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Stability of microbial communities in goat milk during a lactation year: Molecular approaches[☆]

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Abstract

The microbial communities in milks from one herd were evaluated during 1-year of lactation, using molecular methods to evaluate their stability and the effect of breeding conditions on their composition. The diversity of microbial communities was measured using two approaches: molecular identification by 16S and 18S rDNA sequencing of isolates from counting media (two milks), and direct identification using 16S rDNA from clone libraries (six milks). The stability of these communities was evaluated by counting on selective media and by Single Strand Conformation Polymorphism (SSCP) analysis of variable region V3 of the 16S rRNA gene and variable region V4 of the 18S rRNA gene. One hundred and eighteen milk samples taken throughout the year were analyzed. Wide diversity among bacteria and yeasts in the milk was revealed. In addition to species commonly encountered in milk, such as *Lactococcus lactis*, *Lactococcus garvieae*, *Enterococcus faecalis*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus caprae*, *Staphylococcus equorum*, *Micrococcus* sp., *Kocuria* sp., *Pantoea agglomerans* and *Pseudomonas putida*, sequences were affiliated to other species only described in cheeses, such as *Corynebacterium variabile*, *Arthrobacter* sp., *Brachybacterium paraconglomeratum*, *Clostridium* sp. and *Rothia* sp. Several halophilic species atypical in milk were found, belonging to *Jeotgalicoccus psychrophilus*, *Salinicoccus* sp., *Dietzia maris*, *Exiguobacterium*, *Ornithinococcus* sp. and *Hahella chejuensis*. The yeast community was composed of *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Trichosporon beigeli*, *Rhodotorula glutinis*, *Rhodotorula minuta*, *Candida pararugosa*, *Candida intermedia*, *Candida inconspicua*, *Cryptococcus curvatus* and *Cryptococcus magnus*. The analyses of microbial counts and microbial SSCP profiles both distinguished four groups of milks corresponding to four periods defined by season and feeding regime. The microbial community was stable within each period. Milks from winter were characterized by *Lactococcus* and *Pseudomonas*, those from summer by *P. agglomerans* and *Klebsiella* and those from autumn by *Chryseobacterium indologenes*, *Acinetobacter baumannii*, *Staphylococcus*, *Corynebacteria* and yeasts. However, the composition of the community

[☆]Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/Genbank database under the accession numbers EF588263–EF588274, EF621423–EF621473, EF611741–EF611817, EF627478–EF627522, EF633693.

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can vary according to factors other than feeding. This study opens new investigation fields in the field of raw milk microbial ecology.

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Introduction

Many interacting factors can affect the qualities of goat cheese. The most important are technological parameters, the biochemical composition of the milk and the composition of its microbial communities. Even when the milk is prepared using the same technology, these qualities can vary over a 1-year period. Milk fat composition, for example, can change according to feeding regime and lactation [41]. Variation in the composition of goat milk microbial communities has been less extensively studied. Goat milk and cheese microbial communities have more often been described by enumerating a group of microorganisms on various culture media [1,7,29,32,39]. There have been few studies identifying the microorganisms at species level, and these have generally concerned a specific microbial group, mainly lactic acid bacteria [22,27,28,37], *Enterobacteriaceae* [39,45], *Micrococcaceae* [21,34,43,46], yeasts [38,44] or pathogenic microorganisms [16]. The study by Foshino et al. [16] only focused on the effect of farm and lactation period over a 6-months period. Other studies have described variations during cheese manufacture and ripening [18,28,37] at only three or four different times over the lactation. It would be useful to have a more comprehensive view of the microbial composition of milk, and to track its evolution throughout the lactation.

In the past few years, cultivation-independent methods such as Single Strand Conformation Polymorphism (SSCP), Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) have proven to be powerful tools for studying food samples, bringing a more comprehensive and dynamic view of microbial diversity than culture-dependent methods [3,8,12,14]. They also make it easier to compare different microbial communities such as those present in goat milks at different times during 1-year of lactation.

The aim of our study was to investigate the stability of microbial communities, including bacteria and yeasts, of goat milks used for cheese manufacturing and taken from one herd over 1 lactation year. Two approaches were used: (1) molecular identification of isolates and clones (Restricted Fragment Length Polymorphism (RFLP) and 16S rDNA and 26S rDNA sequencing), and (2) 16S rDNA and 18S rDNA SSCP analysis for monitoring changes in the microbial community. The

impact of feeding regime and other factors on milk microbial composition was studied.

Materials and methods

Breeding conditions during the lactation year

Because goat milk production is seasonal, the notion of a “period” is defined by both physiological aspects and seasonal aspects. For herds that are grazed, these aspects are closely related to feeding regime (composition, type, supplementation).

