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Original article

Application of specific starters for the manufacture of Venaco cheese

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Abstract – The application of defined specific starter strains and their influence on microbiological, biochemical and sensory characteristics were studied during ripening of Venaco cheese, a traditional Corsican raw milk cheese manufactured with goat's or ewe's milk. Three defined starter blends, composed of wild strains of lactic acid bacteria, were tested. The first blend was composed of 2 strains of *Lactococcus lactis* subsp. *lactis* (ratio 1:1) and was used as a control. The second was composed of 3 strains, the two *Lactococcus* strains used in the first starter blend in addition to a strain of *Lactobacillus paraplantarum* (ratio 2:2:1). The third blend was also composed of 3 strains, the same two lactococci used in the first blend in addition to a strain of *Leuconostoc mesenteroides* subsp. *mesenteroides* (ratio 2:2:1). The experiment was carried out, in duplicate, at two cheese dairies. The first dairy transforms raw goat's milk and the second transforms raw ewe's milk. DNA fingerprinting of cheese isolates using the Rep-PCR technique showed that strains inoculated in milk established themselves in cheese. Lactococci and *Ln. mesenteroides* subsp. *mesenteroides* strains were present until the end of ripening, while *Lb. paraplantarum* was detected in cheese only during the first 15 d. Indigenous lactic microflora were found throughout ripening, showing a balance between this microflora and the starter strains. Goat's and ewe's milk cheeses made with *Leuconostoc* had the highest level of proteolysis, and those made with *Lactobacillus*, the highest level of lipolysis. These physico-chemical modifications led to significant differences in cheese sensory characteristics assessed by the triangle test.

raw milk / goat's milk cheese / ewe's milk cheese / specific defined starter / *Lactococcus* / *Lactobacillus* / *Leuconostoc*

Résumé – Utilisation de ferments lactiques spécifiques pour la fabrication du fromage Venaco. L'utilisation de levains lactiques sélectionnés spécifiques et leur influence sur les caractéristiques microbiologiques, biochimiques et sensorielles ont été évaluées durant l'affinage du fromage Venaco, un fromage traditionnel corse au lait cru de chèvre ou de brebis. Trois levains définis, composés de souches « sauvages » de bactéries lactiques, ont été testés. Le premier constitué de 2 souches de *Lactococcus lactis* subsp. *lactis* (ratio 1:1) était utilisé comme témoin. Le second était composé de 3 souches, la paire de lactocoques utilisée dans le premier ferment associée à une souche de *Lactobacillus paraplantarum* (ratio 2:2:1). Le troisième ferment comprenait la même paire de lactocoques associée à une souche de *Leuconostoc mesenteroides* subsp. *mesenteroides* (ratio 2:2:1). L'expérimentation a été menée dans deux fromageries, la première transformant du lait cru de chèvre, la seconde du lait cru de brebis. Chaque essai a été répété 1 fois. Le typage génétique des

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isolats collectés des fromages à l'aide de la technique Rep-PCR a montré que les souches inoculées dans le lait s'implantaient dans le fromage. Les souches de lactocoques et de *Ln. mesenteroides* subsp. *mesenteroides* étaient présentes jusqu'à la fin de l'affinage, la souche de *Lb. paraplantarum* jusqu'à 15 j d'affinage. Les bactéries lactiques de la microflore indigène étaient retrouvées durant l'affinage, traduisant un certain équilibre entre cette microflore et les souches du levain. Les fromages de chèvre et de brebis fabriqués avec la souche de lactobacille étaient les plus lipolysés, tandis que l'indice de protéolyse le plus élevé était obtenu avec le ferment contenant la souche de leuconostoc. Ces modifications physico-chimiques conduisaient à des différences significatives des propriétés sensorielles du fromage évaluées par un test triangulaire.

lait cru / fromage de chèvre / fromage de brebis / ferment spécifique défini / *Lactococcus* / *Lactobacillus* / *Leuconostoc*

1. INTRODUCTION

Traditional cheesemaking relies upon the physico-chemical composition of the milk and the diversity of indigenous raw milk microflora to give the cheese sensory characteristics which reflect the *terroir* where the milk is produced [20]. The indigenous microflora contribute significantly to the development of the specific sensory properties of the cheese [21]. However, nowadays, the production conditions (mechanical milking and hygienic practice) result in clean milk with a low microbial load [45].

The indigenous microflora of traditional cheeses such as Fiore Sardo [34], Serra da Estrella [38] and Ibores [42] have been identified and characterized. Wild-type strains, chosen on the basis of their technological properties, have been tested in cheesemaking. Such specific defined starters have been developed for Fiore Sardo [34] and Ibores cheeses [19].

This work seeks to develop specific defined starters for Venaco, a traditional Corsican soft cheese manufactured with raw goat's or ewe's milk. Previous studies on Venaco cheese have shown that lactococci are the main acidifying agent [7] and that facultatively heterofermentative lactobacilli (FH lactobacilli) and leuconostocs compose the main secondary flora [3]. Strains from Venaco cheese were characterized [9] and specific defined starters composed of lactococci were identified [8].

The main functions of the specific defined starters consist in providing adequate acidification and producing compounds which contribute to the development of sensory properties [48]. These starters are generally composed of mesophilic bacteria such as *Lactococcus,* or thermophilic bacteria such as *Streptococcus thermophilus* or *Lactobacillus helveticus*, depending on the cheese variety. These bacteria, called SLAB (Starter Lactic Acid Bacteria), provide acidification. Bacteria from the secondary flora (NSLAB or Non-Starter Lactic Acid Bacteria) such as FH lactobacilli may also be included. During cheese ripening, these two types of bacteria are responsible for a complex series of biochemical reactions which are vital for proper development of both flavor and texture [4]. The interest in using FH lactobacilli as an adjunct starter is that these bacteria can contribute to proteolysis during ripening, with the production of peptides and amino acids [37] which have an impact on cheese flavor [35, 41]. Moreover, some lactococcal cells lyse in cheese and may provide metabolizable carbohydrates for NSLAB [61].

When a leuconostoc is associated with lactococci in a starter, the growth of the leuconostocs is stimulated by lactococci which produce peptides and amino acids necessary for leuconostoc [6, 12]. Moreover, the decrease in pH produced by lactococci improves the activity of the enzymes that transform citrate into flavor compounds such as diacetyl and acetoin [13]. The maximum activity of these enzymes is obtained at pH 5.5.

Table I. Starter composition.

