doi: 10.1111/j.1471-0307.2009.00496.x

ORIGINAL RESEARCH

Characterization of the free fatty acids profile of Pategrás cheese during ripening

MARÍA C PEROTTI, DIEGO J MERCANTI,* SUSANA M BERNAL and CARLOS A ZALAZAR

Instituto de Lactología Industrial (UNL/CONICET), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829, S3000AOM Santa Fe, Argentina

This work compares the free fatty acids (FFA) profiles of Pategrás cheeses manufactured on the pilot scale, with and without the addition of probiotics. Total and FFA in milks and cheeses, respectively, were similar, indicating a nonselective release during ripening. Lipolysis was lo for control cheeses, but somewhat higher for those made in summer, probably because of an elevated psychrotrophic bacterial development. Probiotic bacteria remained highly viable during ripening and had an influence on FFA profiles. A simple sensory profiling of the cheeses showed no differences in flavour and taste and little evidence of treatment effects was found using univariate analysis. Nevertheless, multivariate analysis showed clear differences between probiotic and control products.

Keywords Cheese ripening, Lipolytic activity, Pategrás cheese, Probiotics.

INTRODUCTION

Cheese production in Argentina is important both in terms of size and diversity—487 000 tons in 2007, with an annual internal consumption of 11.15 kg per capita (Centro de la Industria Lechera Argentina 2005). Semihard cheeses amounted to 30% of that production (159 000 tons) (Centro de la Industria Lechera Argentina 2005), of which Pategrás cheese is the leader of the local market. The Argentinean regulations describe Pategrás cheese as a partially cooked cheese, which optionally can contain small eyes well distributed throughout the cheese. The ripening period for this variety ranges, depending on the cheese size, from 30 to 60 days (CAA 2008).

Initially, this variety was commonly manufactured using natural milk starters (Zalazar *et al.* 1999) prepared by warming good quality raw milk at about 65°C, followed by an immediate cooling to 45°C. The milk was held at this temperature until a pH of 5.2 was reached. Finally, the prepared starter was cooled to 8–10°C and stored at this temperature and used within 24 h. With this procedure, a starter containing mainly natural thermophilic streptococci was obtained. Natural milk starters contained a secondary microflora in addition to the predominant lactic acid bacteria. Within this secondary microflora, mainly composed of *Lactobacillus* sp., other bacteria (e.g. *Lactococcus lactis*, *Enterococcus faecium*) could also be present at lower numbers (Powel *et al.* 2003). This complex biodiversity determines characteristics, such as taste and flavour similar to those of raw milk cheeses (Albenzio *et al.* 2001). To improve and standardize the quality of the natural milk starter, the initial milk was sometimes inoculated with 1–2% of the milk starter from the previous day. Now-a-days; however, natural milk starters have been progressively replaced by the widespread use of direct-vat-set (DVS) cultures, either of mesophilic or thermophilic bacteria. The evolution undergone by this technology has several advantages. First, DVS are cultures with relatively constant and well-known acidifying activity. Second, their microbiological composition is not highly variable, and thus the risk of dangerous and uncontrolled contaminations is low. On the other hand, their use could potentially change the typical characteristics of the cheeses and increase the risk of problems related to bacteriophages during the cheesemaking process (Candioti *et al.* 2000).

The use of both high temperature/short time (HTST) pasteurization (72°C × 15 s) and DVS cultures has also reduced the eye development in cheese that resulted from heterofermentative bacteria originating either in raw milk or in natural starters. To counteract that negative effect, it is now usual to add propionibacteria as adjunct cultures during the cheesemaking process (Deborde 2003). In addition, given the increased demand for probiotic products, Pategrás cheese was found to be a suitable carrier for the delivery of high concentrations of probiotic bacteria (Bergamini *et al.* 2005).

In view of the successive technological changes applied to the manufacture of Pategrás cheese and

*Author for correspondence. E-mail: diegojav78@gmail.com

SDT International Journal of Dairy Technology

the lack of information about its ripening process, the objective of this study was to characterize the FFA profile during the ripening of cheeses manufactured with and without the addition of probiotic bacteria. Moreover, considering differences in the lipolysis among cheeses manufactured during different seasons, previously found by others (Fernández-García *et al.* 2006), the main goal was to profile FFA in Pategrás cheeses made only with thermophilic starter culture (control) or with the extra addition of probiotic micro-organisms (experimental). The proteolytic profile of Pategrás cheese has been formerly characterized by Bergamini *et al.* (2006).