Four periods were defined according to season and the related breeding conditions. The winter period P1 encompasses the milk samples from day 45 through 86. At this time the milk volume of the herd was more than 400 l. There was no outside grazing, the feed was dried *alfafa*. Outdoor grazing on *Graminaceae* and dried *alfafa* characterized the spring period (P2; days 87–163) in which the herd yielded 400–500 l milk/day. During the summer period P3 (days 164–269), the milk volume of the herd was 200–400 l and the feed was like in period P1. The autumn period (P4; days 270–330) was characterized by the lower milk volume (100–200 l) and outdoor grazing on *Graminaceae*.

Sample collection and treatment

A total of 118 samples of raw bulk milk (30 ml) were collected between February and November 2005 on an experimental goat farm (Domaine du Pradel, France), at a rate of 3–5 milks per week. Samples were numbered by day of the year (1 for January 1st and so on). Each sample was taken from the refrigerated tank (a mixture of the evening milking refrigerated overnight at 4 °C and the morning milking) and stored at –20 °C before being investigated.

Counts on media and isolate collection

Ten milliliters of milk were homogenized in 90 ml sterile diluent (solution with 0.8% NaCl, 0.1% peptone, 0.1% Tween 80) for 2 min in a Stomacher Laboratory Blender (Interscience, St. Nom la Bretèche, France). The microbial counts of the milks were evaluated on

different media (two replicates per sample) as described by Millet et al. [30].

The bacterial media used (Table 4) were Plate Count Agar with Gram-positive inhibitor (PCAI), Cheese Ripening Bacteria Medium (CRBM), Terzaghi and Sandine M17, Rabbit Plasma Fibrinogen Agar (RPF), facultatively heterofermentative lactobacilli agar (FH), Slanetz and Bartley (SB), Turner–Sandine–Elliker with nalidixic acid (TA), Mayeux–Sandine–Elliker (MSE), and Cetrinin–Fucidin–cephalosporin (CFC) media plates. All colonies growing on the various media were retrieved from plates of two milks (49 and 151). These two milks were selected as they had they belong to two different periods. Moreover, they had the highest counts and the colonies on different media had the highest morphology's diversity. Two hundred and eighty-one bacterial isolates were purified and stored at -20°C in 50/50 (v/v) broth and milk with glycerol (10%).

All yeast colonies growing on Oxytetracyclin Glucose Agar (OGA) medium from the analysis of six different milks, numbered 72 and 81 (from P1), 168, 181 and 209 (from P3), 280 (from P4), were retrieved. Forty-six isolates were purified and stored at -20°C in 50/50 (v/v) Yeast Peptone Glucose broth and milk with glycerol (10%).

Bacteria identification

The identification of bacteria was solely based on 16S rRNA sequence comparison.

Total DNA extraction

Total DNA from bacterial isolates were extracted from 5 ml overnight cultures using Easy DNA Kit with phenol/chloroform (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions and suspended in 50 μl of TE buffer (Tris-EDTA).

PCR of 16S rRNA gene fragments

Ribosomal 16S rRNA gene (1450 bp) of all DNA isolates were amplified using the universal primers W02 and W18 as previously described by Callon et al. [9]. All amplifications were performed with a geneAmp PCR System 9700 (Applied Biosystems, Courtaboeuf, France).

Screening by RFLP analysis

All the 16S PCR products were analyzed by the Restriction Fragment Length Polymorphism (RFLP) method, using the following protocol: in a final volume of 15 μl , 5 μl of 16S PCR products were added to 0.5 μl of EcoRI and Hae III (MP Biochemical, Vannes, France), 1.5 μl of buffer (100 mM KCl, 10 mM tris-HCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 500 $\mu\text{g}/\text{ml}$ BSA, 50% glycerol) and 7.5 μl of water. The

thermal cycler apparatus was programmed for 1 h at 37°C . The amplification products were electrophoresed in 2% standard agarose gel with TBE 0.5 \times and ethidium bromide (10 mg/ml) for 2 h at 120 V, using a 50 bp DNA step ladder as standard. The profiles generated were analyzed with BioNumerics software using UPGMA (Unweighted Pair Group Method with arithmetic Average) analysis and a dendrogram was deduced from the matrix of similarities. The 16S RFLP profiles of isolates were compared to a reference bank of 16S RFLP profiles realized in a first step by the analysis of 60 different species of the laboratory collection. Isolates showing an identical RFLP pattern at 90% of similarity were grouped together and one or several 16S rDNA from each group were analyzed by sequencing.