Starter	Batch	Ratio	Lc43	Lc202	Lb824	Ln845
Lc43-Lc202	43 (control)	1:1	5.0×10^8	5.0×10^8		
Lc43-Lc202-Lb824	824	2:2:1	4.0×10^8	4.0×10^8	2.0×10^8	
Lc43-Lc202-Ln845	845	2:2:1	4.0×10^8	4.0×10^8		2.0×10^8

Counts (cfu·mL^{-1})

Most of the work on the interactions between lactic acid bacteria has been carried out on pasteurized cow's milk in order to be in controlled conditions.

The aim of this work was to study the application of specific starters composed of *Lb. paraplantarum* and *Ln. mesenteroides* subsp. *mesenteroides*, associated with lactococci for the manufacture of Venaco raw milk cheese. The capacity of the starter strains to implant themselves in the cheese and the action of *Lb. paraplantarum* and *Ln. mesenteroides* subsp. *mesenteroides* were studied.

2. MATERIALS AND METHODS

2.1. Strains and starters

The lactic acid bacteria strains used in the cheese starter culture belong to the INRA-LRDE collection (Corté, France) and were previously isolated from milk and Venaco cheese [3, 7]. Starters were composed of strains which have major functions in cheesemaking: lactococci for their acidifying activity, FH lactobacilli and leuconostocs for their capacity for flavor development during ripening. Two strains of *Lc. lactis* subsp. *lactis* (strain Lc43 isolated from goat's cheese and strain Lc202 isolated from goat's milk) were selected according to their acidifying activity, phage-resistance and temperature sensitivity [9]. One strain of *Lb. paraplantarum* (strain Lb824 isolated from ewe's cheese) and one strain of *Ln. mesenteroides* subsp. *mesenteroides* (strain Ln845 isolated from ewe's cheese) were chosen because they are representative of Venaco microflora [3]. Three starters were tested: 2 lactococci (ratio1:1), 2 lactococci + 1 lactobacillus (2:2:1), and 2 lactococci + 1 leuconostoc (2:2:1) (Tab. I).

Before inoculation, strains were grown separately on appropriate autoclaved medium under the following conditions in order to obtain 10^9 cfu·mL^{-1}: lactococci at 30 °C for 16 h in skimmed milk (Merck, Darmstadt, Germany) with 0.75 g·L^{-1} of litmus (Difco, Detroit, MI, USA), lactobacillus at 38 °C for 16 h in FHL broth [31] and leuconostoc at 30 °C for 16 h in MRS broth [16].

2.2. Cheesemaking conditions

The experimentation was carried out in duplicate at one-week intervals at two cheese dairies. The first dairy transforms goat's milk, the second transforms ewe's milk. At each cheese dairy, 20-L batches of raw milk, previously heated to 28–30 °C, were inoculated with (i) the pair of lactococci (batch and cheese 43), (ii) the lactococci and the lactobacillus (batch and cheese 824) and (iii) the lactococci and the leuconostoc (batch and cheese 845) (Tab. I). The milk was inoculated at 1‰ (v/v) in order to obtain 10^6 cfu·mL^{-1}. Cheesemaking and cheese ripening were carried out according to the cheesemakers' usual practices described by Casalta and Zennaro [11]. Cheeses were ripened at 12–15 °C, 85–95% RH, for 45 d.

2.3. Physical and chemical analyses of cheese

The pH of all samples was measured by direct measurement, at room temperature, with a GK2401C Radiometer electrode

(Copenhagen, Denmark), connected to a pHm82 Radiometer pHmeter. The pH was measured in milk and in cheese at 1, 2, 7, 15, 30 and 45 d, inside the sample, at half the radius and halfway up. The following determinations were carried out on cheeses after 2 d and 45 d of ripening: total solids (TS) according to AFNOR [1]; fat (F) as described by Heiss [25]; fat acidity according to IDF [29] modified by Delacroix-Buchet et al. [15]; total nitrogen (TN) according to the Kjeldhal method modified by Gripon et al. [22] and soluble nitrogen (SN) according to Kuchroo and Fox [33]; salt content (NaCl) measured with a Corning 926 chloride analyzer (Halstead, England) and calcium (Ca) as described by Pearce [46] .

2.4. Microbiological analyses

2.4.1. Bacterial counts

Sampling and dilutions were performed according to the IDF standard 122 [30]. Counts of micro-organisms were determined as follows: lactococci on M17 agar [60] (Merck) incubated for 24 h at 30 °C ; FH lactobacilli on FH medium with 50 $mg \cdot L^{-1}$ of vancomycin (Sigma, St Louis, MO, USA) incubated for 72 h at 38 °C; leuconostocs on MRS agar (Merck) with 30 $mg \cdot L^{-1}$ of vancomycin (Sigma) incubated for 72 h at 30 °C; enterococci on Slanetz and Bartley Agar [55] (Merck) incubated for 48 h at 44 °C; salt-tolerant bacteria on MSA [11] (Merck) with 100 $mg \cdot L^{-1}$ of cycloheximide and 5 $g \cdot L^{-1}$ of $CaCO_3$ incubated for 72 h at 30 °C and 96 h at room temperature; yeasts and moulds on YGC agar (Merck) incubated for 96 h at 25 °C.

Determinations were made on raw milk after starter inoculation and on total cheeses after 2, 15, 30 and 45 d of ripening. Counts on milk before inoculation of the starter were also carried out: total viable micro-organisms on Plate Count Agar (Difco) incubated for 72 h at 30 °C, total coliforms on VRBA (Merck) incubated for 24 h at 30 °C. The plates containing between 10 and 300 cfu were chosen for enumeration and isolation.

2.4.2. Isolation of bacteria

A representative sample of colonies (at least the square root of the number of cfu grown per plate) were isolated from the plates used for counting lactococci, FH lactobacilli and leuconostocs. Isolation from M17 was carried out for 3 batches. Isolation from FH medium was performed on batch 824 and from MRS with vancomycin on batch 845. Colonies were isolated from the 2 cheese dairies, from milk after starter inoculation and from cheese at different stages of ripening (2, 15, 30 and 45 d). Colonies were grown on appropriate broth medium (M17 or MRS) and a second isolation was performed on agar medium. The pure cultures were frozen in a 1:1 glycerol – M17 or MRS broth mixture and stored at –80 °C.

2.4.3. Phenotypic characterization

Gram-positive and catalase-negative cocci were tested for their ability to produce CO_2 from glucose (by culturing the isolates in MRS broth tubes with Durham bells), to hydrolyze arginine and to grow in M17 broth at 45 °C (2 d). Gram-positive and catalase-negative rods were tested for their ability to grow in MRS broth at 45 °C (2 d) and 15 °C (7 d), and to produce CO_2 from glucose as described above.

Presumptive lactococci were CO_2-negative and 45 °C-negative cocci. Presumptive leuconostocs were CO_2-positive and arginine hydrolyze-negative. Presumptive FH lactobacilli were 45 °C-negative, 15 °C-positive and CO_2-negative.