MATERIALS AND METHODS

Cheesemaking

Control cheeses were produced at the pilot plant of the Instituto de Lactología Industrial (INLAIN) located in Santa Fe (Argentina), according to the standard process for semihard Pategrás cheese (Zalazar *et al.* 1999). Raw bulk milk [100 L, fat 3.5 ± 0.3% w/v, protein 3.0 ± 0.3% w/v, pH 6.70 ± 0.05, acidity 18 ± 1°D (1°D = 100 mg lactic acid/L)], was supplied by a nearby dairy plant (Milkaut Coop. Ltda., Franck, Santa Fe, Argentina) on every cheesemaking day. Milk fat was standardized at 3.8% (w/v). Milk was pasteurized at 65°C for 20 min, cooled to 37°C and $CaCl_2$ (Merck, Darmstadt, Germany) was added to give a final concentration of 0.02% (w/v). A lyophilized DVS culture of *Streptococcus thermophilus* (Diagramma, Santa Fe, Argentina) was used as primary starter. This culture was dispersed in 100 mL of pasteurized milk, maintained for 5–10 min at 37°C and then added to cheesemaking milk to obtain a concentration of 10^6 colony-forming units (cfu)/mL. After 15 min, 1 g of chymosin (MAXIREN-150; Gist Brocades, Sechim, France; 100% chymosin, rennet strength 150 000 IMCU/mL) was dispersed in 25 mL of distilled water and added to the vat. The pH of the milk was approximately 6.7 at the moment of rennet addition. When the curd reached the appropriate strength, which was assessed empirically by testing its hardness with a spatula, it was cut in several steps (with manual stirring in between) by using a harp with parallel wires spaced at 2 cm, until the curd particles reached a size of 8–10 mm. The mixture of curd particles and whey was gently stirred and heated at the rate of 1°C/min until 45°C for 15–20 min approximately, to reduce the moisture content of curd grains. The whey was extracted from the vat by aspiration with a pump, and the curd was put into cylindrical moulds, which allowed further whey drainage. The moulds were pressed at room temperature (22–25°C) for 18 h (0.2–0.3 kg/cm^2). The cheeses, obtained in wheels of approximately 4 kg, were brined at 12°C for 24 h (20% w/v, pH 5.4) and ripened without packaging at 12°C and 80% relative humidity for 60 days.

Probiotic cheeses were manufactured using the same cheesemaking protocol and primary starter strain and concentration as for the control cheeses. The unique difference was the addition of a mixture of *Lactobacillus acidophilus* (Chr. Hansen, Argentina), *Lactobacillus paracasei* subsp. *paracasei* (DSM, Argentina) and *Bifidobacterium animalis* subsp. *lactis* (DSM, Argentina) lyophilized DVS cultures as adjunct starters. The mixture of the probiotic strains was dispersed in 100 mL of pasteurized milk, kept at 37°C for 5–10 min and added to the vat immediately after the primary starter, in a quantity sufficient to attain counts of 10^6 cfu/mL of each probiotic strain in the cheesemaking milk.

Two vats were used on every cheesemaking day, yielding two cheeses (replicates) per trial, either for control or probiotic cheeses. Control cheese manufacture was carried out over the four seasons, during a period of 2 years and 4 months (December 2002–April 2005). Five control cheesemaking trials were made in spring, five in summer, six in autumn and four in winter (overall 20 cheesemaking trials). Probiotic cheeses were manufactured in six cheesemaking trials during the same period, but only in two seasons (spring and autumn); in this context our objective was simply to assess differences caused by the addition of probiotic cultures.

Cheese sampling

Sampling of pilot scale cheeses was carried out after 3 and 60 days of ripening according to the International Dairy Federation Standard (1995). Each sample was obtained by cutting two cylindrical portions of approximately 10 g each, from the centre of the cheese to the lateral surface. The outer 10 mm of the cheese cylinders were discarded. In view of the gradient of moisture and salt concentration along the radius of the cheese, each cylinder was in turn cut into small pieces, which were mechanically mixed with a spatula. Equivalent fractions of the two cheeses made on the same cheesemaking day (replicates), were mixed together and stored at −18°C until analysed.

Cheese composition and microbiology

Cheeses were analysed for moisture (IDF 1982), protein (IDF 1993), fat-in-dry matter (FDM) and pH at 60 days of ripening, according to the methods of the American Public Health Association (APHA) (Bradley *et al.* 1993).

Lactic acid and probiotic bacteria were enumerated according to the methods reported by Vinderola and Reinheimer (2000) throughout ripening. *Lactobacillus acidophilus* and *L. paracasei* were differentially enumerated in B-MRS (Britania S.A.,

Buenos Aires, Argentina) supplemented with 0.15% (w/v) of bile (Sigma, Steinheim, Germany)] agar (1.2% w/v) incubated aerobically for 72 h at 37°C. Bifidobacteria were selectively counted on LP-MRS (Britania S.A) supplemented with 0.2% (w/v) of lithium chloride and 0.3% (w/v) sodium propionate agar (1.2% w/v) incubated anaerobically (GasPak System-Oxoid, Basingstoke, Hampshire, England) for 72 h at 37°C.

