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Incorporation of probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* ssp.) in Argentinean ovine cheese

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Abstract The market of cheeses from ewe's milk has been growing steadily in Argentina. The nutritional benefits of these products can be enhanced by adding probiotic cultures. In the present study, the survival of a mix of probiotic microorganisms (*Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* Bb12) in a semi-hard ovine cheese, manufactured with a technology previously developed in our institute was evaluated. Besides, the effect of its incorporation on the chemical composition and ripening parameters of cheeses, including the fatty acid composition and CLA production, was investigated. Experimental cheeses made with Bb12 and La-5 retained counts of the probiotic strains at the required therapeutic level (10^7 CFU g^{-1}). No significant differences were detected between experimental and control cheeses with respect to the different parameters evaluated: gross composition, nitrogen fractions, lipolysis, fatty acids profiles of cheese fat including CLA, and volatile fraction. Results demonstrated that the ovine cheese matrix seems to be suitable for delivery of probiotic microorganisms.

Keywords Ovine cheese · Probiotic bacteria · Proteolysis · Lipolysis · CLA · Volatile compounds

1 Introduction

Probiotics are bacteria, generally lactobacilli and bifidobacteria, associated with a plethora of health promoting properties. It is suggested that the concentration of viable

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probiotics in the product should reach 10^7 CFU g^{-1} or mL^{-1} at the time of consumption (Reid 2008). In addition, certain probiotic bacteria have demonstrated to produce conjugated linoleic acid (CLA), which has gained considerable attention because of its anticarcinogenic property, among others (Coakley et al. 2003).

Cheese is one of the most diffused vehicles to hold efficiently probiotic bacteria, and thus introduce them into the human diet (Saxelin et al. 2003). For a long time the cow's milk cheeses were mostly studied for the incorporation of probiotics (Bergamini et al. 2010a; Gomes et al. 2011; Ong et al. 2007; Souza and Saad 2009). However, today the supplementation of ovine and caprine cheeses with probiotic bacteria is a usual practice as can be seen in the widespread literature about this topic (Albenzio et al. 2013; dos Santos et al. 2012; Özer et al. 2009; Santillo et al. 2012; Santillo and Albenzio 2008). This trend is due to the increasing interest in giving added value to this food that already has benefits inherent to its nutritional and organoleptic properties.

In Argentina, the cheese production from small ruminant's milk is very low and is still performed following artisanal procedures in farmhouses. However, the ovine cheeses are gaining popularity and acceptance on the regional market. Therefore, the development of a probiotic cheese from ewe's milk results in an interesting alternative for diversifying the existing products. Likewise, this type of cheese could impact positively on regional economies.

For this reason, the aim of this study was to evaluate the survival of a probiotic bacteria mixture (*Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* Bb12) incorporated in a semi-hard ovine cheese and its effect on the final product characteristics such as chemical parameters, proteolysis, lipolysis, CLA content and volatile profiles.

2 Materials and methods

2.1 Cheese-making

Milk from the flock of Pampinta breed sheep was provided by the Escuela de Agricultura, Ganadería y Granja (Esperanza, Santa Fe, Argentine). Two replicates of control (C) and experimental (E) cheeses were made on the same day, using for each replicate a total of 20 L of milk (4 vats of 5 L each). Therefore, eight cheeses of each type (C and E) were obtained.

Milk was in-batch pasteurized at 65°C for 20 min and cooled to 36°C. Then, a solution of calcium chloride (Merck, Germany) was added to a final concentration of 0.02 % (w/v). A mix of lyophilized culture composed by *Streptococcus thermophilus* (60 %), *Lactobacillus helveticus* (20 %) and *Lactobacillus bulgaricus* (20 %) (Chr. Hansen, Argentine) was inoculated at 1 % (v/v) as primary starter. A probiotic culture mix of *L. acidophilus* La-5 and *B. animalis* ssp. *lactis* Bb12 (1:1.4 inoculum ratio) (Chr. Hansen, Argentine) was also incorporated into the E cheeses in order to achieve an estimated concentration of at least 10^7 CFU mL^{-1} for each strain. After 15 min, chymosin (Chymax, Chr. Hansen, Argentine) was added at a concentration of 0.3 $mL L^{-1}$ and the whole was left undisturbed. When the curd reached the appropriate strength (approx. 20 min), which was assessed empirically by testing its hardness with a spatula, it was cut into 5-mm pieces. The mixture of whey and curd particles was heated under stirring at

1 °C min⁻¹ to 44 °C for 5 min. Finally, the curd was moulded and pressed during 18 h to facilitate whey expulsion, and salted in a 20 % w/v brine (pH 5.4 at 12 °C) for 4 h. Cheeses were ripened at 12 °C and 80 % relative humidity for 45 days. After 15 days, cheeses of approx. 600 g were vacuum packed in plastic bags. Analyses of four cheeses of each type were conducted at the end of ripening.