Phenotypic characterization was carried out on isolates collected from goat's and ewe's milk and cheese.

2.4.4. Genomic characterization according to Rep-PCR

Total DNA was extracted from 1.0 mL of fresh culture (18 h at 30 °C on M17 or

MRS) in the exponential growth phase using guanidine thiocyanate 4 mol·L^{-1}, pH 7.5, containing N-laurylsarcosine (87:13) (Berthier, personal communication). DNA was then precipitated with ethanol. After drying, the DNA pellet was re-suspended in 40 µL TE buffer. Primer set Rep-1R-Dt/Rep2-D [63] was used (Genosys Biotechnologies Ltd, Cambridge, UK) to generate highly specific and reproducible genomic fingerprints [50]. PCR reactions were performed in a final volume of 20 µL containing 1× PCR buffer, 5 µL DNA, 1.0 mmol·L^{-1} $MgCl_2$, 0.25 µmol·L^{-1} of each primer, 0.2 mmol·L^{-1} dNTP, and 1 unit *Taq* DNA polymerase (Appligene Oncor, Illkirch, France). The PCR was undertaken in a 9600 Gene Amp System Thermal Cycler (Perkin-Elmer Applied Biosystems, Courtaboeuf, France). The cycling program used was 5 min at 94 °C, followed by 30 amplification cycles of 1 min at 94 °C, 1 min at 40 °C, 6 min ramping to 72 °C and 1 min at 72 °C [5]. The PCR products were separated by electrophoresis on 1% agarose gel in TBE buffer at 90 V. Fingerprints were stained in ethidium bromide solution (2.0 µg·mL^{-1}). Gels were photographed and the photo was scanned using a GS670 Molecular Imager System (Biorad, Ivry-sur-Seine, France). The Bionumerics version 2.0 software package (Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate the Pearson similarity coefficient between fingerprints and to cluster the fingerprints according to the UPGMA method. Isolates were considered to be equivalent to the starter strain if their profiles showed at least 80% similarity to the starter strain profile.

Strains isolated from ewe's milk and cheese were identified according to the following plan: 120 strains presumed lactococci, isolated on M17 (40 from each batch, 10 per stage), 40 presumed FHL from batch 824, isolated on FH (10 per stage) and 40 presumed leuconostocs from batch 845, isolated on MRS with vancomycin (10 per stage). Genetic characterization was only carried out on strains isolated from ewe's milk and cheese.

2.5. Sensory evaluation

Sensory analysis was performed at the end of the ripening, according to the recommendations of IDF standard 99C [28]. A panel of 12 to 14 trained tasters from the INRA Dairy Research Unit of Jouy-en-Josas carried out a triangle test [62] in order to detect sensory differences between cheeses. Cheeses were presented with their rind. The tasters based their judgement upon appearance, texture and flavor.

3. RESULTS

3.1. Physical and chemical characteristics

In all cheeses, the pH mean followed a similar tendency (Fig. 1). It declined significantly during the first day. The pH drop was less significant during the second day. The minimum value was reached after one week, following which it slowly increased. Acidification was greater in goat's cheese, the lowest pH mean achieved being 4.75 compared with a pH mean of 4.90 in ewe's cheese. In the same way, the subsequent increase in pH was more significant in goat's cheese: a pH mean after 45 d of 5.64 in comparison with a pH mean of 5.46 in ewe's cheese.

pH evolution depended on the starter used: at 15 and 30 d, cheese 43 showed the highest pH (at 30 d, 5.26 in goat's cheese and 5.41 in ewe's cheese), cheese 824 the lowest pH (at 30 d, 5.04 in goat's cheese and 5.13 in ewe's cheese), while cheese 845 pH was intermediate. In goat's cheese, another difference appeared at the end of ripening: while the pH rise was linear in cheese made with the lactococci pair (final pH 5.37), there was a significant pH increase between 30 and 45 d with the 2 other starters (final pH 5.72 with Lb824 and 5.83 with Ln845). In ewe's cheese,

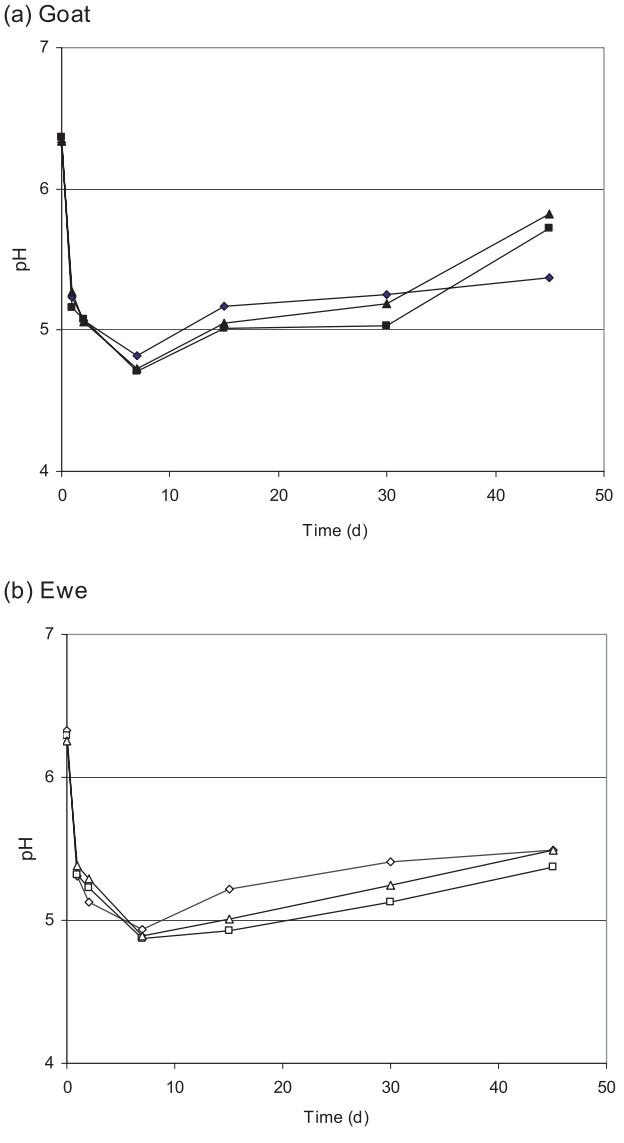

Figure 1. pH mean (n = 2) change; (a) goat's cheese made with Lc43-Lc202 (♦), Lc43-Lc202-Lb824 (■), Lc43-Lc202-Ln845 (▲); (b) ewe's cheese made with Lc43-Lc202 (◇), Lc43-Lc202-Lb824 (□), Lc43-Lc202-Ln845 (△).

differences in pH at the end of ripening were slight (5.38 in the cheese made with Lb824 and 5.50 in the other two).

