Microbial dynamics during the ripening of a mixed cow and goat milk cheese manufactured using frozen goat milk curd

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ABSTRACT

To overcome the seasonal shortage of goat milk in mixed milk cheese manufacture, pasteurized goat milk curd and high-pressure-treated raw goat milk curd manufactured in the spring were held at -24°C for 4 mo, thawed, and mixed with fresh cow milk curd for the manufacture of experimental cheeses. Control cheeses were made from a mixture of pasteurized cow and goat milk. The microbiota of experimental and control cheeses was studied using culture-dependent and culture-independent techniques. Bacterial enumeration by classical methods showed lactic acid bacteria to be the dominant population in both control and experimental cheeses. In total, 681 isolates were grouped by partial amplified rDNA restriction analysis (ARDRA) into 4 groups and identified by *16S rRNA* gene sequencing as *Lactococcus lactis* ssp. *lactis* (563 isolates), *Leuconostoc pseudomesenteroides* (72 isolates), *Lactobacillus* spp. (34 isolates), and *Lc. lactis* ssp. *cremoris* (12 isolates). Temporal temperature gradient gel electrophoresis (TTGE) analysis of cheese showed (1) the predominance of *Lc. lactis* in all cheeses; (2) the presence of *Leu. pseudomesenteroides* population in all cheeses from d 15 onward; (3) the presence of a *Lactobacillus plantarum* population in control cheese until d 15 and in experimental cheeses throughout the ripening period. Due to the most diverse and complete set of peptidases present in the genus *Lactobacillus*, the prevalence of this population in experimental cheeses could give rise to differences in cheese flavor between experimental and control cheeses.

Key words: microbial dynamics, mixed-milk cheese, *16S rDNA* gene, temporal temperature gradient gel electrophoresis analysis

INTRODUCTION

Mixed-milk (cow, goat, ewe) cheese varieties constitute more than 40% of Spanish cheese production (MARM, 2008). The quality and organoleptic charac-

teristics of these varieties are positively influenced by the presence of milk from small ruminants (Ha and Lindsay, 1991; Molina et al., 1999). However, because seasonal production of milk by these species has large fluctuations between summer and winter, it is difficult to maintain a standardized composition in cheese manufacture throughout the year.

To overcome seasonality and its effects on production of pure ewe or goat milk cheese, several approaches have been tested, including the use of skim milk powder (IDF, 2002) and the frozen storage of milk (Muir, 1984), curd (Alichanidis et al., 1981; Sendra et al., 1999, 2002), fresh cheese (Fontecha et al., 1994; Casla et al., 1995), or fully ripened cheese (Tejada et al., 2000, 2002). The most successful attempts have been those that preserved ewe or goat milk in the form of curd, fresh cheese, or fully ripened cheese at temperatures below zero, although some defects such as a softer texture, milder taste, and a lower number of viable microorganisms were reported. We developed a new procedure for the manufacture of Hispánico cheese, a semi-hard Spanish variety made from an 80:20 mixture of cow and ewe milk, which stands as the most important representative of Spanish mixed-milk varieties. Hispánico cheese was manufactured by mixing fresh cow milk curd with thawed frozen-stored ewe milk curd (Picon et al., 2010). The procedure yielded satisfactory results for cheese sensory and textural characteristics, and no significant differences were found for counts of lactic acid bacteria (**LAB**).

The negative effect of milk pasteurization on the activity of native milk enzymes and the sensory quality of cheese is well known (Grappin and Beuvier, 1997). The pressurization of raw milk curd at low temperature might be used as an alternative to pasteurization. High-pressure (**HP**) processing at low or moderate temperature causes destruction of microbial vegetative cells, thus reducing microbial loads without detrimental effects on flavor or nutritional components. Application of HP has been proposed as a method to obtain model cheeses with controlled microflora (Trujillo et al., 2000) and for accelerating cheese ripening (Saldo et al., 2002).

In the present work, pasteurized goat milk curd or HP-treated raw goat milk curd, which were held at

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−24°C for 4 mo, were thawed, cut into small pieces, and added to cheese vats containing fresh cow milk curd in its own whey, to manufacture experimental 70:30 cow and goat milk cheeses. High-pressure processing as well as the freezing and thawing steps might influence the microbial population present in cheese, which in turn could affect cheese characteristics. The microbiota of experimental cheeses was monitored throughout ripening by culture-dependent and culture-independent (temporal temperature gradient gel electrophoresis; **TTGE**) techniques, and compared with control cheese made from a mixture of pasteurized cow and goat milk.