The free fatty acids (FFA) in cheese

Free fatty acids $C_{6:0}$ to $C_{18:2}$ were analysed by gas chromatography as previously described by Perotti *et al.* (2005a). A cheese sample (4 g) was mixed with 50% (v/v) H_2SO_4 to reduce the pH below 2, and then homogenized and dehydrated with approximately 25 g of anhydrous Na_2SO_4. Lipid extraction was carried out for 2 h in a Twysselmann continuous solid–liquid extractor, using *n*-hexane at 60°C. FFAs were separated from triacylglycerols by titration with 0.1N aqueous sodium hydroxide. The aqueous layer containing the FFA sodium salts was evaporated to dryness. Derivatization of fatty acid salts was performed in~screw cap tubes by adding 8 mL of ethanol–H_2SO_4 (5% v/v) to dry salts, and heating to 70–72°C for 1 h. After cooling, 6 mL of distilled water were added to facilitate further separation of phases and 1 mL *n*-hexane was added to extract ethyl esters. One microlitre of the upper solution was injected into a gas chromatograph (manual injection with a Hamilton syringe) and triplicate analyses were performed for each sample.

Chromatographic analyses were carried out on a Perkin Elmer gas chromatograph Model 9000 fitted with Turbochrom v. 4.0 software, equipped with a split/splitless injector, a flame ionization detector (FID) and a fused silica capillary column PE-Wax (Perkin Elmer Corp., Waltham, MA, USA; polyethylene glycol, 30m × 0.25 mm × 0.25 μm). The oven temperature was programmed as follows: 50°C (4 min), a first ramp from 50 to 150°C at a constant rate of 10°C/min, 150°C (3 min), a second ramp from 150 to 230°C at a constant rate of 10°C/min and a final temperature of 230°C (5 min). The injector was maintained at a constant temperature of 220°C in split mode (split ratio 1/52) and the FID temperature was 275°C. The pressure at the top of the column was 22 psi, and nitrogen was employed as carrier gas (3.3 mL/min, constant flow).

The FFA were quantified by means of the internal standards method. Calibration curves were prepared by combining increasing concentrations of a mixture of caproic ($C_{6:0}$), caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) fatty acids, with fixed concentrations of enantic ($C_{7:0}$) and margaric ($C_{17:0}$) fatty acids (internal standards). Both standards were subsequently added (0.4 mg of $C_{7:0}$ and 2.0 mg of $C_{17:0}$) to all cheese samples (4 g) immediately before starting lipids extraction.

Fatty acid composition of milk

Fatty acid composition, $C_{4:0}$ to $C_{18:2}$, was determined in milks used for cheesemaking according to Perotti *et al.* (2005b). Milk fat isolation was carried out by centrifuging milk at 800 *g* for 5 min and filtering at 50°C. Anhydrous milk fat (150 mg) was dissolved with 10 mL *n*-hexane and 1 mL of this solution was transferred to a reaction screw cap tube. Transesterification of lipids was accomplished at 70–72°C for 3 h using ethanol–H_2SO_4 (5% v/v). Fatty acid ethyl esters were dissolved in the upper phase of hexane and analysed by GC (0.7–1.0 μL) using the same chromatographic conditions as for cheese FFA analysis.

Sensory analysis

A simple sensory analysis of cheeses was performed by a nontrained panel, and was aimed to detect differences between cheeses with and without the addition of probiotic bacteria. The panellists were asked to classify samples as 'good', 'fair' or 'disagreeable', based on overall quality, and allowed to describe defective characteristics if there were any.

Statistical analysis

Data from fat and protein content, dry matter and individual and total FFA were analysed by the one-way ANOVA procedure of Statgraphics Plus software (v 3.0; Statistical Graphics Corp., Warrenton, VA, USA). Differences among mean values were detected by the Least Significant Difference Multiple Range Test. The data set for the fatty acid profiles of the cheese was complex. To aid interpretation, sample means from the control and probiotic cheese were combined and analysed together using Principal Component Factor Analysis (PCA) (Varimax rotation) on the correlation matrix. Varimax rotation was applied after factor extraction with the aim of obtaining an easier interpretation of the extracted PCs. Interpretation of the result was aided by correlation of the ratings for the individual fatty acids with the Factor Scores. The effect of treatment factors (type, season and ripening time) was investigated by Analysis of Variance of the Factor Scores. Linear Discriminant Analysis (with cross-validation) was then applied using selected Factor Scores as predictors to establish if control cheeses could be discriminated from probiotic ones on the basis of fatty acids profile. Multivariate statistical analyses of the chromatographic data were performed using the software SPSS v. 10.0 (SPSS Inc., Chicago, IL, USA).

Table 1 Gross chemical composition and pH values of cheeses at 60 days of ripening

Cheese type	*Moisture (% w/w)*	*Fat-in-dry matter (% w/w)*	*Protein (% w/w)*	*pH*
Control cheeses (n = 20)	44.5 ± 1.4[a]	52.3 ± 2.9[a]	21.8 ± 0.8[a]	4.86 ± 0.04[a]
Probiotic cheeses (n = 6)	44.3 ± 1.2[a]	52.3 ± 2.0[a]	21.9 ± 0.5[a]	4.85 ± 0.05[a]

Values in the same column with different superscript letters differ ($P < 0.05$).