The biochemical composition of the cheeses after 2 and 45 d is reported in Table II. At 2 d, total solids (TS) in goat's cheese (46.6 g·100 g^{-1}) were higher than in ewe's cheese (43.3 g·100 g^{-1}). In all cheeses, the ratio NaCl/Moisture (NaCl/M) was between 3% and 4%. By 45 d, TS had increased to 58.8 g·100 g^{-1} in goat's cheese and to 51.6 g·100 g^{-1} in ewe's cheese. Differences according to the starter were slight, except with starter Lc43-Lc202-Lb824 in goat's cheese, which gave the highest TS content (60.9 g·100 g^{-1}). Ca

Table II. Physico-chemical characteristics of the cheeses after 2 and 45 d of ripening. Mean and standard deviation values of the 2 trials.

Cheeses at d2

Milk	Cheese	TS	F	MDC	NaCl	NaCl/M
Goat's	43	45.3 ±0.5	20.7 ±0.2	68.9 ±0.6	1.6 ±0.2	3.0 ±0.3
	824	46.0 ±0.1	21.5 ±0.0	68.7 ±0.1	1.7 ±0.1	3.2 ±0.1
	845	48.5 ±0.8	21.2 ±0.2	67.2 ±1.1	1.7 ±0.1	3.3 ±0.1
Ewe's	43	44.1 ±0.9	18.7 ±0.9	68.6 ±0.8	2.2 ±0.2	3.8 ±0.2
	824	42.4 ±1.1	18.2 ±0.9	70.3 ±1.3	1.9 ±0.1	3.3 ±0.1
	845	43.5 ±1.0	19.2 ±0.8	69.9 ±1.0	1.9 ±0.0	3.3 ±0.1

Cheeses at d45

Milk	Cheese	TS	F	MDC	Fat Acidity	TN	SN/TN	Ca	NaCl	NaCl/M
Goat's	43	57.8 ±0.4	29.3 ±0.6	59.7 ±1.5	19.5 ±0.6	3.1 ±0.1	20.3 ±0.3	0.41 ±0.01	3.0 ±0.1	7.1 ±0.1
	824	60.9 ±0.3	31.7 ±0.9	57.3 ±0.6	22.6 ±0.8	3.2 ±0.1	26.4 ±0.2	0.31 ±0.02	2.6 ±0.3	6.6 ±0.7
	845	57.8 ±0.1	30.5 ±0.9	60.7 ±0.6	16.9 ±0.5	3.1 ±0.0	35.6 ±0.5	0.36 ±0.05	3.0 ±0.3	7.1 ±0.9
Ewe's	43	51.5 ±0.8	26.3 ±0.7	65.8 ±1.1	13.0 ±1.7	3.5 ±0.2	18.3 ±0.5	0.60 ±0.07	2.4 ±0.2	4.9 ± 0.5
	824	51.2 ±0.5	27.2 ±0.2	67.1 ±1.0	23.7 ±1.7	3.5 ±0.2	24.5 ±0.6	0.55 ±0.01	2.3 ±0.0	4.7 ±0.2
	845	52.2 ±0.3	26.8 ±0.2	65.3 ±0.9	10.6 ±0.9	3.5 ±0.1	30.2 ±0.2	0.45 ±0.00	2.1 ±0.0	4.4 ±0.1

43: cheese made with starter Lc43-Lc202 ; 824: cheese made with starter Lc43-Lc202-Lb824; 845: cheese made with starter Lc43-Lc202-Ln845.
TS: total solids (g·100 g^{-1}); F: fat (g·100 g^{-1}); MDC: moisture on defatted cheese (g·100 g^{-1}); Fat acidity (mg of lauric acid in 1 g of fat); TN : total nitrogen (g·100 g^{-1}); SN/TN: soluble nitrogen in total nitrogen (g·100 g^{-1} TN); Ca : calcium (g·100 g^{-1}); NaCl: sodium chloride (g·100 g^{-1}); NaCl/M: salt in moisture (g NaCl·100 g^{-1} H_2O).

content appeared to be higher in ewe's cheese (mean = 0.53 g·100 g^{-1}) than in goat's cheese (0.36 g·100 g^{-1}). Cheeses with the highest values were those made with the two lactococci. NaCl content was higher in goat's cheese (mean = 2.9 g·100 g^{-1}) than in ewe's cheese (2.3 g·100 g^{-1}). For the 2 types of cheese, the highest fat acidity (above 20 mg lauric acid in 1 g of fat) was obtained with the starter including Lb824, the lowest with the starter including Ln845. The SN/TN ratio was higher in goat's cheese (mean = 27.4 g·100 g^{-1}) than in ewe's cheese (24.3 g·100 g^{-1}). This ratio was different according to the starter: the two lactococci gave the lowest value, the Lb824 associated with the lactococci a medium value, while the highest was obtained with Ln845 associated with the lactococci.

3.2. Microbiological characteristics

3.2.1. Milk bacteriological quality before starter inoculation

In goat's milk, the mean total count was 6.3×10^5 cfu·mL^{-1} and total coliforms was 6.3×10^2 cfu·mL^{-1}. In ewe's milk these populations reached about 1.2×10^5 cfu·mL^{-1} and 3.2×10^2 cfu·mL^{-1}, respectively.

3.2.2. Evolution of the microbial counts

Mean microbial population changes are shown in Figures 2 to 7. Populations had a similar evolution. They largely increased between 0 and 2 d. Then, they tended to stabilize or in most cases, decrease slightly.

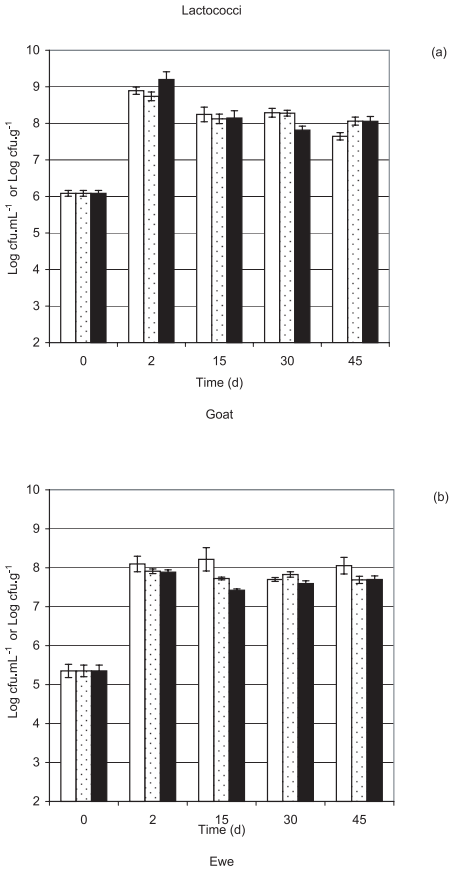

Figure 2. Lactococci mean change and standard deviation (n = 2); (a) goat's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■); (b) ewe's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■).