MATERIALS AND METHODS

Milk

Raw cow and goat milk were purchased from a local dairy company. The mean composition of cow milk, determined with a MilkoScan Minor (Foss Electric A/S, Hillerød, Denmark), was 3.38% fat, 3.10% protein, and 4.42% lactose. Raw goat milk used for curd manufacture had a mean composition of 5.60% fat, 3.55% protein, and 4.45% lactose, whereas that used in the manufacture of control cheeses was 5.69% fat, 3.59% protein, and 4.53% lactose.

Curd Manufacture

Curd was manufactured in 2 trials, in which pasteurized (15 s at 75°C) or raw goat milk (30 L each in each trial) was heated at 32°C, and 30 mL of a 10% CaCl₂ solution was added. Milk was inoculated with a commercial mesophilic starter (3 units of CHN-11, Chr. Hansen A/S, Hørsholm, Denmark) added directly to the vat. After 20 min, rennet (6 mL of Maxiren, 165 international milk clotting units/mL; DSM, Delft, the Netherlands) was added. Forty minutes later, the curd was cut into 5-mm pieces, and the curd-whey mixture was stirred and heated at a rate of approximately 0.5°C/min to a temperature of 35°C. After 15 min at this temperature, the whey was drained off and the curd was pressed in the vat. Curd blocks (approximately 7 cm wide by 12 cm long by 5 cm deep) were packed in plastic film bags (BB325, Cryovac, Barcelona, Spain) and immersed in an ice-water mixture at 2°C for 15 min. Raw milk curd blocks were vacuum packed and subjected to an HP treatment (400 MPa for 5 min) in a 3.5-L-capacity discontinuous high pressure apparatus (model ACIP 6000, ACB, Nantes, France). After the HP treatment, curd blocks were immersed again in an ice-water mixture at 2°C for 15 min. Both pasteurized milk and HP-treated raw milk curd blocks were frozen in a multiple plate freezer at −52°C. When a tempera-

ture of −24°C was reached, the curd blocks were transferred to a freezer cabinet and held at −24°C for 4 mo.

Cheese Manufacture

Control cheese was manufactured in 2 trials from a mixture of pasteurized (15 s at 75°C) cow milk (40 L) and goat milk (15 L). Additions of CaCl₂, starter cultures, and rennet were in the same proportions as described above. After 40 min, the curd was cut into 5-mm cubes and scalded at 37°C for 15 min. After whey drainage, curd pieces were distributed into cylindrical molds. Three cheeses of approximately 2 kg were pressed overnight at 20°C and 1.5 kg/cm² pressure, salted for 24 h at 12°C in a brine of 160 g of NaCl /L, and ripened at 12°C and 85% relative humidity for 60 d. Cheeses were coated with 2 layers of pimarinic-acid-containing polyvinyl acetate on d 7.

In each cheesemaking trial, 2 vats of experimental cheeses were made. In each vat, 40 L of pasteurized (75°C for 15 s) cow milk with added CaCl₂ and starter cultures was coagulated, as described above. After 40 min, the curds were cut into 5-mm cubes and scalded at 37°C for 10 min. At that time, frozen pasteurized goat milk curd or frozen HP-treated raw goat milk curd, previously thawed at 2°C overnight, was cut into 2-mm pieces and added to the vats, in an amount equivalent to 15 L of goat milk. The mixtures of curds were scalded at 37°C for 5 min before the whey was drained off. The remainder of the manufacture and ripening process was as described above for the control cheese.

Chemical and Microbiological Analyses

Cheese pH and DM content were determined in duplicate as described previously (Ávila et al., 2005). For microbiological analyses, experimental and control cheeses were sampled at d 0 (curd), 1, 15, 30, and 60. Cheese homogenates (in sterile 2% sodium citrate solution) and decimal dilutions (in sterile 0.1% peptone water) were prepared as described previously (Nuñez et al., 1985).

Counts of aerobic mesophilic bacteria were determined in duplicate on plate count agar (Liofilchem, Roseto, Italy) supplemented with 0.1% skim milk (Biolife, Milano, Italy), after incubation for 48 h at 30°C, as described previously (Ávila et al., 2005). Counts of LAB were determined in duplicate on M17 agar (Biolife), after incubation for 48 h at 30°C. Counts of gram-negative bacteria were determined in duplicate on plate count agar-monomycin-KCl agar (Biolife), after incubation for 48 h at 30°C. Counts of coliforms were determined in duplicate on violet red bile agar (Oxoid, Basingstoke, UK), using the pour-plate and overlay

technique. Bacteria were enumerated after incubation for 24 h at 37°C. Counts of staphylococci were determined in duplicate on mannitol salt agar (Oxoid). Colonies with yellow or red zones were enumerated after incubation for 24 h at 37°C.