RESULTS AND DISCUSSION

Cheese composition and microbiology

Table 1 shows the gross chemical composition and pH values for the control and probiotic cheeses after 60 days of ripening. The values obtained for FDM and moisture were within the ranges established by the Argentinean food regulations standing for this type of cheese (45.0–59.9% w/w for fat and 36.0–45.9% w/w for moisture, CAA 2008). Fat content is an important parameter to be considered for the FFA profile analysis, and showed no significant differences between control and probiotic cheeses. Protein content and pH values were comparable to those obtained by Bergamini *et al.* (2006).

The *S. thermophilus* population in all cheeses was higher than 8 log cfu/g at 3 days of ripening, and higher than 9 log cfu/g from day 15 until the end of ripening. In cheeses with probiotic addition, counts of the three strains assayed were higher than 6.5 log cfu/g in 3-day-old cheeses, and increased at 60 days of ripening (8.00 log cfu/g for *L. acidophilus*, 7.46 log cfu/g for *B. animalis* subsp. *lactis* and 9.11 log cfu/g for *L. paracasei* subsp. *paracasei*). These levels were higher than the minimum required for a probiotic dairy product to exert the health and nutritional benefits ascribed to these bacteria (Stanton *et al.* 1998; De Vuyst 2000).

The free fatty acids profiles of cheeses

The nine individual concentrations of fatty acids ($C_{6:0}$ to $C_{18:2}$; mg/kg) were determined for 3 and 60-day-old Pategrás cheeses manufactured in different seasons, either for control (Table 2) or probiotic cheeses (Table 3). Although a slight increase in total FFA concentrations was noticed in cheeses without probiotics throughout the ripening period (60 days), even the highest values were quite low (Table 2) when compared with the FFA concentrations found in cheeses where lipolysis plays an important role, for example Parmesan (4993 mg of fatty acids/kg of cheese) and Romano (6754 mg of fatty acids/kg of cheese) (Fox *et al.* 1993). This fact is in agreement with the characteristics of Pategrás cheese, as its manufacture protocol involves both the use of pasteurized milk, where enzymatic activity of milk native lipoprotein lipase is rather low, and a nonlipolytic coagulant. Moreover, *S. thermophilus* strains present very low lipolytic activity (Choisy *et al.* 1997).

On the other hand, seasonal variations were found ($P < 0.05$) in total FFA concentrations, both at the beginning and at the end of ripening. The values from summer were higher than those from winter, autumn and spring, which is probably related to the higher psychrotrophic bacterial counts found in raw milk in Argentina during the summer. Differences for total mesophilic and psychrotrophic flora between raw milks collected in the Santa Fe (Argentina) area during summer and winter were found by Reinheimer *et al.* (1985), and are shown in Table 4. The influence exerted by the endo and exoenzymes of the psychrotrophic bacteria on the cheeses has been demonstrated in previous work by our group (Zalazar *et al.* 1986). Cheese quality can be affected by either proteases or lipases causing low yields, flavour defects, rancidity and soapy flavours (McPhee and Griffiths 2003). Extracellular lipases and proteases may survive normal pasteurization and sterilization processes, and are likely to remain active in cheeses all through the ripening period (Weihrauch 1988).

Some important conclusions were obtained from the analysis of FFA profiles of the control cheeses. First, it was observed that significantly higher concentrations of palmitic and oleic fatty acids, which as a whole represent more than 60% of the total FFA, were in most cases found in summer ($P < 0.05$) (Table 2). On the contrary, stearic and myristic acids, which were also found at relatively high levels, had less seasonal variation, as well as the rest of the minor FFA. Regarding the importance of ripening time, the nine FFA levels slightly increased, which was evident from the comparison between FFA profiles for 3- and 60-day-old cheeses (Table 2).

The FFA profiles of the cheeses made in two different seasons with the addition of probiotic bacteria (Table 3) exhibited a small increase in total FFA levels during ripening, but this effect was statistically significant only for cheeses manufactured in spring. FFA profiles of probiotic cheeses were similar to those of control ones, either at 3 or 60 days of ripening. In all the cheeses, FFA profiles were characteristic for this type (Zalazar *et al.* 1999). The percentages of each FFA with respect to their sum (values between brackets in Tables 2 and 3) were also similar to the ones determined in the milk used for the manufacture of all (control and experimental) cheeses, in the respective seasons (Table 5). These results support the theory of

Table 2 Concentrations of the free fatty acids $C_{6:0}$ to $C_{18:2}$ in control Pategrás cheeses manufactured in the four seasons, at 3 and 60 days of ripening (mg of fatty acids/kg of cheese)