2.2 Microbiological analyses

Cheese samples were prepared according to standard FIL-IDF 122C:1996.

Presumptive mesophilic and thermophilic cocci were enumerated on M17 agar (Microbiol, Cagliari, Italy) incubated for 72 h at 30 °C aerobically, and at 45 °C anaerobically, respectively. Presumptive mesophilic lactobacilli were enumerated on FH agar (Isolini et al. 1990), anaerobically incubated at 30 °C for 72 h. Presumptive thermophilic lactobacilli were enumerated on MRS agar (Microbiol, Cagliari, Italy) pH 5.4, anaerobically incubated at 45 °C for 72 h.

The VRBA MUG medium (Microbiol, Cagliari, Italy), aerobically incubated at 37 °C for 18 h, was used for total Coliform bacteria enumeration.

To evaluate the concentration of viable probiotic bacteria cells, the PCR culture technique (PCR-CT) (Comunian et al. 2010) was applied. One millilitre of ten-fold dilutions of the E and C cheeses prepared for the conventional microbiological analysis was anaerobically incubated in MRS broth pH 5.4, at 45 °C for 72 h for La-5, and anaerobically incubated in MRS broth plus cystein (0.5 g.L⁻¹) and dicloxacillin (1 g.L⁻¹), at 37 °C for 72 h for Bb12.

DNA from 1 mL of each culture, grown from each decimal dilution incubated, was extracted by microwave oven treatment (Mannu et al. 2000) and amplified using the 23-10C/Laci-1 primers pairs to detect *Lb. acidophilus*, and Bflact2/Bflact5 for *B. animalis* ssp. *lactis*, according to PCR protocols reported in literature (Song et al. 2000; Ventura et al. 2001).

2.3 Cheese composition and nitrogen fraction analyses

Cheeses were subjected to the analyses of gross composition and nitrogen fractions, according to standardized methods: pH (potentiometric method, pHmeter ORION 420 A, Orion Research Inc., USA); moisture (FIL-IDF 4A:1982); fat (Soxhlet extraction, FIL-IDF 172:2001); total nitrogen (TN) (Kjeldhal method, FIL-IDF 20B:1993); soluble nitrogen at pH 4,6 (Ns-4,6), in TCA 12 % (Ns-TCA) and in PTA 2.5 % (Ns-PTA) (Gripon et al. 1975), and sodium chloride (by titration, Titrator Mettler DL 70, Mettler-Toledo, USA).

2.4 Fatty acid composition of cheese fat matter

The fatty acid (FA) profile of cheese fat was determined using the method of Jiang et al. (1996) for fat matter extraction, and the procedure described in FIL-IDF 182:1999 for the preparation of fatty acid methyl esters (FAME). Briefly, a sample of grated cheese (3 g) was extracted with isopropanol/hexane and the organic layer was separated by centrifugation; this procedure was repeated three times. The lipid fraction was collected in a pear-shaped flask and placed in a Rotavapor kept at

30 °C until dryness. A portion of the fat (50 mg) underwent a basic transmethylation with KOH in methanol and the FAME were obtained. The esters were analyzed by a Varian 3900 gas chromatograph (Varian, Palo Alto CA, USA) fitted with a capillary column SP-2560 (100 m×0.25 mm i.d.×0.25 µm film thickness; Supelco Inc., USA). The injector and FID detector temperatures were 290 °C and the split ratio was 1/50. A constant helium flow rate of 1 mL min⁻¹ was employed. The temperature program of the column was set as follows: 45 °C (4 min), from 45 °C to 175 °C at 13 °C min⁻¹, held at 175 °C (27 min), from 175 °C to 215 °C at 4 °C min⁻¹ and held at 215 °C (35 min). The quantification was done with internal standards added to the fat sample at the methylation step: methyl pentanoate for quantifying C_{4:0}-C_{7:0}; methyl nonanoate for C_{8:0}-C_{11:0}; methyl tridecanoate for C_{12:0}-C_{17:0} and methyl nonadecanoate for C_{18:0}-C_{22:6}. The data were processed using the Star Chromatography Workstation 6.00 (Varian, Palo Alto CA, USA) and the results expressed as percentage of total fatty acids (TFA).