Isolation of Microorganisms

To analyze the total population, 30 colonies from each cheese sampling were randomly chosen from plate count agar supplemented with 0.1% skim milk and M17 plates. Each colony was purified by streaking 3 times on the same medium from which it was collected. All isolates were stored in liquid cultures with 20% glycerol at -40°C.

DNA Extraction from Pure Cultures and Cheese Samples

Total genomic DNA from pure cultures in the late exponential growth phase was isolated with the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich Inc., St Louis, MO), following the manufacturer's recommendations. Total microbial DNA from curd and cheese samples was isolated from 2% sodium citrate homogenates with the QIAamp DNA Stool kit (Qia-gen GmbH, Hilden, Germany), in accordance with the manufacturer's instructions.

Molecular Identification of Isolates

Total genomic DNA from isolates was used as template to amplify a 348-bp fragment of the *16S rRNA* gene with the primer set Y1 and Y2 (Ward et al., 1998). The PCR products were examined by electrophoresis in 2% agarose gels in 0.5× Tris-borate-EDTA buffer (45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA, pH 8.0) using a 100-bp DNA ladder (Invitrogen, Groningen, the Netherlands) as molecular size marker.

Partial amplified rDNA restriction analysis (**ARDRA**) was used to classify the isolates (Ward et al., 1998). The amplified products were digested with restriction endonucleases *CfoI* and *RsaI* (Roche Applied Science, Mannheim, Germany) and *MboII* (New England Biolabs Inc., Ipswich, MA) under conditions recommended by the manufacturers. Amplicons that were not digested with any of these endonucleases were subjected to digestion with endonucleases *MseI*, *BfaI*, and *AluI* (New England Biolabs Inc.) as described previously (Rodas et al., 2003). Restriction fragment patterns were analyzed in 2% (wt/vol) agarose in 0.5× Tris-borate-EDTA buffer using a 100-bp DNA ladder (Invitrogen) as molecular size marker.

Total genomic DNA from selected isolates was used as template to amplify a 798-bp fragment of the *16S rRNA* gene using the universal primers W01 (Ogier et al., 2002) and 800R (Sawada et al., 1993). The reaction mixture (50 µL) contained 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 0.4 mM deoxyribonucleotide triphosphate (dNTP; each one), 0.1 µM primer (each one), 1.25 units of Platinum Taq DNA polymerase (Invitrogen), and approximately 50 ng of bacterial DNA. The amplification program consisted of an initial denaturation step of 94°C for 5 min, 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 5 min. Amplicons were purified with the GenElute PCR Clean-up Kit (Sigma-Aldrich Inc.) and sequenced in both directions with each of the amplification primers. Sequences were compared with those in the GenBank database (<http://blast.ncbi.nlm.nih.gov>) using the BLAST program (Altschul et al., 1997).

Lactobacillus isolates belonging to the *plantarum* species were identified at the species level by the multiplex PCR assay with *recA* gene-derived primers (Torriani et al., 2001).

PCR Amplification and TTGE Conditions

Total DNA from curd and cheese samples was used as template material to amplify the V3 region of the bacterial *16S rRNA* gene by performing a nested PCR, with primers W01 and W012 in the first step and HDA1GC and HDA2 in the second step, following the procedure described by Ogier et al. (2002).

The PCR products previously obtained were subjected to TTGE analysis in a DCode apparatus (BioRad, Richmond, CA). Polyacrylamide gels (8% acrylamide, 7 M urea) were prepared and run in 1× Tris-acetate-EDTA (**TAE**) buffer (diluted from a 50× TAE stock, BioRad). Samples were subjected to electrophoresis for 16 h at 41 V with a temperature gradient from 63 to 70°C at an increase rate of 0.4°C/h (Ogier et al., 2002). Gels were stained with ethidium bromide (0.5 µg/mL in 1× TAE) for 15 min, rinsed in 1× TAE buffer for 20 min, and photographed under UV light.

Statistical Analysis

Statistical treatment of data was performed by using the SPSS Win 14.0 program (SPSS Inc., Chicago, IL). Analyses of variance were carried out considering 2 main effects: cheese type (2 experimental cheeses and 1 control cheese) and cheese age. Means were compared using Tukey's test at a significance level of $P < 0.05$.