	3 days of ripening				*60 days of ripening*			
FFA	*Spring*	*Summer*	*Autumn*	*Winter*	*Spring*	*Summer*	*Autumn*	*Winter*
C6:0	22.5 ± 7.1^{a} (1.3)	33.3 ± 5.7^{a,b,c} (1.3)	26.6 ± 7.3^{a,b} (1.3)	28.1 ± 10.5^{a,b,c} (1.4)	34.8 ± 9.9^{a,b,c} (1.6)	38.7 ± 10.7^{b,c} (1.3)	36.8 ± 13.3^{b,c} (1.4)	39.8 ± 14.2^{c} (1.7)
C8:0	21.2 ± 4.4^{a} (1.3)	29.3 ± 4.0^{a,b,c} (1.2)	25.6 ± 5.7^{a,b} (1.2)	26.7 ± 6.6^{a,b} (1.3)	28.9 ± 6.2^{a,b} (1.3)	32.2 ± 7.0^{b,c} (1.1)	30.7 ± 7.6^{b,c} (1.2)	37.8 ± 8.5^{c} (1.6)
C10:0	46.0 ± 8.4^{a} (2.7)	66.8 ± 14.3^{b} (2.7)	58.6 ± 16.5^{a,b} (2.8)	65.7 ± 13.6^{a,b} (3.2)	62.8 ± 10.9^{a,b} (2.9)	73.8 ± 20.8^{b,c} (2.6)	67.4 ± 17.6^{b} (2.5)	89.8 ± 17.2^{c} (3.7)
C12:0	59.7 ± 11.3^{a} (3.5)	86.5 ± 22.3^{a,b} (3.5)	72.8 ± 23.3^{a,b} (3.5)	84.6 ± 18.7^{a,b} (4.1)	76.5 ± 6.0^{a,b} (3.5)	97.7 ± 32.0^{b,c} (3.4)	81.7 ± 21.7^{a,b} (3.1)	116.8 ± 19.7^{c} (4.9)
C14:0	185.8 ± 33.6^{a} (11.0)	266.1 ± 74.0^{a,b,c,d} (10.8)	219.6 ± 62.8^{a,b} (10.7)	215.8 ± 12.7^{a,b} (10.5)	245.7 ± 33.2^{a,b,c} (11.2)	347.7 ± 102.5^{d} (12.0)	322.5 ± 89.1^{c,d} (12.1)	288.3 ± 63.9^{b,c,d} (12.0)
C16:0	567.2 ± 81.5^{a} (33.6.)	818.6 ± 138.0^{c,d} (33.2)	637.2 ± 72.7^{a,b} (31.0)	638.8 ± 52.8^{a,b} (31.1)	709.6 ± 74.7^{a,b,c} (32.3)	955.3 ± 226.6^{d} (33.1)	808.5 ± 164.3^{c,d} (30.4)	745.4 ± 89.7^{b,c} (31.1)
C18:0	235.9 ± 52.8^{a} (14.0)	307.0 ± 40.3^{b,c} (12.4)	242.3 ± 24.6^{a} (11.8)	253.9 ± 22.3^{b,c} (12.4)	280.8 ± 18.8^{a,b,c} (12.8)	330.3 ± 55.6^{c} (11.4)	289.5 ± 62.9^{b,c} (10.9)	277.6 ± 29.8^{a,b,c} (11.6)
C18:1	476.5 ± 243.1^{a} (28.3)	773.9 ± 124.8^{b,c} (31.3)	690.5 ± 69.5^{b} (33.6)	643.2 ± 134.4^{a,b} (31.3)	660.1 ± 91.1^{a,b} (30.0)	906.4 ± 133.7^{c} (31.4)	916.2 ± 184.6^{c} (34.4)	694.9 ± 109.9^{b} (29.0)
C18:2	71.4 ± 42.8^{a} (4.2)	87.7 ± 29.6^{a,b} (3.6)	81.4 ± 18.2^{a,b} (4.0)	96.4 ± 14.2^{a,b} (4.7)	98.7 ± 16.3^{a,b} (4.5)	105.3 ± 27.2^{a,b} (3.6)	108.1 ± 29.3^{b} (4.1)	106.8 ± 39.2^{a,b} (4.5)
Total	1686.3 ± 428.7^{a}	2469.2 ± 355.7^{b,c}	2054.5 ± 214.5^{a,b}	2053.1 ± 213.4^{a,b}	2197.9 ± 76.0^{b}	2887.4 ± 541.5^{c}	2661.4 ± 518.7^{c}	2397.1 ± 333.3^{b,c}

In total, the free fatty acids were calculated as the sum of the nine fatty acids separately quantified.

Values in the same row with different superscript letters differ ($P < 0.05$).

Values between brackets represent the percentages of each the free fatty acids respect to the total amount of the free fatty acids.

Table 3 Concentrations of the free fatty acids $C_{6:0}$ to $C_{18:2}$ in probiotic Pategrás cheeses manufactured in spring and autumn, at 3 and 60 days of ripening (mg of fatty acids/kg of cheese)