2.5 Free fatty acid analyses

Free fatty acids (FFA) were analyzed by De Jong and Badings (1990) method. Briefly, a sample of grated cheese (1 g) was treated with Na₂SO₄ and H₂SO₄ and the fat was extracted with ethyl ether/heptane. The FFA were isolated from the rest of lipid components by solid phase extraction (SPE) using an aminopropyl column as adsorbent (Bond Elut; Varian Inc., USA); thereof desorption was carried out using ethyl ether acidified with formic acid. The FFA were analyzed by a Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto CA, USA) coupled with a capillary column HP-FFAP (50 m×0.32 mm i.d.×0.50 µm film thickness; J&W Scientific, USA). The injector and FID detector were set to 290 °C and the split ratio was 1/50. A constant helium flow rate of 2 mL min⁻¹ was employed. Oven temperature was programmed from 65 to 240 °C at a rate of 10 °C min⁻¹ and held at 240 °C for 40 min. The quantification was carried out with internal standards added to the cheese sample at the extraction step: pentanoic acid for quantifying C_{2:0}-C_{6:0}; nonanoic acid for C_{8:0}-C_{10:0}; tridecanoic acid for C_{12:0}-C_{16:1} and nonadecanoic acid for C_{17:0}-C_{18:3}. The data were processed using the HP Chemstation (Hewlett-Packard, Palo Alto CA, USA) and the results expressed as mmol kg⁻¹ of cheese.

2.6 Volatile compound analyses

Volatile compounds were isolated by solid phase microextraction (SPME). Briefly, a sample of grated cheese (3 g) was weighted into 10 mL vials, which were hermetically closed with a Viton[®] septa and crimp seals and kept at 4°C for 24 h. Then, the vials were thermostated at 40°C for 15 min in an autosampler device (Varian 8200 CX, Palo Alto CA, USA) fitted with a DVB/Car/PDMS 50/30 µm fiber (Supelco Inc., Bellefonte PA, USA). Finally, the fiber was exposed into the sample headspace for 15 min at 40 °C.

Analytes were thermally desorbed from the fiber into an injector port at 250°C for 5 min (splitless mode).

A Varian 3800 gas chromatograph directly coupled with a Saturn 2000 ion trap mass detector (Varian, Milan, Italy) was used for the analyses of volatile compounds.

Chromatographic separation was performed on a capillary column DB-WAX 52CB (60 m×0.32 mm i.d.×0.5 μm film thickness; Agilent Technologies, Palo Alto CA, USA), with the following conditions: 45 °C (4 min), from 45 °C to 150 °C at 5 °C min⁻¹, held at 150 °C (3 min), from 150 °C to 250 °C at 10 °C min⁻¹ and held at 250 °C (5 min). Helium was used as carrier gas at a constant flow of 1 mL min⁻¹. The flow coming out from the column was split using two deactivated transfer lines (0.15 mm i.d.) towards two detectors: the MS for the identification of the analytes and the FID for the integration of their areas (split ratio 2:3, MS/FID). FID detector was held at 290°C and the operative conditions of MS were: ionization by electronic impact (EI) at 70 eV; mass acquisition range, 20–300 m/z; scan rate, 3 scan s⁻¹.

The tentative identification of the volatile compounds was performed on the basis mass spectral data (NIST98 and Wiley libraries, USA) and confirmed by comparison with retention index of authentic standard (when available). Those compounds positively identified were integrated by FID and the area values expressed as arbitrary units.

All determinations were performed in duplicate.

2.7 Statistical analyses

Statistical analysis was performed using Minitab statistical package, release 15 (Minitab Inc., USA). The results of physicochemical composition, fatty acid profiles and volatile compounds were compared by one-way analyses of variance (ANOVA). Moreover, the possible correlation between the probiotic strain counts and the level of thermophilic and mesophilic cocci and lactobacilli was calculated as Pearson correlation coefficient.

