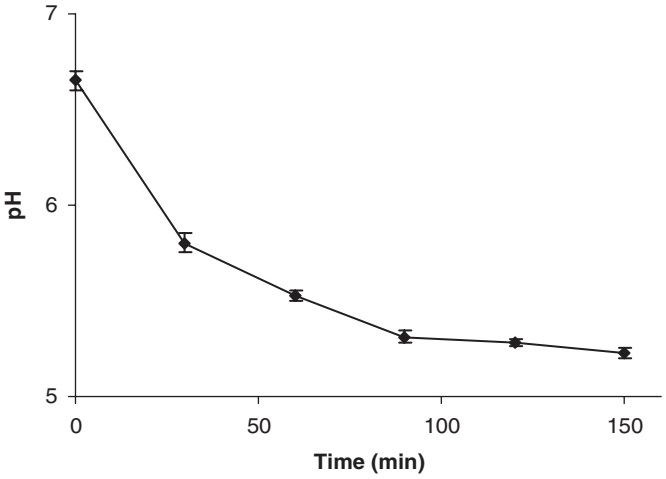

Fig. 1. Acidification curve for miniature cheeses manufactured with a starter culture of *Streptococcus thermophilus*.

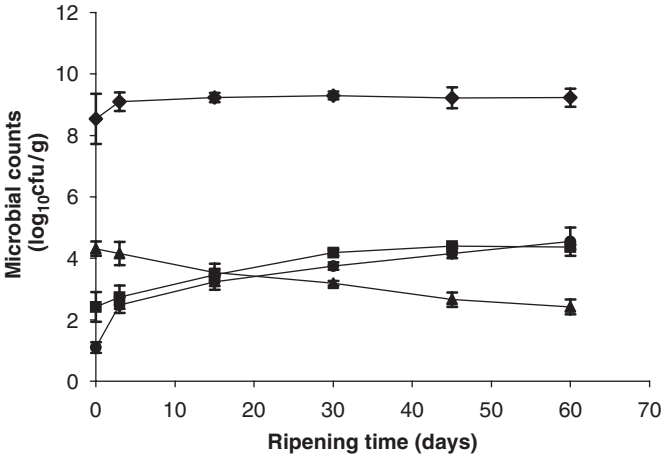

Fig. 2. Evolution of microbial populations during ripening of miniature Cremoso cheeses. Streptococci from starter culture (♦), nonstarter lactobacilli (■), coliform bacteria (▲), and moulds and yeasts (●). Average and standard deviation values for 16 cheeses are reported.

These results allowed us to prolong the ripening of the model cheese up to 60 d in order to increase the possibilities of detecting biochemical changes due to enzymatic agents present in the curd. However, ripening time was not further extended for two reasons: (i) moulds and yeasts increased from very low values to $\sim 10^4$ cfu/g, (ii) cheeses became too soft and even liquefied. A significant moulds and yeasts population is not characteristic of Cremoso Argentino cheese and may conduce to erroneous conclusions about proteolysis and lipolysis patterns, as well as aroma production. On the other hand, too soft or fluid Cremoso cheeses are considered substandard.

Proteolysis patterns of the miniature cheeses were similar to previously reported for standard Cremoso cheeses. In