	3 days of ripening		*60 days of ripening*	
FFA	*Spring*	*Autumn*	*Spring*	*Autumn*
C6:0	19.4 ± 2.8[a]	19.5 ± 6.8[a,b]	35.4 ± 12.8[b]	22.4 ± 2.8[a,b]
	(1.0)	(1.0)	(1.4)	(1.0)
C8:0	19.6 ± 2.2[a]	18.2 ± 4.3[a]	31.2 ± 8.5[b]	19.5 ± 0.7[a]
	(1.0)	(0.9)	(1.2)	(0.9)
C10:0	48.8 ± 5.2[a]	41.8 ± 7.7[a]	78.1 ± 17.0[b]	42.2 ± 2.1[a]
	(2.4)	(2.2)	(3.1)	(1.9)
C12:0	62.6 ± 6.2[a]	54.9 ± 9.3[a]	101.8 ± 21.0[b]	58.5 ± 7.0[a]
	(3.1)	(2.8)	(4.0)	(2.7)
C14:0	217.7 ± 39.3[a]	187.8 ± 16.8[a]	297.2 ± 55.8[b]	244.8 ± 37.1[a,b]
	(10.9)	(9.7)	(11.6)	(11.2)
C16:0	625.1 ± 49.1[a]	594.8 ± 81.7[a]	793.5 ± 131.3[b]	695.0 ± 5.5[a,b]
	(31.2)	(30.8)	(31.0)	(31.8)
C18:0	282.8 ± 27.2[a]	249.0 ± 25.7[a]	353.0 ± 49.4[b]	265.3 ± 17.3[a]
	(14.1)	(12.9)	(13.8)	(12.1)
C18:1	613.4 ± 46.6[a]	678.7 ± 61.2[a,b]	740.8 ± 88.5[b]	742.1 ± 67.3[a,b]
	(30.6)	(35.2)	(29.0)	(34.0)
C18:2	111.9 ± 16.9[a,b]	85.9 ± 4.8[a]	126.1 ± 13.5[b]	93.5 ± 5.8[a]
	(5.6)	(4.4)	(4.9)	(4.3)
Total	2001.3 ± 186.5[a]	1930.6 ± 218.4[a]	2557.1 ± 373.5[b]	2183.3 ± 20.2[a,b]

In total, the free fatty acids were calculated as the sum of the nine fatty acids separately quantified.
Values in the same row with different superscript letters differ ($P < 0.05$).
Values between brackets represent the percentages of each the free fatty acids respect to the total amount of the free fatty acids.

Table 4 Mesophilic and psychrotrophic bacterial counts for raw milks collected from Santa Fe area in summer and winter

Season	*Mesophilic microflora (log cfu/mL)*	*Psychrotrophic microflora (log cfu/mL)*
Summer	7.63	6.98
Winter	7.26	5.91

Mean values of 32 milk samples (adapted from Reinheimer *et al.* 1985).

a nonselective release of fatty acids from triglycerides during ripening (Fox *et al.* 1993).

Principal component analyses were applied to data for control and experimental cheese samples. The Kaiser–Meyer–Olkin of sampling adequacies were > 0.5, which allowed an explanation of the data variability with a reduced number of PCs (Hair *et al.* 1999). The first four PCs were selected according to the Cattell criterion and explained 98.4% of the total variability contained in the original data set. Table 6 shows the results before and after rotation. The nine variables studied were considered in the PCA, as their communalities for the first four factors were very high (> 0.6). The Component Score Coefficient Matrix was useful to interpret the meaning of these factors in terms of the original attributes: the individual fatty acids. PC1 was therefore associated with short and medium chain fatty acids ($C_{6:0}$ to $C_{12:0}$), and PC2, PC3 and PC4 with oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and stearic ($C_{18:0}$) acids respectively (values in italics, Table 7). Furthermore the Factor Score Coefficient confirmed the results found by correlating the Factor Score with the original variables, allowing a definitive assignment of the factor scores. ANOVA (general linear model) on the Factor Scores showed a significant effect of cheese type on PC4, season on PC2 and PC4, ripening time on PC2 and cheese type × ripening time on PC4 (Table 8).

Discriminate linear analysis is useful to classify or assign objects to a small number of groups predicted by a number of variables that should be uncorrelated to achieve successful classification. In this study it was applied to validate the previous analysis, using Factor Scores as predictor variables, and either time of ripening, season of manufacture or cheese type as classification factors. Samples were then classified into the group they best matched, and the analysis was performed in terms of the percentages of them assigned to the correct class. One discriminant function ($P < 0.05$) was calculated for ripening time and cheese type, and three functions for season. The former correctly classified, after cross-validation, 91% of the cases (11 of 12 samples) into the two pre-defined groups

Table 5 Total fatty acids in cheesemaking milk collected in different seasons (percentage of each fatty acid respect to the total amount of fatty acids)