3 Results and discussion

3.1 Microbiological analyses

Cell numbers of presumptive thermophilic and mesophilic lactobacilli and cocci counted in cheese samples are reported in Fig. 1.

Thermophilic and mesophilic cocci counts did not vary for E and C cheeses (about 10⁹ CFU g⁻¹), while slight and high variations occurred in mesophilic and thermophilic lactobacilli average counts (up to 2 and 4 log CFU g⁻¹) respectively, as one out of the four cheeses of each type showed low or very low level of these bacteria. The high variability observed in lactobacilli counts both in C and E cheeses could be attributed to the pronounced decline of this microbial population during ripening as reported by Candioti et al. (2010) for this type of cheese, which can occur at different rates.

No coliform bacteria were detected in any of the cheeses.

As regards probiotics estimated by PCR-CT, they were not detected in C cheeses. A high average concentration (7.75±0.96 log CFU g⁻¹) of La-5 was found in all E cheeses, while Bb12 average counts were about one log CFU g⁻¹ lower (6.50±1.91 log CFU g⁻¹). Indeed, a very low level (10⁴ CFU g⁻¹) of Bb12 was recorded for one out of the four E cheeses, which was the same sample that showed a low lactobacilli count. Probably, an unexpected event that prevented the development/survival of these

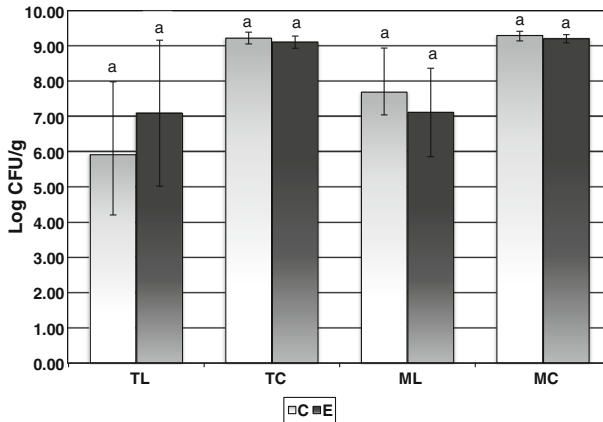


Fig. 1 Counts (average \pm SD) of thermophilic lactobacilli and cocci (TL and TC), and mesophilic lactobacilli and cocci (ML and MC) found in experimental (E) and control (C) cheeses. **a** Letters on vertical bars of standard error indicate no significant statistical differences ($P > 0.05$)

bacteria occurred during manufacture or ripening process of this cheese. Regarding this, in previous assays we studied the viability of La-5 and Bb-12 strains in ovine cheeses made with two different primary starters: *S. thermophilus* and a mix of *S. thermophilus*, *L. bulgaricus* and *L. helveticus*, being observed that the survival improved by the presence of lactobacilli (data not shown).

On the other hand, no statistically significant differences ($P > 0.05$) were found in counts of the investigated lactic microflora colonizing cheeses made with and without probiotic bacteria. Thus, the incorporation of the probiotic strains to cheese did not seem to affect the counts of the lactic microflora.

The results suggest that the technology used for cheese manufacture can allow the survival of La-5 and Bb12 strains during ripening.

3.2 Chemical composition and proteolysis

The chemical parameters and nitrogen fractions of cheeses are shown in Table 1.

As can be seen, no significant differences ($P > 0.05$) were detected between E and C cheeses regarding the gross composition and the indices of proteolysis. Therefore, the inclusion of La-5 and Bb12 did not modify neither the physicochemical characteristics

Table 1 Chemical composition and nitrogen fractions (average \pm SD) of experimental (E) and control (C) cheeses at the end of ripening (45 days)