In goat's milk, the lactococci population (Fig. 2a) started at 10^6 cfu·mL^{-1} and reached 10^9 cfu·g^{-1} in cheese after 2 d. Counts decreased between 2 and 15 d and stabilized at 10^8 cfu·g^{-1}. In ewe's milk (Fig. 2b), the initial population was 2.2×10^5 cfu·mL^{-1}. The population increased over the first 2 days (10^8 cfu·g^{-1}). Then, counts were relatively stable. In the two cheese types, differences resulting from the starter were slight (lower than 1 log). Cheese 845 showed the lowest counts at 30 d in goat's cheese, and 2, 15 and 45 d in ewe's cheese.

Non-starter FH lactobacilli counts were 10^4 cfu·mL^{-1} in goat's milk (Fig. 3a). Inoculation of strain 824 raised the population to 10^5 log cfu·mL^{-1}. In the 3 goat's cheeses, the population reached 10^7 cfu·g^{-1} after 2 d and then varied from 10^7 to 10^8 cfu·g^{-1}. Differences resulting from the starter were lower than 1 log. In ewe's milk (Fig. 3b), the non-starter initial population was lower (10^3 cfu·mL^{-1}). As in goat's milk, it reached 10^5 cfu·mL^{-1} after inoculation. In cheese 824, FH lactobacilli exceeded 10^8 cfu·g^{-1}after 15 d and decreased to 10^6 cfu·g^{-1} after 30 d. In the other two cheeses, the increase was lower (between 10^6 and 10^7 cfu·g^{-1}after 15 d). At the end of ripening, cheese 43 had the highest population (2.0×10^7 cfu·g^{-1}) and cheese 824 the lowest (4.7×10^5 cfu·g^{-1}). After 30 d, counts in ewe's cheeses were lower than in goat's cheeses.

In goat's milk, the non-starter leuconostoc population was below 10^5 cfu·mL^{-1} (Fig. 4a). Inoculation of strain Ln845 raised the count to 10^6 cfu·mL^{-1} in cheese 845. In cheeses 43 and 824, the count increased (above 10^8 log cfu·g^{-1} at 2 d) while in cheese 845, the increase was lower (10^7 log cfu·g^{-1} at 2 d). After 15 d, counts were between 10^8 and 3.2×10^8 cfu·g^{-1} in the 3 cheeses. As for the FH lactobacilli in ewe's milk (Fig. 4b), the non-starter population was lower (about 3.2×10^2 cfu·mL^{-1}) than in goat's milk. Inoculation of strain Ln845 raised the population to 2.2×10^5 cfu·mL^{-1}. Unlike goat's cheese, the population strongly increased in cheese 845 (10^8 cfu·g^{-1} at 2 d) and stabilized. In the other two cheeses, counts reached 10^8 cfu·g^{-1} only after 15 d. It is worth noting that FH lactobacilli could be found on MRS with vancomycin (data not shown).

During the first few days, the difference in counts due to the inoculation with FH lactobacilli or leuconostocs was higher in ewe's than in goat's cheese. Nevertheless,

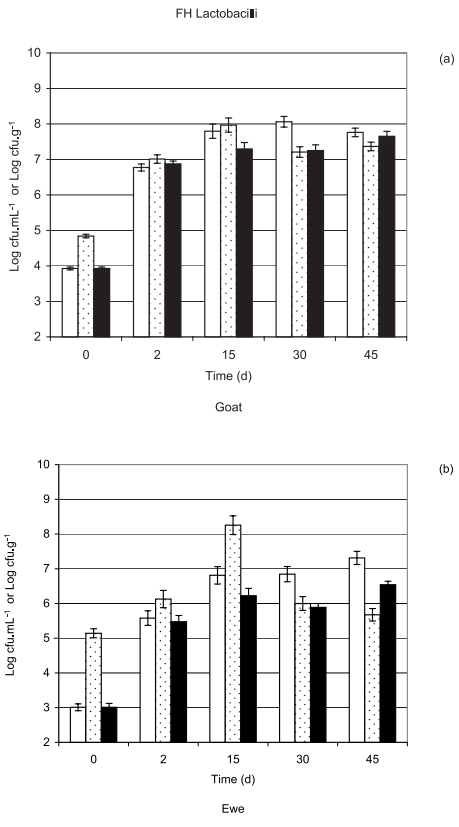

Figure 3. Facultatively heterofermentative lactobacilli mean change and standard deviation (n = 2); (a) goat's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■); (b) ewe's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■).

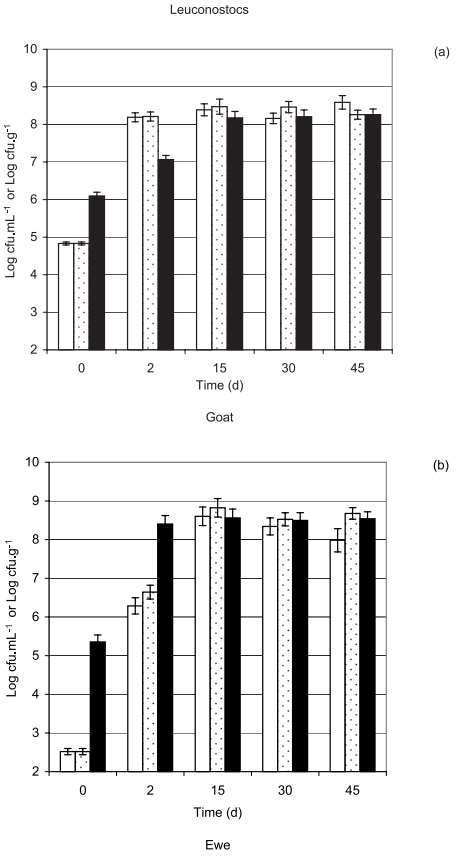

Figure 4. Leuconostocs mean change and standard deviation (n = 2); (a) goat's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Lb845 (■); (b) ewe's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■).

this difference did not hold out during ripening.

Table III shows the multiplication coefficients (count at 2 d / count at 0 d) of lactococci, FH lactobacilli and leuconostocs between 0 and 2 d, in cheese where the respective strains were inoculated. The lactococci coefficients were close, according to the milk and the starter. In goat's cheese, FH lactobacilli had a higher multiplication coefficient than the leuconostocs, while in ewe's cheese, this result was vice versa.