FFA	*Summer*	*Autumn*	*Winter*	*Spring*
C4:0	4.60 ± 0.57^{a,b}	4.44 ± 0.54^{a}	5.02 ± 1.03^{b}	5.10 ± 0.45^{b}
C6:0	2.15 ± 0.14^{a,b}	2.11 ± 0.15^{a}	2.32 ± 0.20^{b,c}	2.47 ± 0.09^{c}
C8:0	1.02 ± 0.08^{a}	1.11 ± 0.07^{a}	1.27 ± 0.07^{b}	1.27 ± 0.11^{b}
C10:0	1.83 ± 0.24^{a}	2.09 ± 0.16^{a}	2.63 ± 0.24^{b}	2.48 ± 0.36^{b}
C12:0	2.38 ± 0.34^{a}	2.78 ± 0.20^{a,b}	3.42 ± 0.37^{c}	3.12 ± 0.53^{b,c}
C14:0	8.82 ± 0.42^{a}	9.35 ± 0.50^{a}	9.93 ± 0.34^{b}	10.16 ± 0.48^{b}
C16:0	24.94 ± 0.73^{a}	25.16 ± 0.42^{a}	25.29 ± 0.65^{a}	25.14 ± 0.93^{a}
C18:0	12.76 ± 0.50^{c}	12.21 ± 0.43^{b,c}	11.35 ± 0.54^{a}	11.52 ± 0.72^{a,b}
C18:1	29.92 ± 1.19^{b}	29.04 ± 1.15^{b}	26.65 ± 1.74^{a}	25.55 ± 1.03^{a}
C18:2	1.59 ± 0.16^{a}	1.92 ± 0.20^{b}	2.32 ± 0.14^{c}	2.41 ± 0.16^{c}

Values in the same row with different superscript letters differ ($P < 0.05$).

Table 6 Variance explained by principal component analysis

	% of variance explained by factor				*% of cumulative variance*
	1	*2*	*3*	*4*	
Before rotation	74.5	13.0	8.2	2.7	98.4
After rotation	43.5	29.4	16.3	9.1	98.4

Table 7 Component Score Coefficient Matrix

FFA	*PC1*	*PC2*	*PC3*	*PC4*
C6:0	*0.259*	0.065	−0.224	0.006
C8:0	*0.356*	−0.039	−0.113	−0.193
C10:0	*0.347*	−0.163	0.076	−0.177
C12:0	*0.328*	−0.211	0.034	0.018
C14:0	−0.030	0.341	0.029	−0.122
C16:0	−0.075	0.231	−0.370	0.691
C18:0	−0.182	−0.266	0.024	*1.376*
C18:1	−0.180	*0.792*	0.101	−0.820
C18:2	−0.123	−0.060	*1.126*	−0.636

Table 8 ANOVA of PC scores

	Significance of treatment			
PC	*Type*	*Season*	*Ripening*	*Type × ripening*
1	NS	NS	NS	NS
2	NS	*	*	NS
3	†	NS	NS	NS
4	*	**	NS	*

NS, not significant; *$P < 0.05$; **$P < 0.01$;
†$P < 0.075$.

according to cheese type, but only 58% of them with respect to ripening time. On the other hand, the three functions calculated for season classified a total of 75% of cases into the four pre-defined groups by cross-validation.

Sensory analysis

The sensory analysis showed no differences between probiotic and control cheeses (both varieties were qualified as 'good'), suggesting that the probiotic strains used in our study would not have an influence on the traditional flavour and taste of Pategras cheese or, otherwise, such influence is too weak to be detected by nontrained people.

CONCLUSIONS

In this work, it was shown that lipolysis in Pategrás measured as the increase of FFA levels during ripening is similar to cheeses with a very low incidence of this enzymatic activity (Por Salut, 700; Edam, 356 and Monterrey, 736 mg FFA/kg of cheese) (Fox *et al.* 1993), somewhat lower than in cheeses with noticeable lipolysis such as Romano (6754 mg FFA/kg of cheese) and Parmesano (4993 mg FFA/kg of cheese) and considerably lower than in cheeses with a high degree of lipolysis such as Roquefort (32 453 mg FFA/kg of cheese).

Statistical treatment of data correctly classified a high percentage of samples according to cheese type (with or without probiotic addition), even when the degree of lipolysis was low in both types and no differences were appreciated by the sensory analysis. The same conclusion is valid for the differences found for seasonal factors. Given that very limited information about FFA profiles is currently available for this typical Argentinean semihard cheese, our results will be useful to better understand its ripening process.

Our results, combined with previous ones about proteolysis (Bergamini *et al.* 2005) and the sensory properties of Pategrás cheese, confirm the feasibility of incorporating probiotic bacteria in this variety without significantly changing its physicochemical and sensory characteristics.

Finally, most dairy companies in Argentina currently incorporate propionibacteria in the manufacture of Pategrás cheese. As propionibacteria are associated with a relatively high lipolytic activity, it would be interesting to evaluate their influence on FFA profiles during ripening in cheese manufactured at our pilot plant.

ACKNOWLEDGEMENTS

This study was made possible by the financial support of Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT01 09-08040 BID 1201 OC/AR). Authors gratefully acknowledge Lic. Silvina Rebechi for her help during the analysis of fatty acids composition of milk. Authors also wish to thank Milkaut S.A. for the raw milk supply.

REFERENCES

Albenzio M, Corbo M R, Rehman S U, Fox P F, De Angelis M, Corsetti A, Sevi A and Gobetti M (2001) Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food Microbiology* **67** 35–48.

Bergamini C V, Hynes E R, Quiberoni A, Suárez V B and Zalazar C A (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 V, Hynes E R and Zalazar C A (2006) Influence of probiotic bacteria on the proteolysis profile of a semi-hard cheese. *International Dairy Journal* **16** 856–866.