Parameters	C ($n=4$)	E ($n=4$)	Significance
pH	5.0 \pm 0.1	4.9 \pm 0.1	ns
Moisture (%)	40.8 \pm 0.8	40.7 \pm 0.6	ns
Fat (%)	28.4 \pm 0.9	29.3 \pm 0.6	ns
Protein (%)	24.7 \pm 0.9	23.9 \pm 1.2	ns
NaCl in moisture (%)	4.8 \pm 0.3	4.3 \pm 0.2	ns
Ns 4.6/NT	16.05 \pm 1.14	18.08 \pm 2.11	ns
Ns TCA/NT	13.67 \pm 1.98	15.47 \pm 1.85	ns
Ns PTA/NT	5.06 \pm 0.41	5.18 \pm 0.31	ns

ns not significant ($P > 0.05$)

nor the proteolytic patterns of the cheeses. However, as reported, this feature is strain dependent. In Minas fresh cheese, no differences in yield and chemical composition were found between cheeses added with *L. acidophilus* in comparison to cheeses without addition (Buriti et al. 2005). Kılıç et al. (2009) pointed out that the use of a mix of *L. fermentum* and *L. plantarum* in a typical Turkey sheep cheese (Beyaz) did not affect the gross composition and the proteolysis process during ripening. On the contrary, Brearty et al. (2001) found higher moisture content and a more extensive proteolysis in Cheddar cheeses with Bb12 compared with controls. Likewise, different types of cheeses made with the incorporation of *Bifidobacterium* were characterized by higher levels of proteolysis than traditional cheeses (Corbo et al. 2001; Shahab Lavasani et al. 2012).

3.3 Fatty acid composition of cheese fat

A total of 59 fatty acids constituting the milk fat fraction were identified. They included the main ($C_{4:0}$ - $C_{18:3}$) and minor ($C_{11:0}$ - $C_{22:6}$) fatty acids, with even and odd numbered carbon chains, cis and trans octadecenoic isomers, $C_{18:1}$ (4t, 5t, 6t/8t, 9t, 10t, 11t, 12t, 13t/14t, 15t/10c, 11c, 12c, 13c, 14c/16t) and cis/trans isomers of the octadecadienoic acid, $C_{18:2}$ (conjugated: 9c-11t, 9t-11c, 11t-13c, 11t-13t, 9t-11t and not conjugated: 9c-13t, 9c-12c, 11t-15c, 9c-12t). The percentage values (g FA 100 g⁻¹ TFA) were pooled according to carbon chain length, in short- (SCFA), medium- (MCFA) and long-chain fatty acids (LCFA), as well as in saturated, monounsaturated and polyunsaturated fatty acids (Table 2). For each group there were no significant differences ($P>0.05$) between E and C cheeses, suggesting that the inclusion of probiotic bacteria did not influence the composition of fatty acids that make up triglycerides of cheese fat. Similar results were obtained by Abd El-Salam et al. (2011), who did not observe changes in the fatty acid profile of Ras cheese made with a mix of probiotic bacteria (*Lb. casei* and *Lb. acidophilus*).

Table 2 Fatty acid composition of cheese fat (average \pm SD) of experimental (E) and control (C) cheeses at the end of ripening (45 days) (% g FA 100 g⁻¹ TFA)

Fatty acids	C (n=4)	E (n=4)	Significance
SCFA	11.38 \pm 0.08	11.31 \pm 0.01	ns
MCFA	45.43 \pm 0.13	45.43 \pm 0.27	ns
LCFA	43.19 \pm 0.10	43.27 \pm 0.26	ns
SFA	66.52 \pm 0.06	66.44 \pm 0.20	ns
UFA	33.48 \pm 0.06	33.56 \pm 0.20	ns
MUFA	28.11 \pm 0.04	28.16 \pm 0.14	ns
PUFA	5.37 \pm 0.04	5.40 \pm 0.07	ns
$C_{18:2}$ 9c,11t	1.65 \pm 0.01	1.66 \pm 0.01	ns

Short-chain fatty acids (SCFA): $C_{4:0}$ - $C_{10:0}$ and $C_{7:0}$; Medium-chain fatty acids (MCFA): $C_{12:0}$ - $C_{16:0}$, $C_{11:0}$, $C_{13:0}$, $C_{13:0ai}$, $C_{15:0}$, $C_{15:0i}$, $C_{15:0ai}$, $C_{14:1}$, $C_{17:0}$, $C_{17:0i}$, $C_{17:0ai}$, $C_{17:1}$, $C_{16:1-7c}$ and $C_{16:1-9c}$; Long-chain fatty acids (LCFA): $C_{18:0}$ - $C_{22:6}$

SFA saturated fatty acids; UFA unsaturated fatty acids; MUFA monounsaturated fatty acids; PUFA polyunsaturated fatty acids. ns not significant ($P>0.05$)