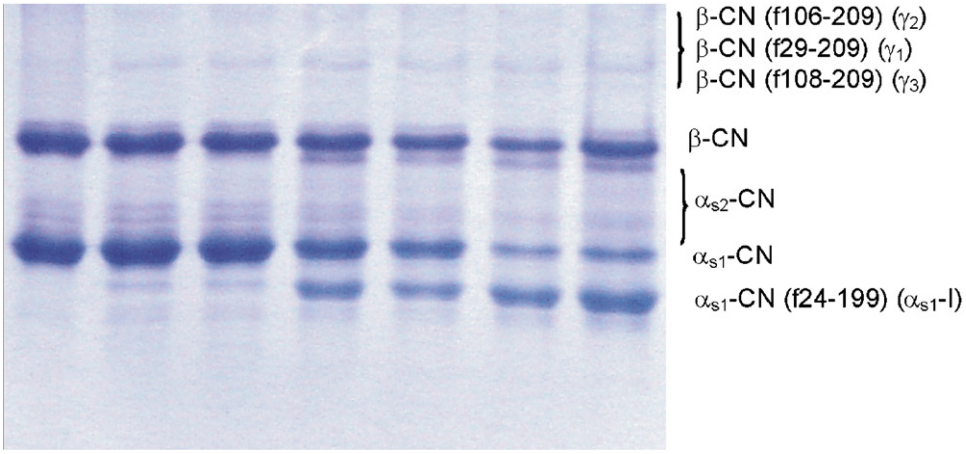

Fig. 3. Urea-PAGE of miniature cheeses. 1: standard caseinate, 2 and 3: 3-d-old cheeses, 4-5: 30-d-old cheeses, 6-7: 60-d-old cheeses. Two replicates cheeses are shown as an example.

Table 2
Nitrogen content of soluble fractions at pH 4.6 (pH 4.6-SN), in thricloroacetic acid 0.73 mol/l (TCA-SN) and in phosphotungtic acid 0.009 mol/l (PTA-SN) expressed as a proportion of total nitrogen (TN) for miniature cheeses after 3, 30 and 60 d of ripening

Ripening time (d)	3	30	60
N fraction			
pH 4.6-SN/TN (g/1000 g)	55±2	118±11	151±23
TCA-SN/TN (g/1000 g)	23±2	50±10	67±7
PTA-SN/TN (g/1000 g)	9±6	10±2	10±2

Average values and standard deviation of 16 replicate cheeses (four cheeses by cheese-making day). Significant differences were not detected among cheese-making days ($P>0.05$).

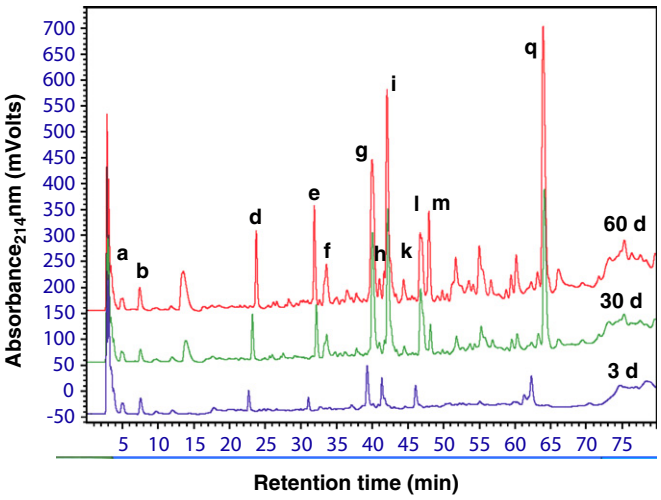

Fig. 4. Peptide profiles of water soluble extract of cheeses by RP-HPLC, after 3 (3 d), 30 (30 d) and 60 (60 d) day of ripening. One cheese sample was chosen as an example, the other chromatograms were similar.

Fig. 3, primary proteolysis of the cheeses described by urea-PAGE is shown. The cheeses were characterized by an extensive hydrolysis of α_{s1} casein and the appearance of peptide α_{s1} (f24–199), formerly known as α_{s1}–I. This transformation has been previously reported as the main biochemical change during ripening of Cremoso Argentino (Hynes, Delacroix-Buchet et al., 1999; Hynes et al., 2001).

Nitrogen fractions also showed the same trend (Table 2). The amount of pH 4.6-SN increased during all ripening up to 150 g/1000 g, which is in agreement with results previously reported (Hynes et al., 2001; Hynes, Zalazar et al., 1999). Fractions TCA-SN and PTA-SN, which represent small to medium peptides and oligopeptides and amino acids, respectively, did not evidence an advanced degree of secondary proteolysis in the products. This is also coincident with former reports for Cremoso cheese, which were about 70 g/1000 g and 10 g/1000 g for TCA-SN and PTA-SN, respectively (Hynes, Zalazar et al., 1999). Although standard ripening time was doubled for miniature cheeses, no increase of the nitrogen content in soluble fractions was observed. Commercial and pilot plant standard Cremoso cheeses are not ripened longer than 30 d.