Bradley R L, Arnold E, Barbano D M, Semerad R G, Smith D E and Vines B K (1993) Chemical and physical methods. In *Standard Methods for the Examination of Dairy Products*, 16th edn, pp 433–453. Marshall R, ed. Washington, DC: American Public of Health Association.

CAA (2008) *Código Alimentario Argentino, Capítulo VIII: Alimentos Lácteos, Artículo 630 – (Dec. 111, 12.1.76)*, pp 82–83 [Internet document] URL http://www.anmat.gov.ar/codigoa/CAPITULO%20VIII%20(actualiz%206-7-02).pdf. Accessed 16/08/2006.

Candioti M, Hynes E, Meinardi C, Sabbag N and Zalazar C (2000) Uso de fermentos seleccionados directos en la elaboración de queso Cremoso Argentino. *Industria Lechera* **80** 18–25.

Centro de la Industria Lechera Argentina (2005) *Índices y Estadísticas*. Buenos Aires, Argentina [Internet document] URL http://www.cil.org.ar. Accessed 11/06/2006.

Choisy C, Desmazeaud M J, Gripon J C, Lamberet G and Lenoir J (1997) La biochimie de l'affinage. In *Le fromage*, pp 86–105. Eck A and Gillis J C, eds. Paris: Tec & Doc Lavoisier.

De Vuyst L (2000) Technology aspects related to the application of functional starter cultures. *Food Technology and Biotechnology* **38** 105–112.

Deborde C (2003) *Propionibacterium* spp. In *Encyclopedia of Dairy Sciences*. Vol. 4, pp 2330–2339. Roginski H, Fuquay J W and Fox P F, eds. Cornwall: Academic Press.

Fernández-García E, Carbonell M, Calzada J and Nuñez M (2006) Seasonal variation of the the free fatty acids contents of Spanish ovine milk cheeses protected by a designation of origin: a comparative study. *International Dairy Journal* **16** 252–261.

Fox P F, Law J, McSweeney P and Wallace J (1993) Biochemistry of cheese ripening. In *Cheese: Chemistry, Physics and Microbiology. Vol. 1: General Aspects*, 2nd edn, pp 389–438 and 422. Fox P F, ed. London: Chapman & Hall.

Hair J, Anderson R, Tatham R and Black W (1999) *Análisis Multivariado*. Madrid: Prentice Hall.

IDF (1982) *Cheese and Processed Cheese. Determination of the Total Solids Content (reference method). FIL–IDF Standard 4A*. Brussels, Belgium: International Dairy Federation.

IDF (1993) . *Milk. Determination of Nitrogen Content. FIL–IDF Standard 20B*. Brussels, Belgium: International Dairy Federation.

IDF (1995) *Milk and Milk Products. Methods of Sampling. FIL–IDF Standard 50C*. Brussels, Belgium: International Dairy Federation.

McPhee J D and Griffiths M W (2003) Psychrotrophic Bacteria. In *Encyclopedia of Dairy Science*, pp 2340–2344. Roginski H, Fuquay J and Fox P, ed. London: Academic Press.

Perotti M C, Bernal S M, Meinardi C A and Zalazar C A (2005a) The free fatty acids profiles of Reggianito Argentino cheese produced with different starters. *International Dairy Journal* **15** 1150–1155.

Perotti M C, Rebechi S R and Bernal S M (2005b) Detección de adulteraciones en grasa láctea a través del tratamiento por regresión lineal múltiple de perfiles de ácidos grasos totales. *Grasas y Aceites* **56** 67–74.

Powel I, Brome M C and Limsowtin G K (2003) Starter cultures: general aspects. In *Encyclopedia of Dairy Sciences*. Vol. 1, pp 261–268. Roginski H, Fuquay J W and Fox P F, eds. Cornwall: Academic Press.

Reinheimer J, Bargagna M, Candioti M, Ramanzín M and Zalazar C (1985) Microflora psicrotrofa de leche cruda. Su naturaleza y niveles de contaminación en la zona de Santa Fe. *La Alimentación Latinoamericana* **153** 76–80.

Stanton C, Gardiner G, Lynch P B, Collins J K, Fitzgerald G and Ross R P (1998) Market potential for probiotics. *International Dairy Journal* **8** 491–496.

Vinderola C G and 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.

Weihrauch J L (1988) Lipids of milk: deterioration. In *Fundamentals of Dairy Chemistry*, 3rd edn, pp 215–278. Wong N P, ed. New York: Van Nostrand Reinhold.

Zalazar C A, Meinardi C A, Reinheimer J A and Bernal S M (1986) Efectos del mantenimiento en frío de leche sobre la elaboración de quesos de pasta semidura. *La Alimentación Latinoamericana* **160** 87–90.

Zalazar C, Meinardi C and Hynes E (1999) *Quesos Típicos Argentinos. Una revisión general sobre producción y características*. Santa Fe: Centro de publicaciones, Universidad Nacional del Litoral.