Isomers of conjugated linoleic acid (or CLA) are particularly interesting from a nutritional point of view, being $C_{18:2\ 9c,11t}$ the most representative compound of this group. Cheese is a good source of CLA as it is naturally present in milk from ruminants; moreover, additional quantities could be produced during processing and ripening of cheeses. Abd El-Salam and El-Shibiny (2012) performed an overview from the literature about the CLA content in different cheese varieties. They reported a wide range of concentration values ($0.05\text{--}2.90\text{ g }100\text{ g}^{-1}$ TFA) attributed to the type of milk, dietary feeding of lactating animals, environmental factors, cheese manufacturing conditions and biochemical changes that occur in cheese during ripening process. In fact, certain cheese-related microorganisms including probiotic bacteria have demonstrated the ability to produce CLA; however, a wide interspecies variation has been observed (Coakley et al. 2003).

In our study, the values of $C_{18:2\ 9c,11t}$ did not show an increase by the presence of La-5 and Bb12. This could be due to the fact that these strains do not possess a significant ability to produce CLA or that the aging of cheeses (45 days) was not enough for its formation. Similar results were reported by dos Santos et al. (2012) for caprine cheese with *L. acidophilus* La-5. By contrast, Albenzio et al. (2010) and Santillo et al. (2009) demonstrated that the addition of *L. acidophilus*, *B. lactis* and *B. longum* in ovine cheese produced higher levels of CLA at 60 days of ripening.

3.4 Lipolytic pattern

The concentrations of the even-numbered FFA ($C_{4:0}\text{--}C_{18:3}$) and the acetic and propionic acids ($C_{2:0}$ and $C_{3:0}$, respectively) quantified in cheese samples are reported in Table 3.

It has been suggested that certain probiotic strains are able to liberate fatty acids into the cheese matrix (Collins et al. 2003). However, in the current study the level of lipolysis, measured as total of FFA, did not change significantly ($P>0.05$) among cheeses. These values ranged from 4000 to 4400 mg kg^{-1} , and were similar to those reported by Bergamini et al. (2010b) and Hernandez et al. (2009).

As regard the profiles, no significant differences were found ($P>0.05$) between E and C cheeses for the most acids quantified. Only myristic ($C_{14:0}$) and palmitic acids ($C_{16:0}$) differed ($P<0.05$), having E cheeses slightly higher values compared to C cheeses. The most abundant acids were oleic ($C_{18:1}$), palmitic ($C_{16:0}$), myristic ($C_{14:0}$) and butyric ($C_{4:0}$). The relative abundance of the different groups of FFA (short-, medium- and long-chain FFA) were unaffected by the presence of probiotic bacteria. This finding was in accordance with those reported by Corbo et al. (2001), who did not observe appreciable differences in the lipolytic pattern of Canestrato Pugliese cheeses supplemented with bifidobacteria in comparison to traditional cheeses. By contrast, Albenzio et al. (2010) found higher contents of SCFFA in cheeses containing bifidobacteria and higher levels of LCFFA in cheeses made with *L. acidophilus*, compared to control cheeses. Likewise, in white cheeses made with microencapsulated cells of La-5 and Bb12, the quantities of medium- and long-chain FFA were much higher than in control cheeses in which probiotics were used as free cells (Özer et al. 2009).

In addition to the acids derived from lipolysis, other acids were also determined. In fact, very volatile acids ($C_{2:0}$ and $C_{3:0}$) were detected in both cheeses; they could arise from the citrate and lactate metabolisms or the amino acids catabolism (McSweeney

Table 3 Concentrations of free fatty acids (FFA) and acetic and propionic acids in experimental (E) and control (C) cheeses (mmol kg⁻¹ cheese)