Table 1. Dry matter content (%) and cheese pH during ripening of experimental mixed cow and goat milk cheeses manufactured by mixing fresh cow milk curd with frozen pasteurized goat milk curd or frozen high-pressure (HP)-treated raw goat milk curd, and control cheese made from a mixture of pasteurized cow and goat milks

Parameter ¹	Age (d)	Control cheese	Experimental cheeses (30% thawed goat milk curd)	
			Pasteurized milk curd	HP-treated curd
DM	1	46.00 ± 1.35 ^a	46.04 ± 0.44 ^a	45.71 ± 0.49 ^a
	15	51.10 ± 0.44 ^a	50.64 ± 0.51 ^a	50.47 ± 0.20 ^a
	30	57.01 ± 0.22 ^a	53.06 ± 0.56 ^b	53.07 ± 0.19 ^b
	60	58.72 ± 1.81 ^a	55.49 ± 0.27 ^b	55.41 ± 0.91 ^b
pH	1	5.08 ± 0.03 ^b	5.13 ± 0.01 ^a	5.16 ± 0.01 ^a
	15	5.09 ± 0.01 ^a	5.03 ± 0.05 ^b	5.08 ± 0.03 ^a
	30	4.99 ± 0.02 ^b	5.08 ± 0.01 ^a	5.03 ± 0.04 ^{ab}
	60	5.00 ± 0.02 ^b	5.11 ± 0.04 ^a	4.99 ± 0.02 ^b

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹Mean ± SD ($n = 6$) of triplicate determinations in 2 cheesemaking trials.

RESULTS

DM Content and pH

Dry matter content (Table 1) increased gradually in all cheeses during ripening due to moisture loss. No significant differences were recorded between experimental and control cheeses during the first 15 d. However, control cheeses lost more moisture from d 30 onward than did experimental cheeses.

Cheese pH showed minor differences among cheeses, which did not exceed 0.12 units during ripening (Table 1). No significant differences were recorded between the experimental cheese made with HP-treated curd and the control cheese from d 15 onward.

Bacterial Counts

Counts of the different bacterial groups are shown in Table 2. No significant differences in aerobic mesophilic bacteria or LAB counts were found between experimental and control cheeses throughout ripening. Values of approximately 10^7 cfu/g were recorded for curd, which increased to 10^9 cfu/g at d 1 and slowly decreased during the 60-d ripening period. Counts of both groups were very close, indicating that LAB was the dominant population.

Gram-negative bacteria counts were significantly ($P < 0.05$) higher in experimental cheese made with frozen curd from pasteurized goat milk than in experimental cheese made with frozen HP-treated curd or in control cheese.

Coliform counts during the first 24 h were also significantly higher in experimental cheeses made with frozen curd from pasteurized goat milk than in experimental cheese made with frozen HP-treated curd or in

control cheese. However, no significant differences were observed in coliform counts between cheeses from d 15 onwards. No significant differences in staphylococci counts were found between experimental and control cheeses throughout the ripening period.

Grouping of Isolates by Partial ARDRA

A fragment of approximately 349 bp of the *16S rRNA* gene was amplified by PCR using DNA from 317 isolates from cheesemaking trial 1 and 364 isolates from trial 2 as template. The fragment was subjected to ARDRA, with endonucleases *CfoI*, *RsaI*, and *MboII*. Four different patterns were obtained (Figure 1): the most numerous group (238 of 317 and 325 of 364 isolates) comprised isolates whose *16S rRNA* gene fragment was digested by *CfoI* and *RsaI*, but not by *MboII*. Isolates whose *16S rRNA* gene fragment was only digested by *CfoI* were the second most abundant group (52 of 317 and 20 of 364). The third group comprised those isolates whose *16S rRNA* gene fragment was slightly larger and could not be digested by any of these 3 endonucleases (27 of 317 and 7 of 364). The smallest group comprised those whose *16S rRNA* gene fragment was digested only by *MboII*; these isolates were found only in cheeses manufactured in trial 2 (12 of 364). The first and last patterns (Figure 1A, patterns a and b) were described previously as *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*, respectively (Ward et al., 1998). Amplified fragments from isolates of the second group were subjected to digestion with *BamHI*. All fragments possessed this restriction site (Figure 1A, pattern c). They were presumptively identified as members of the genus *Leuconostoc*. The greater size of the amplified fragment in isolates from group 3 indicated that they belonged to the genus *Lactobacillus*. To

Table 2. Counts of aerobic mesophilic bacteria, lactic acid bacteria (LAB), gram-negative bacteria, coliforms, and staphylococci during ripening of experimental mixed cow and goat milk cheeses manufactured by mixing fresh cow milk curd with frozen pasteurized goat milk curd or frozen high-pressure (HP)-treated raw goat milk curd, and control cheese made from a mixture of pasteurized cow and goat milk¹