Enterococci counts in milk were about 10^4 cfu·mL^{-1} (Fig. 5). During the first 2 days, populations increased and then stabilized at about 10^6 and 10^7 cfu.g^{-1} in the majority of cheeses.

Salt-tolerant bacteria (Fig. 6) and yeasts and moulds (Fig. 7) grew during the first 15 days, reaching about 10^7–10^8 cfu·g^{-1}, while growth of the other microflora occurred

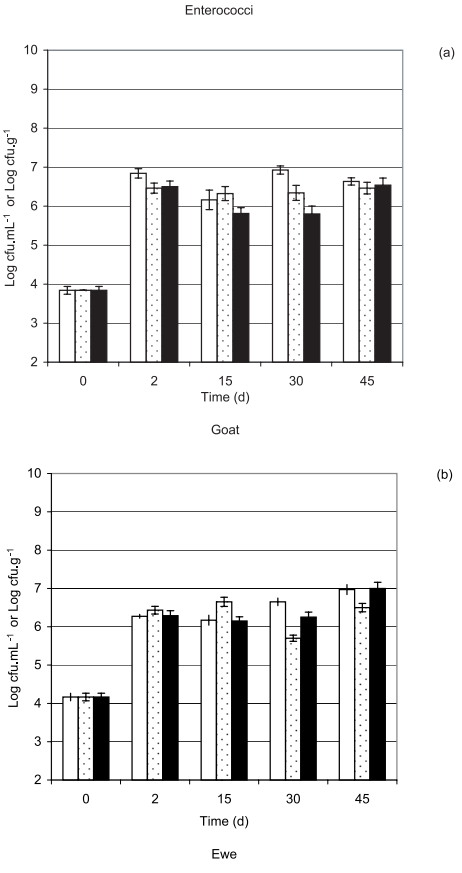

Figure 5. Enterococci mean change and standard deviation (n = 2); (a) goat's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■); (b) ewe's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■).

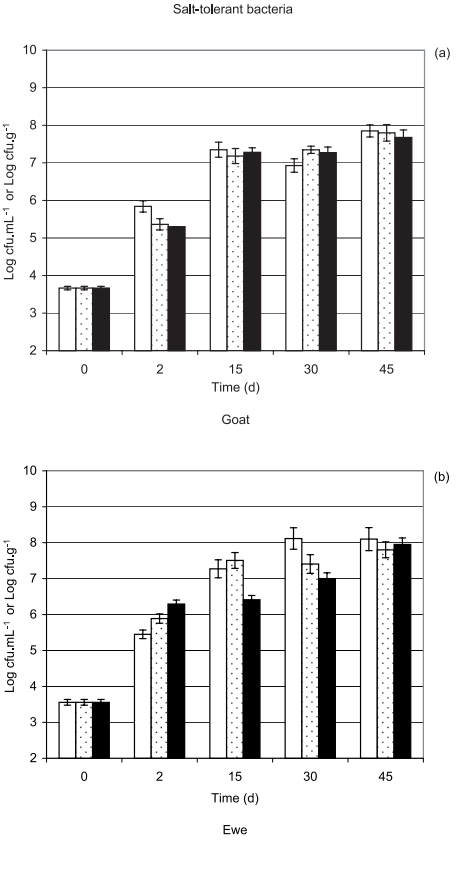

Figure 6. Salt-tolerant bacteria mean change and standard deviation (n = 2); (a) goat's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■); (b) ewe's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■).

mainly during the first 2 days. Population of yeasts and moulds was lower in ewe's milk (1.7×10^2 cfu·mL^{-1}) than in goat's milk (about 4.5×10^3 cfu·mL^{-1}) and in ewe's cheese at 2 d (about 10^4 vs. 10^5 cfu·g^{-1}). During ripening, this difference was lower. Differences according to the starter were slight, except in ewe's cheese, where growth of salt-tolerant bacteria during ripening was slower in cheese 845.

3.2.3. Phenotypic identification of isolates

A total of 507 isolates was collected and identified, 257 from goat's milk and cheese and 250 from ewe's milk and cheese. A total of 493 Gram-positive, catalase-negative isolates were composed of 342 cocci and 151 rods. Table IV shows the repartition according to the medium, the batch and the ripening stage. In goat's cheese,

Table III. Multiplication coefficient between 0 and 2 d (count at d 2/count at d 0) of the micro-organisms inoculated as starters. Mean and standard deviation values of the 2 trials.

Milk	Cheese	Lactococci	FH Lactobacilli	Leuconostocs
Goat's	43	1.47 ± 0.10		
	824	1.44 ± 0.15	1.45 ± 0.23	
	845	1.52 ± 0.10		1.19 ± 0.20
Ewe's	43	1.52 ± 0.17		
	824	1.48 ± 0.12	1.19 ± 0.20	
	845	1.48 ± 0.10		1.57 ± 0.10

43: cheese made with starter Lc43-Lc202; 824: cheese made with starter Lc43-Lc202-Lb824; 845: cheese made with starter Lc43-Lc202-Ln845.

Table IV. Isolates' phenotypic characterization.

Milk	Presumptive genus	Medium	Cheese	Ripening stage					
				15 d		30 d		45 d	
				ratio	%	ratio	%	ratio	%
Goat's	*Lactococcus*	M17	43	16/17	94	12/16	75	13/17	76
			824	10/12	83	2/12	17	9/12	75
			845	10/12	83	7/12	58	10/12	83
	F.H. lactobacillus	FH medium	824	10/12	83	11/12	92	11/12	92
	Leuconostoc	MRS with vancomycin	845	5/12	42	3/12	25	1/12	8
Ewe's	*Lactococcus*	M17	43	8/10	80	7/11	64	8/12	66
			824	8/10	80	8/10	80	6/12	50
			845	8/11	73	7/11	64	7/12	58
	F.H. lactobacillus	FH medium	824	12/12	100	11/12	91	11/12	92
	Leuconostoc	MRS with vancomycin	845	12/12	100	10/10	100	1/10	10

43: cheese made with starter Lc43-Lc202; 824: cheese made with starter Lc43-Lc202-Lb824; 845 cheese made with starter Lc43-Lc202-Ln845.
Ratio: $\frac{\text{number of isolates belonging to the genera according to phenotypic characterization}}{\text{total of identified isolates}}$

lactococci were dominant among the isolates, except in cheese 824 at 30 d. FH lactobacilli were dominant in cheese 824 at all stages. Leuconostocs were not dominant in cheese 845. In ewe's cheese, lactococci were also dominant but to a lesser extent than in goat's cheese after 45 d. FH lactobacilli were dominant in cheese 824. Leuconostocs were dominant for up to 30 d in cheese 845.