	FFA	C (n=4)	E (n=4)	Significance
	C _{2:0}	4.54±0.53	4.69±0.32	ns
	C _{3:0}	0.04±0.00	0.03±0.02	ns
	iC _{4:0}	0.07±0.02	n.d.	*
	C _{4:0}	1.93±0.14	1.90±0.07	ns
	iC _{5:0}	0.13±0.03	0.10±0.01	ns
	C _{6:0}	0.67±0.07	0.66±0.03	ns
	C _{8:0}	0.61±0.05	0.62±0.02	ns
	C _{10:0}	0.98±0.05	1.03±0.02	ns
	C _{12:0}	0.62±0.04	0.65±0.02	ns
	C _{14:0}	1.71±0.05	1.80±0.05	*
	C _{15:0}	0.20±0.01	0.21±0.01	ns
	C _{16:0}	4.15±0.22	4.50±0.17	*
	C _{16:1}	0.30±0.02	0.33±0.01	ns
	C _{17:0}	0.09±0.01	0.10±0.00	ns
	C _{18:0}	1.22±0.13	1.26±0.04	ns
	C _{18:1}	4.74±0.59	5.03±0.23	ns
	C _{18:2}	0.42±0.05	0.45±0.01	ns
	C _{18:3}	0.17±0.02	0.16±0.01	ns
	SCFFA	3.42±0.24	3.30±0.11	ns
	MCFFA	3.31±0.12	3.48±0.10	*
	LCFFA	11.10±1.00	11.85±0.29	ns
	Total	17.83±1.00	18.62±0.36	ns

n.d. not detected; *ns* not significant ($P>0.05$); * significant differences ($P<0.05$); Short-chain free fatty acids (SCFFA): C_{4:0}-C_{8:0}; Medium-chain free fatty acids (MCFFA): C_{10:0}-C_{14:0}; Long-chain free fatty acids (LCFFA): C_{16:0}-C_{18:3}

and Sousa 2000). While the propionic acid had levels negligible, a high concentration of acetic acid was found in all samples, and there were no differences between E and C cheeses. In contrast to our results, Ong et al. (2006) reported that the level of acetic acid in probiotic cheeses was significantly higher than in control cheeses; this fact was attributed to the presence of bifidobacteria.

On the whole, our results suggest that the addition of La-5 and Bb12 probiotics in cheeses did not modify substantially the degradation of fat matter during ripening, or the ripening time of 45 days was not long enough to favour the lipolysis process.

3.5 Volatile compound profile

The headspace analysis of cheese samples by SPME-GC/FID/MS allowed us to identify 38 volatile compounds (8 acids, 14 alcohols, 7 ketones, 6 esters and 3 aldehydes). They were detected both in C and E cheeses, with differences in the amounts of some individual compounds (taking into account the area values) depending on the type of cheese. Figure 2 shows the relative proportions of each group of volatile compounds expressed as percentages of the total area of identified compounds.

Acids were the predominant group of compounds in all cheeses. They represented about 50 % of all volatiles, and significant differences were not detected ($P>0.05$) between E and C cheeses.

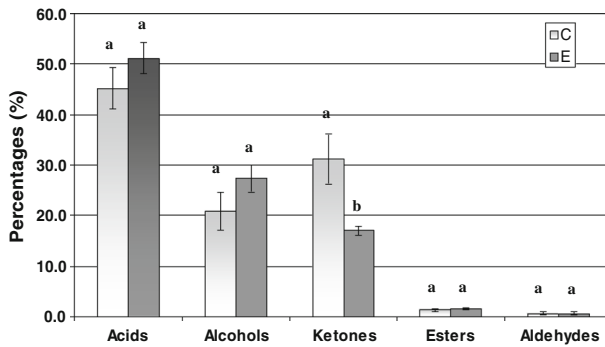


Fig. 2 Percentages of the different chemical families of compounds calculated respect to the total area values (average \pm SD) in experimental (E) and control (C) cheeses. **a, b** Different letters on vertical bars of standard error indicate significant statistical differences ($P < 0.05$)

Alcohols and ketones were other prevalent groups in the volatile profiles of cheeses. The major differences between C and E cheeses were observed in the area values of some components of these chemical families (Figs. 3 and 4). Concerning alcohols, a wide diversity of compounds were identified, among them: primary linear-chain alcohols (ethanol, 1-butanol, 1-pentanol, 1-hexanol, 1-octanol), secondary linear-chain alcohols (2-propanol, 2-pentanol and 2-heptanol) and branched-chain alcohols (2-methyl 1-propanol, 3-methyl 1-butanol and 2-methyl 3-pentanol). Ethanol was the most abundant alcohol in all samples, which are consistent with data reported in literature for different varieties of ewe milk cheeses, some of them added with probiotic strains (Bergamini et al. 2010b; Irigoyen et al. 2007; Özer et al. 2009). Although the percentage of alcohol group did not differ significantly ($P > 0.05$) between both types of cheese, certain primary alcohols were quantitatively higher in E cheeses than C cheeses (Fig. 3). Primary linear-chain alcohols derive from the reduction of corresponding aldehydes produced from fatty acids and/or amino acids catabolism (McSweeney and Sousa 2000). They have weak aroma intensities therefore, it is expected a minor contribution to cheese flavour.