Bacterial group ²	Age (d)	Control cheese	Experimental cheeses (30% thawed goat milk curd)	
			Pasteurized milk curd	HP-treated curd
Aerobic mesophilic bacteria	Curd	6.89 ± 0.07 ^a	6.93 ± 0.19 ^a	6.88 ± 0.07 ^a
	1	9.01 ± 0.05 ^a	9.12 ± 0.07 ^a	8.95 ± 0.16 ^a
	15	8.86 ± 0.30 ^a	8.80 ± 0.08 ^a	8.67 ± 0.32 ^a
	30	8.89 ± 0.08 ^a	8.68 ± 0.15 ^{ab}	8.41 ± 0.29 ^b
	60	7.65 ± 0.23 ^a	7.82 ± 0.11 ^a	7.88 ± 0.16 ^a
LAB	Curd	7.38 ± 0.14 ^a	7.38 ± 0.08 ^a	7.39 ± 0.22 ^a
	1	9.34 ± 0.03 ^a	9.39 ± 0.10 ^a	9.31 ± 0.02 ^a
	15	8.90 ± 0.28 ^a	8.89 ± 0.06 ^a	8.80 ± 0.41 ^a
	30	8.77 ± 0.21 ^a	8.83 ± 0.18 ^a	8.56 ± 0.32 ^a
	60	7.78 ± 0.32 ^a	7.85 ± 0.06 ^a	7.77 ± 0.28 ^a
Gram-negative bacteria	Curd	1.30 ± 0.00 ^b	3.12 ± 0.31 ^a	1.80 ± 0.71 ^b
	1	1.30 ± 0.00 ^b	4.42 ± 0.04 ^a	1.80 ± 0.71 ^b
	15	2.30 ± 0.00 ^b	3.25 ± 0.07 ^a	3.47 ± 0.08 ^a
	30	1.30 ± 0.00 ^b	2.60 ± 0.00 ^a	1.30 ± 0.00 ^b
	60	1.95 ± 0.91 ^b	4.20 ± 0.02 ^a	2.69 ± 0.13 ^b
Coliforms	Curd	ND ³	2.02 ± 0.26 ^a	ND
	1	ND	2.29 ± 1.48 ^a	ND
	15	ND	1.20 ± 0.85 ^a	1.20 ± 0.85 ^a
	30	ND	ND	ND
	60	ND	ND	ND
Staphylococci	Curd	1.80 ± 0.71 ^a	2.58 ± 0.03 ^a	1.80 ± 0.71 ^a
	1	2.45 ± 0.21 ^a	1.80 ± 0.71 ^a	2.65 ± 0.01 ^a
	15	1.30 ± 0.00 ^a	1.80 ± 0.71 ^a	2.49 ± 0.11 ^a
	30	1.30 ± 0.00 ^a	1.80 ± 0.71 ^a	1.30 ± 0.00 ^a
	60	1.80 ± 0.71 ^a	1.30 ± 0.00 ^a	1.80 ± 0.71 ^a

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

²Mean counts are expressed as log cfu/g of cheese.

²Mean ± SD ($n = 4$) of duplicate determinations in 2 cheesemaking trials.

³Not detected.

ascertain if more than one species was present in this group, digestions with endonucleases *MseI*, *BfaI*, and *AluI* were also performed. Group 3 isolates could be divided into 3 subgroups (Figure 1B). Patterns a and b were each found in 1 isolate from cheesemaking trial 1, whereas the rest of the isolates showed pattern c.

and control cheeses from d 15 onward in cheesemaking trial 1, whereas they were isolated mainly at the end of ripening in trial 2. Presumptive *Lactobacillus* were isolated from both experimental cheeses in trial 1 and only from experimental cheese made with HP-treated curd in trial 2.

Diversity of the Culturable Fraction During Ripening

Figure 2 shows the microbial distribution of the isolates in control and experimental cheeses during ripening. Differences between the cheesemaking trials were recorded. On d 1, only *Lc. lactis* was isolated from experimental and control cheeses. Most of them belonged to *Lc. lactis* ssp. *lactis*, but a few *Lc. lactis* ssp. *cremoris* were isolated from cheeses manufactured in trial 2 (Figure 2B). *Lactococcus lactis* was the most abundant population in most of the cheeses, with the exception of 60-d-old control cheese and experimental cheese made with HP-treated curd in trial 1. Presumptive *Leuconostoc* isolates were found in experimental

Identification of Microbial Isolates

Sequencing of an approximately 800-bp fragment of the *16S rRNA* gene obtained by amplification with primers W01 and 800R identified the isolates with the pattern shown in Figure 1A, section a, as *Lc. lactis* ssp. *lactis* (100% homology), the pattern shown in Figure 1A, section b, as *Lc. lactis* ssp. *cremoris* (99% homology), the pattern shown in Figure 1A, section c, as *Leuconostoc pseudomesenteroides* (100% homology), the pattern shown in Figure 1B, section a, as *Lactobacillus brevis* (99% homology), the pattern shown in Figure 1B, section b, as *Lactobacillus casei* or *Lactobacillus paracasei* (both 100% homology), and Bc pattern shown