3.2.4. Genomic characterization according to Rep-PCR

The Rep-PCR fingerprints and the corresponding dendrogram of strains Lc43, Lc202 and isolates collected from ewe's cheese 43 at 45 d on M17 are shown in Figure 8.

Figure 9 shows strain diversity among isolates harvested from M17 according to Rep-PCR. In cheese 43, strains Lc43 and

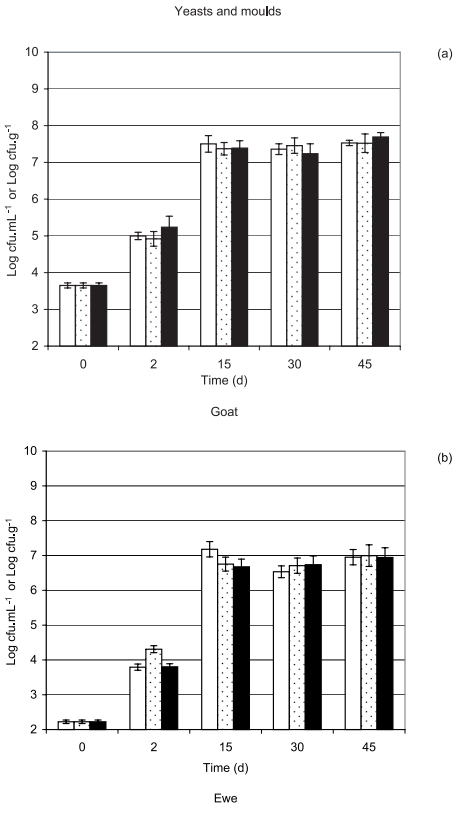

Figure 7. Yeasts and moulds mean change and standard deviation (n = 2); (a) goat's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■); (b) ewe's cheese made with Lc43-Lc202 (□), Lc43-Lc202-Lb824 (⊡), Lc43-Lc202-Ln845 (■).

Lc202 established themselves in cheese. They comprised the majority after 2 and 15 d. After 30 d, they represented 50% of the isolates. At this stage, isolates of lactococci (about 50%) presented a different profile from those of starter strains and were considered as strains from the indigenous microflora. Strain Lc43 was dominant during the first stages (2 and 15 d) and then, its proportion decreased on behalf of strain Lc202 and strains from the indigenous microflora. In cheese 824, the strains Lc43 and Lc202 were also present but to a lesser extent than in cheese 43. Strain Lc202 was still present after 45 d while strain Lc43 was no longer detected after 15 d. In cheese 845, after 2 d, the starter strains were present with indigenous microflora strains. After 2 and 15 d, the proportion of strain Lc202 was greater than in the other cheeses. The 2 starter strains were present after 45 d with a higher proportion of strain Lc202. The strains from indigenous microflora were present from 2, 15 or 30 d, depending on the cheese. In cheese 824, strain Lb824 represented the totality of the isolates from FHL medium after 2 and 15 d and, 0% after 30 and 45 d. In cheese 845, strain Ln845 represented the totality of the isolates harvested from MRS with vancomycin after 2 and 15 d, about 90% after 30 d, and still present after 45 d (10% of the isolates) (data not shown). So, strains Lb824 and Ln845 established themselves in cheese and were present for up to 15 d and 45 d, respectively.

3.3. Sensory characteristics

The results of the triangular test are shown in Table V. All the trials with the adjunct of *Lactobacillus* or *Leuconostoc*, except ewe's cheese 824 in the repetition, were significantly different ($P < 0.05$) from cheese 43.

4. DISCUSSION

Microbial quantitative differences according to milk origin were important. During the first 2 days, the growth of leuconostocs was faster in raw ewe's milk. Therefore, under the experimental conditions, ewe curd seems to fit the nutritional and physiological needs of leuconostocs better. The higher content of citrate in ewe's milk [32] could favor the growth of leuconostocs.

Biochemical differences between goat's and ewe's cheeses are in accordance with the observations made by Casalta et al. [10] in Bastelicaccia cheese: goat's cheese presents a higher dry matter, salt content and proteolysis index. The lower fat content in goat's milk and the higher NaCl content

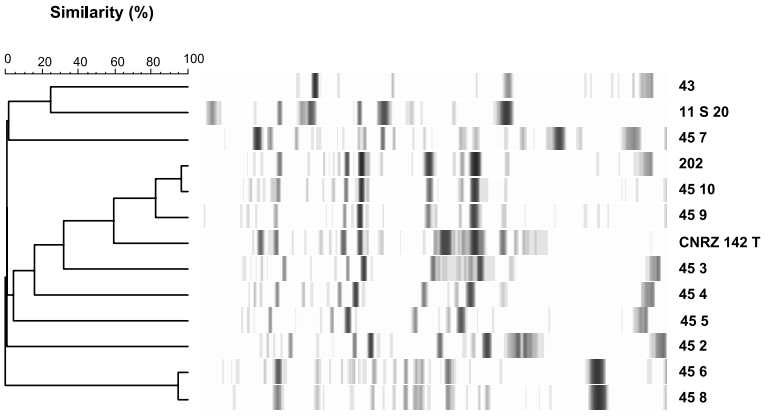

Figure 8. Rep-PCR fingerprints and corresponding dendrogram of lactococci. Type strain CRNZ 142T (*Lactococcus lactis* ssp. *lactis*), strain collection 11S20 (*Lactococcus lactis* ssp. *cremoris*), starter strains (43 and 202), strains collected from ewe's cheese 43 at 45d on M17 (45 followed with the isolate number).

Table V. Triangle test done with a panel of trained tasters.

Milk		Tested cheeses	Number of tasters	Number of right answers	Significance level
Goat's		43 versus 824	12	8	*
		43 versus 845	12	12	***
	Repetition	43 versus 824	12	8	*
		43 versus 845	12	9	**
Ewe's		43 versus 824	14	12	***
		43 versus 845	14	12	***
	Repetition	43 versus 824	12	6	NS
		43 versus 845	12	11	***

43: cheese made with starter Lc43-Lc202; 824: cheese made with starter Lc43-Lc202-Lb824; 845: cheese made with starter Lc43-Lc202-Ln845.
Significance levels: NS: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

and acidification level in goat's cheese led to an enhanced drainage of whey from this cheese, resulting in higher total solids.

The higher NaCl content in goat's cheese, by providing a decrease in a_w, could favor FH lactobacilli which are resistant to these constraints [40]. It could also lead to a faster establishment of yeasts on the cheese surface, the first micro-organisms which develop on the surface of smeared cheeses [51], goat's cheese showing a higher yeast and mould count than ewe's cheese after 2 d.