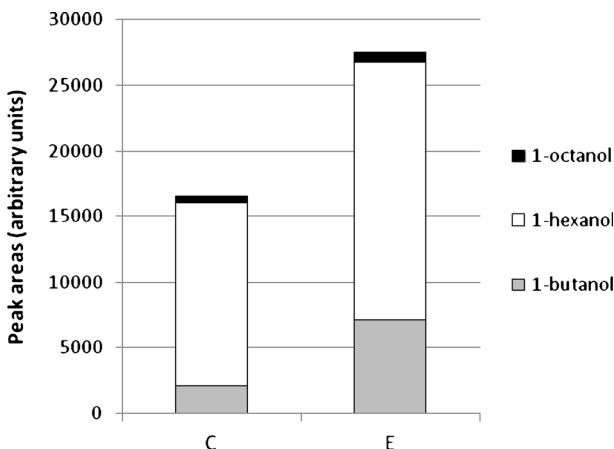


Fig. 3 Area values (in arbitrary units) of some alcohols found in experimental (E) and control (C) cheeses

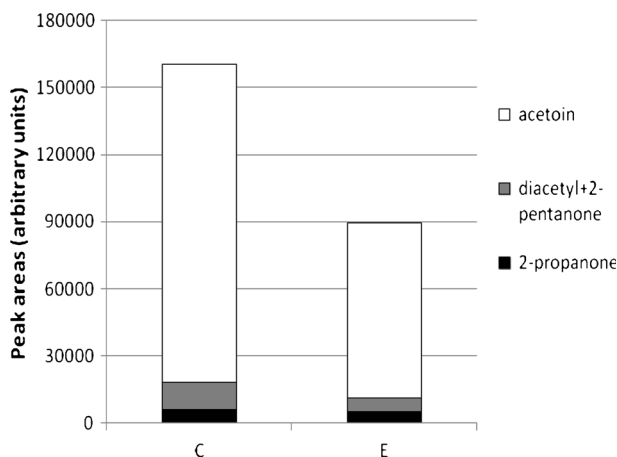


Fig. 4 Area values (in arbitrary units) of some ketones found in experimental (E) and control (C) cheeses

In contrast to the alcohols, the percentage of ketones group was significantly higher ($P < 0.05$) in controls than cheeses made with added probiotics. The area values of three ketones: acetoin, 2-pentanone+diacetyl (unresolved peak) and 2-propanone, were significantly higher in C cheeses (Fig. 4). The area values of remaining ketones were similar in both types of cheese. The main ketone was acetoin (3-hydroxy 2-butanone), as reported in other ovine cheeses (Di Cagno et al. 2003). It is produced from reduction of diacetyl or may be synthesized from pyruvate, lactose or citrate by lactic acid bacteria (McSweeney and Sousa 2000).

Compared with the other chemical families, esters and aldehydes were minority fractions in the volatile profile of all cheeses. Among them, ethyl butanoate and acetaldehyde were the most abundant. Significant differences were not detected between C and E cheeses ($P > 0.05$). Likewise, the levels of the individual compounds from these groups were not modified by the presence of probiotics. In particular, some authors have suggested that *Bifidobacterium* strains are able to produce acetaldehyde (Özer et al. 2009). In our case, this ability was not observed.

Taking into account that E cheeses showed similar volatile profiles to C cheeses, except for ketones group, it is expected that probiotic bacteria evaluated in this study do not affect the sensory characteristic of the cheeses.

4 Conclusions

The data obtained in the present study were promising. It was found that *Lb. acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* Bb12 strains reached satisfactory levels in the ovine cheeses analysed; thus, this matrix seems to be adequate for the survival of these probiotics. Moreover, the added probiotic cultures did not modify the different parameters evaluated: gross composition, nitrogen fractions, lipolysis, fatty acids profiles of cheese fat including CLA and volatile profile of cheeses.

Therefore, the results showed that the production of a functional ovine cheese is feasible, which could impact positively on the regional economies.

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