Figure 1. Patterns obtained by partial amplified rDNA restriction analysis (ARDRA) with endonucleases *CfoI* (1), *RsaI* (2), *MboII* (3), *BamHI* (4), *MseI* (5), *BfaI* (6), and *AluI* (7). Panel A = *Lactococcus lactis* ssp. *lactis* (a), *Lc. lactis* ssp. *cremoris* (b), and *Leuconostoc* spp. (c); panel B: *Lactobacillus* spp. (a, b, c).

in Figure 1B, section c, as *Lactobacillus plantarum*, *Lactobacillus pentosus*, or *Lactobacillus paraplantarum* (all 99% homology).

All isolates with the pattern shown in Figure 1B, section c, were subjected to a multiplex PCR that amplified part of the *recA* gene and rendered a PCR product with a species-specific size. A PCR product of 318 bp was obtained for all isolates tested (data not shown), which classified them as members of *Lb. plantarum* species.

TTGE Analysis of the Isolates

Figure 3 shows the TTGE profiles of the different culturable groups and that of the starter used in cheese-making trials (CHN-11). Pure cultures gave rise to one major band on TTGE gels, with the exception of *Lb. casei/paracasei*, where 2 major bands could be observed. In spite of the different sequences of the amplified 16S rDNA gene fragment of *Leu. pseudomesenteroides* and *Lb. brevis*, their TTGE profiles were identical.

Figure 2. Microbial distribution in control (C) and experimental (PaM = manufactured with frozen pasteurized goat milk curd and PrC = manufactured with frozen high-pressure-treated raw goat milk curd) cheeses in cheesemaking trial 1 (A) and trial 2 (B) during ripening: *Lactococcus lactis* ssp. *lactis* (black bars), *Lc. lactis* ssp. *cremoris* (striped bars), *Leuconostoc* spp. (gray bars), and *Lactobacillus* spp. (white bars).

The TTGE profile of starter CHN-11 (lane 7) showed a prominent band corresponding to *Lc. lactis* together with a faint band corresponding to *Lb. plantarum*.

TTGE Analysis of Cheese Samples

The DNA extracted from curd and cheese samples was subjected to PCR-TTGE analysis. Combined amplicons of identified strains isolated from cheese samples were used as markers. The TTGE profiles obtained for the control cheese and the experimental cheese made with pasteurized milk curd (Figure 4) were simple, as would be expected from cheeses made with pasteurized milk. However, no greater complexity was observed for the experimental cheese made with HP-treated curd. The most prominent band in all cheese samples was that corresponding to *Lc. lactis*. A faint band corre-

sponding to the *Lb. plantarum* group could be observed in control cheeses at d 0, 1, and 15 of ripening and in experimental cheeses throughout the ripening period. The band corresponding to *Leu. pseudomesenteroides* or *Lb. brevis* was visible at d 15, 30, and 60 in both experimental and control cheeses.

DISCUSSION

The lower DM content observed in experimental cheeses compared with the control cheese has been previously reported in experimental Hispánico cheeses made with HP-treated ewe milk curd (Alonso et al., 2011). However, experimental Hispánico cheese made

Figure 3. Temporal temperature gradient gel electrophoresis (TTGE) profiles of the V3 region of 16S rRNA gene fragments of pure cultures. Fragments were separated by TTGE on a denaturing 8% (wt/vol) acrylamide gel. M = combined amplicons of identified strains used as control: *Lactococcus garvieae* (a), *Leuconostoc citreum* (b), *Lactococcus lactis* (c), and *Streptococcus thermophilus* (d). Lane 1 = *Lc. lactis* ssp. *lactis*; lane 2 = *Lc. lactis* ssp. *cremoris*; lane 3 = *Leuconostoc pseudomesenteroides*; lane 4 = *Lactobacillus plantarum*; lane 5 = *Lactobacillus brevis*; lane 6 = *Lactobacillus casei/paracasei*; and lane 7 = starter CHN-11 (Chr. Hansen A/S, Hørsholm, Denmark).

Figure 4. Temporal temperature gradient gel electrophoresis profiles of the V3 region of *16S rRNA* gene fragments that were amplified by PCR from extracts of cheese samples. M = combined amplicons of identified strains used as control: *Lactobacillus plantarum* (a), *Leuconostoc pseudomesenteroides* (b), *Lactococcus lactis* (c), and *Lactobacillus casei/paracasei* (d). Lanes 1 to 5 = control cheese samples at 0, 1, 15, 30, and 60 d of ripening; lanes 6 to 10 = experimental cheese made with frozen pasteurized goat milk curd at 0, 1, 15, 30, and 60 d of ripening; lanes 11 to 15 = experimental cheese made with frozen high-pressure-treated raw goat milk curd at 0, 1, 15, 30, and 60 d of ripening.

using frozen ewe milk curds had similar DM content values to those of control cheese (Picon et al., 2010).