Higher buffering properties of ewe's milk could explain differences in pH decrease [2]. The faster pH increase in goat's cheese could come from higher lactic acid consumption due to a greater yeast population.

Phenotypic tests on isolates indicated that among colonies which grew on M17 and FHL media, lactococci and FHL represented the dominant groups in most cheese samples. On the other hand, on MRS + vancomycin, leuconostocs represented the minority of the isolates in goat's cheese

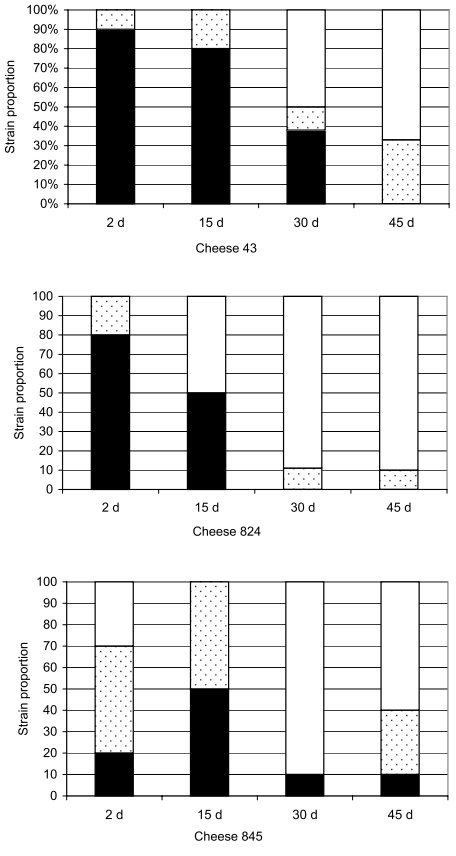

Figure 9. Strain diversity among isolates from ewe's cheese on M17 according to Rep-PCR; profile Lc43 (■), profile Lc202 (⊡), other profiles of lactococci (□).

and the majority of the isolates up to 30 d in ewe's cheese. This result is in agreement with the fact that ewe curd seems to suit the nutritional and physiological needs of leuconostocs better.

The genomic characterization of the isolates from ewe's cheese, using Rep-PCR, showed that the starter strains established themselves in the cheese: lactococci and leuconostocs were present up to d45, FH lactobacilli up to d15. These results show the compatibility between the starter strains under the experimental conditions. The strains considered from the indigenous lactic microflora were also found and became dominant at the end of ripening.

At this stage, the strain isolated from milk (Lc202) was represented with a higher proportion than the strain isolated from cheese (Lc43). This fact shows a difference in the strain's capacity for developing in cheese.

The growth of the starter strains had a major impact on levels of lipolysis and proteolysis. The higher lipolysis level with the adjunct of *Lb. paraplantarum* agrees with the results that Madkor et al. [39] obtained with *Lb. casei* in Cheddar cheese. Similar observations were reported by El Soda et al. [18] for Ras cheese. Moreover, Menendez et al. [43] showed that the use of *Lactobacillus* increased volatile free fatty acids and long-chain free fatty acid content during ripening of Arzùa-Ulloa cheese. Therefore, although lactic acid bacteria have minor levels of lipolytic activity when compared with other micro-organisms such as *Acinetobacter* and *Candida* [44], intracellular lipases found in *Lb. plantarum* [52] and *Lb. casei* [54] increase the level of lipolysis in cheese. Studies on cheese ripening showed the role of mesophilic lactobacilli in proteolysis. Hynes et al. [26] found a higher SN/TN ratio when *Lb. plantarum* is added to *Lc. lactis* subsp. *lactis* in miniature washed–curd cheeses. The adjunct lactobacilli in experimental Cheddar cheese significantly increase the free amino acid level [57]. Lynch et al. [36] previously explained this impact by the mesophilic lactobacilli peptidase activity. Scolari et al. [53] came to the same conclusion for Grana cheese. Therefore, this intracellular activity could explain the higher proteolysis level in Venaco cheese when *Lactobacillus* is associated with lactococci in the starter.

One of the main functions expected of leuconostocs is proteolysis [56]. These bacteria are as rich as the other lactic acid bacteria in intracellular proteolytic enzymes [17]. The highest proteolysis level, provided with leuconostocs, could be explained by the activity of these enzymes. Another

explanation could be a positive interaction between leuconostocs and surface microflora, observed by Daniellot [14].

The lower pH observed after 15 and 30 d with the adjunct of lactobacilli and, to a lesser extent, with leuconostocs has been shown previously [3, 14]. The higher acidification provided by the inoculation of these strains could explain the lower Ca content obtained in the cheese.

The faster pH rise in goat's cheese from 30 to 45 d with the adjunct of lactobacilli or leuconostocs could be related to proteolysis, as molecules like ammonia produced by lactobacilli or leuconostocs [59] contribute to raising the pH. This rise in pH could favor yeast proteolytic action. As a matter of fact, optimal pH for *Geotrichum candidum* proteinases is 5.5–6.0 [23, 24], and that of the *Debaromyces* endopeptidases is 5.8 [58].

Lb. plantarum used with *Lc. lactis* starter had a strong effect on washed-curd cheese flavor attributes [27]. According to Pelaez et al. [47], one possible explanation for the improved flavor scores obtained with the use of *Lactobacillus* as starter adjunct, in Spanish semi-hard goat's milk cheese, is due to a conversion of amino acids into volatile flavor compounds.

The role of leuconostocs in cheese sensory characteristics could also originate from the production of flavour compounds from citrate, as suggested by Poveda et al. [49].

Therefore, physico-chemical changes, provided by the added lactobacilli or leuconostocs, led to significant differences in cheese sensory characteristics, underlined by the triangular test.

5. CONCLUSION

This work shows that the strains inoculated as starters durably establish themselves in cheese. The presence of lactic acid bacteria from indigenous microflora, particularly at the end of ripening, shows a balance between this microflora and the starter strains. The implanting of adjunct FH lactobacilli and leuconostocs has an effect on cheese lipolysis and proteolysis, which can explain differences in cheese sensory characteristics. An impact on pH was also shown. Like each raw milk cheese ecosystem, the Venaco one is complex. The use of strains as adjuncts, without greatly modifying this ecosystem, has an impact on cheese sensory quality. Further experiments should be carried out in order to assess the impact of adjunct FH lactobacilli and leuconostocs on sensory attributes of cheese. Moreover, the direct action of starter strains and their interaction with other groups of micro-organisms, particularly surface microflora, remains unknown.

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