Fig. 4 shows typical peptides profiles for cheeses at three stages of ripening: 3, 30 and 60 d. Multi-variate analysis was applied to the ensemble of chromatograms: 12 peaks (labelled with characters) were selected from the peptide profiles and their areas were used as input variables for PCA; peaks with no label were not selected (Bergamini et al., 2006). Two PCs were retained, which explained 97.3% of the variation. Sample grouping by cheese age was detected, which was expressed mostly along PC1 axis, but no other subjacent structure could be identified (Fig. 5). Loading of all the variables were always positive in PC1, while some peaks showed positive loadings on PC2 and others, negative values (Table 3). Samples of 30- and 60-d-old cheeses were more disperse along PC2 than 3-d-old cheeses; however, no grouping by the cheese-making day was detected. PC2 only expressed 4.7% of the total variation of the data.

Cheeses obtained under controlled microbiological conditions have been described as lacking of full mature flavour (Crow, Curry, & Hayes, 2001). On the contrary, results of the sniffing test did not show significant

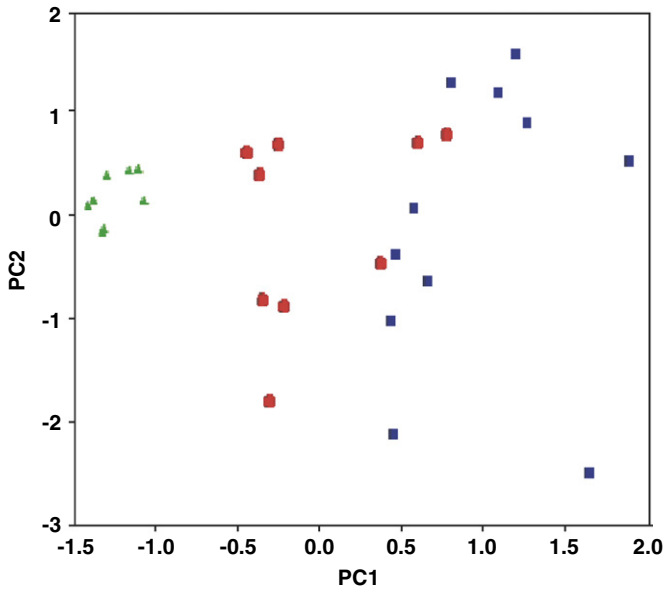

Fig. 5. Principal component analysis of peptide profiles. Score plot of the cheese samples at the beginning of the ripening (3 d, ▲), and after 30 (●) and 60 d (■). Cheeses with the same makers are replicates.

Table 3
Principal component analysis: loadings of the independent variables (peak areas from peptide profiles) on PC1 and PC2

Peak	PC1	PC2
a	0.641	0.515
b	0.750	0.476
d	0.861	0.310
e	0.955	0.124
f	0.868	−0.082
g	0.978	0.184
h	0.745	−0.003
i	0.991	0.104
k	0.955	0.183
l	0.896	−0.073
m	0.802	−0.399
q	0.957	−0.286

differences between the aroma of miniature and standard Cremoso cheeses ($P > 0.05$).

4. Conclusions

Miniature Cremoso cheese is an appropriated model to study biochemical changes in a real food matrix, such as the influence of single strains of lactic acid bacteria on proteolysis and aroma development, or the impact of nonmicrobial enzymes (e.g. rennet, plasmin, pregastric lipase) on caseins and triglycerides hydrolysis. The model is simple, reproducible and representative of standard Cremoso cheese. The high moisture content and the possibility of prolonging ripening time up to 60 d may contribute to detect biochemical activities that would probably take longer in other cheese varieties. However, ripening temperature is a drawback as Cremoso requires 4–8 °C, which probably slows down all biochemical events. New assays aimed to check if mini cheeses can be ripened at 8 °C without significant changes in 'shelf-life' will be performed. Even if it is not possible to extrapolate results obtained in a soft cheese to other food environments, the model may be useful for screening purposes, especially for lactic cultures.

Two examples of the application of this miniature cheese model currently in course in our laboratory are: (i) the study of single strains of mesophilic lactobacilli isolated from cheese as adjunct cultures and (ii) the investigation of residual rennet on soft cheese proteolysis and meltability.

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