Counts of LAB were similar to those obtained for Hispánico cheese, a mixed bovine and ovine pasteurized milk cheese, which peaked at d 1 and decreased slightly during the ripening period (Garde et al., 2003). Although freezing of curd may affect the viability of LAB (Alichanidis et al., 1981), freezing and thawing of goat milk curd before mixing with cow milk curd had a negligible effect on LAB counts in curd and 1-d-old cheeses, as has been reported for cheeses manufactured with frozen ewe milk curds (Picon et al., 2010). Levels of gram-negative bacteria, coliforms, and staphylococci in control and experimental cheeses were lower than those recorded for raw milk cheeses (Arqués et al.,

2006). The usefulness of the HP treatment to inactivate undesirable microorganisms (O'Reilly et al., 2000) was demonstrated because counts of gram-negative, coliforms, and staphylococci in experimental cheeses manufactured with HP-treated raw goat milk curd were not significantly different from those in control cheese. Gram-negative bacteria, and in particular coliforms, are more sensitive than gram-positive bacteria to HP treatment (Arqués et al., 2006). Coliforms were present at higher levels in experimental cheese manufactured with pasteurized goat milk curd than in the other cheeses during the first 24 h (Table 1). The origin of this contamination was not traced, but the low level (2 log cfu/g) does not necessarily imply inadequate milk pasteurization or faulty curd manufacturing practices.

Counts of staphylococci were $<2 \log \text{ cfu/g}$ from d 30 onward in control and experimental cheeses (Table 1). All isolated staphylococci were coagulase-negative and, therefore, supposedly nonpathogenic. By isolation of DNA from some *Staphylococcus* colonies, and sequencing of a fragment of their *16S rRNA* gene, we showed that all isolates belonged to *Staphylococcus hominis*, a harmless commensal species present on human and animal skin.

The commercial starter used in cheesemaking was a multiple mixed-strain culture containing, according to manufacturers' specifications, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, and *Lc. lactis* ssp. *lactis* biovar *diacetylactis*. In spite of the direct addition to the vat, *Lc. lactis* ssp. *cremoris* strains were only isolated from cheese manufactured in trial 2 and, unexpectedly, the *Leuconostoc* strains isolated from cheese belonged to the species *pseudomesenteroides*. The ratio or numbers of the different species of LAB in the starter was not stated by the manufacturer. However, from the TTGE profiles obtained after amplification of the *16S rRNA* region from the DNA directly extracted from starter CHN-11 (Figure 3, lane 7), some information can be inferred. *Lactococcus lactis* must be present at a ratio close to 99% (the predominant band), *Lb. plantarum* at a ratio of at least 1% (visible as a faint band), and *Leu. pseudomesenteroides* at a ratio less than 1% (no visible band). The different subspecies of *L. lactis* cannot be distinguished by TTGE analysis (Ogier et al., 2002). The TTGE profile of the starter also showed a band corresponding to the *Lb. plantarum* group, which was not a declared component of the starter. Studies showing evidence of mistakes during production of wine starters have also been reported (Fernández-Espinar et al., 2001). Detection of species by TTGE may be limited either by low DNA concentrations or by the presence of high concentrations of competing DNA. The limit of detection for this technique has been established when the minority species accounts for 1:100 or less of the total DNA concentration (Muyzer et al., 1993; Ogier et al., 2002).

The TTGE profiles of cheese samples comprised 2 or 3 bands, as could be expected from pasteurized milk cheeses (Randazzo et al., 2006). However, very complex ecosystems with important shifts in the microbial community structure have been shown during the ripening of natural fermented raw milk cheeses (Randazzo et al., 2002; Flórez and Mayo, 2006).

The intensity of an individual band in a TTGE profile is a semiquantitative measurement of the abundance of that sequence in the population (Muyzer et al., 1993). The TTGE profiles showed *Lc. lactis* to be the most abundant population. Culturing has shown *Lactococcus*

strains to be dominant during manufacture and ripening of many cheese varieties (Nuñez, 1978; Fernández del Pozo et al., 1988; Beresford et al., 2001), as has been shown by culture-independent techniques (Flórez and Mayo, 2006; Randazzo et al., 2006; Dolci et al., 2008).

Because the *Leu. pseudomesenteroides* amplicon was present in both control and experimental cheeses from d 15 onward, an increase in the *Leuconostoc* population must have taken place during the first 15 d of ripening. However, in Ragusano cheese, the *Leu. mesenteroides* amplicon was present in milk but disappeared during fermentation and did not reappear during cheese ripening (Randazzo et al., 2002). The intensity of this band was much fainter than that of *Lc. lactis*, indicating a lower level of the *Leuconostoc* population than the *Lactococcus* population. *Leuconostoc pseudomesenteroides* strains were also isolated by culturing techniques from cheeses manufactured in cheesemaking trial 1 at d 15, 30, and 60, and from cheeses manufactured in cheesemaking trial 2, mainly at the end of ripening. The bands corresponding to the amplified V3 region of the *16S rDNA* gene of *Leu. pseudomesenteroides* and *Lb. brevis* appeared at the same height. This has been also described by DGGE analysis in Cabrales cheese (Flórez and Mayo, 2006).

The *Lb. plantarum* band was present in control cheese only at the beginning of ripening and in experimental cheeses throughout the ripening period. Moreover, at the end of ripening, *Lactobacillus* strains were isolated by culture techniques only from experimental cheeses. *Lactobacillus* spp. were not selectively enumerated, but their levels may be estimated from the TTGE profiles and from the identified microbial isolates. The presence of the *Lb. plantarum* band in most of the DNA samples directly extracted from cheese indicates that this species represented at least 1% of the total population, equivalent to $7 \log \text{ cfu/g}$, in all cheeses until d 15. Because no *Lb. plantarum* band was visible in 30- and 60-d-old control cheeses, *Lb. plantarum* counts were at that time probably less than 1% of the total population. In addition, taking into account the number of identified microbial isolates, the fact that no *Lb. plantarum* were isolated from plates of control cheese indicates that they represented less than 3% of the total population. Contrarily, 20 to 55% of the identified isolates from 60-d-old experimental cheeses were *Lb. plantarum*.

Most of the *Lactobacillus* isolates belonged to the *plantarum* species, with the exception of 1 *Lb. brevis* isolate in a 60-d-old experimental cheese manufactured with pasteurized milk curd and 1 *Lb. casei/paracasei* isolate in a 60-d-old experimental cheese manufactured with HP-treated raw milk curd. Mesophilic lactoba-

cilli (*Lb. casei*, *Lb. plantarum*, and *Lb. brevis* being the dominant species) are found in most ripened cheese varieties, being deliberately added to cheese milk as starters or occurring as adventitious bacteria in cheese (Khalid and Marth, 1990). All 3 species have been frequently isolated from dairy products by using culture-independent techniques (Ogier et al., 2002; Randazzo et al., 2002; Flórez and Mayo, 2006). The use of selected strains of homofermentative *Lb. casei* or *Lb. plantarum* as adjunct cultures is claimed to improve and accelerate flavor development (Ortigosa et al., 2005; Williams et al., 2006; Kocaoglu-Vurma et al., 2008). The genus *Lactobacillus* has the most diverse and complete set of peptidases (Mengjin and Siezen, 2006) and contributes to the formation of key volatile compounds.

Because the genus *Lactobacillus* contains the most diverse and complete set of peptidases, the prevalence of this population in experimental cheeses could give rise to differences in cheese flavor between experimental and control cheeses. No amplicons related to *Lb. casei/paracasei* or to gram-negative bacteria, coliforms, or staphylococci were found by TTGE analysis and this was probably due to the low levels reached by these populations compared with the *Lc. lactis* population.

CONCLUSIONS

Mixed cow and goat milk cheeses manufactured by mixing frozen pasteurized goat milk curd or HP-treated raw goat milk curd, previously thawed, with fresh cow milk curd showed no significant differences in aerobic mesophilic bacteria or LAB counts compared with control cheese made from a mixture of pasteurized cow and goat milk. The LAB were dominant in both control and experimental cheeses. A total of 681 isolates were grouped by partial ARDRA into 4 groups and identified by *16S rRNA* gene sequencing as *Lc. lactis* ssp. *lactis* (563 isolates), *Leu. pseudomesenteroides* (72 isolates), *Lactobacillus* spp. (34 isolates), and *Lc. lactis* ssp. *cremoris* (12 isolates). The TTGE analysis of cheese samples showed (1) the predominance of the *Lc. lactis* population in all cheeses; (2) the presence of *Leu. pseudomesenteroides* population in all cheeses from d 15 onward; and (3) the presence of *Lb. plantarum* in control cheese until d 15 and in experimental cheeses throughout the ripening period. Because *Lactobacillus* has the most diverse and complete set of peptidases, which contribute to the formation of key volatile compounds, their presence in experimental cheeses throughout the ripening period could give rise to differences in cheese flavor compared with that of control cheeses.

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