Species PCR amplifications

DNA of Gram-positive and catalase negative isolates were amplified with species-specific primers for *Enterococcus faecalis*: ddIE1-ddIE2 [13], *Lactococcus lactis/cremoris*: LhisF5-Lhis6R [5], *Leuconostoc mesenteroides*: Lnm1-Lnm2 [10], *Lactobacillus casei/paracasei*: paracasei 16S-16 reverse [6] and *Lactococcus garvieae*: Lg1-Lg2 [49].

Partial 16S rRNA gene sequencing

A 50 μl of bacterial 16S rRNA gene was purified on column and sequenced using the W34 primer [50] by GeneCust (Evry, France). The 450 bp of the 5' ends obtained for the seventy-eight 16S rRNA genes of the isolates were compared to sequences available in the GeneBank database, using the BLAST program [2]. Sequences with a percentage similarity of 97% or higher were considered to represent the same species.

Yeast identification

Yeast isolates were identified by phenotypic tests [4,20] and the D1/D2 domain of 26S rRNA encoding gene (Primers NL1-NL4) sequencing, as described by Callon et al. [8].

DNA extraction and purification from milk

The milks were thawed at 25°C . The DNA extraction method was derived from that of Duthoit et al. [14] for DNA extraction from cheese, with the following modifications: 10 ml of milk were incubated at 37°C for 2h30 with 150 μl of 10 mg/ml pronase E (Merck, Darmstadt, Germany). One ml of 20% sodium dodecyl sulfate was added, and the incubation was continued for 1 h. After centrifugation at 8500g for 20 min at 4°C , the fat layer was removed and the sample was incubated 10 min at 30°C . Then the samples were centrifuged at 8500g for 10 min at room temperature and the microbial

pellet was resuspended in 130 μ l of 4 M guanidine thiocyanate-0.1 M Tris (pH 7.5), 20 μ l of 10% *N*-lauroylsarcosine. The phenol/chloroform extraction and DNA precipitation were performed as described by Duthoit et al. [14].

16S rRNA gene cloning

Genomic DNA extracted from six milks spread over the lactation year (milks 90, 95, 103, 116, 151 and 188) was used as template. Amplification and cloning of 16S rRNA genes were carried out as described by Duthoit et al. [14] using w02 and w18 primers, except that Pfu DNA polymerase and 10 \times Pfu buffer (Stratagene, St. Quentin en Yvelines, France) were used. PCR products were ligated into pCR4Blunt-TOPO and transformed into *Escherichia coli* TOP10 OneShot as specified by the manufacturer (Invitrogen, Cergy Pontoise, France). Plasmid inserts were amplified with universal plasmid primers T3 and T7 [14] as specified by the manufacturer. Three hundred and forty-one clones were obtained. A second 16S rDNA PCR was applied with WO2-W18 like for isolates. The 16S PCR products corresponding to the clone inserts were screened by RFLP as described above for isolates and compared to a reference bank of 16S RFLP profiles. Several 16S rDNA from each RFLP cluster were sent to GeneCust for sequencing under the same conditions as described for the isolates. Seventy-nine clones were sequenced.

SSCP-PCR amplification

Total DNA from 118 milks was used as a template to amplify the V3 region of the bacterial 16S rRNA gene, using the primers w34 and w49 as described by Duthoit et al. [14], except that Pfu DNA polymerase and 10 \times Pfu buffer were used. PCR was performed on genomic DNA extracted from the milk, on PCR product from plasmid insert amplification, and on DNA extracted from pure culture. W34 was labeled with 5'-fluorescein phosphoramidite (NED). The primers were synthesized by Applied Biosystems.

Total DNA from 49 milks (1 or 2 per week) were analyzed by yeast SSCP analysis. The V4 region of the 18S rRNA gene was amplified with Lev1 and Lev2 primers as described by Callon et al. [8].

SSCP electrophoresis

Samples were denatured at 95 $^{\circ}$ C for 3 min and SSCP capillary electrophoresis in non-denaturing condition was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems), as previously described [14]. The migration of the DNA fragments depends on their

conformation which one depends on their sequence. All SSCP profiles of milks were lined up using an internal DNA molecular weight standard Genescan-300HD ROX (Applied Biosystems) and an external standard (a same milk analyzed in each run with the others milks). The areas of the peaks were calculated. To analyze the different profiles, we calculated the relative area of the peak *i* to the sum of the peak areas, $P_i = a_i / \sum a_i$, where a_i is the area of one peak.

SSCP peaks assignments

The peaks in the V3 16S rDNA profiles of the milks were assigned to different species according to their coelution with the clones in the library or strains isolated from goat milks and identified by 16S rDNA sequencing. The V3 regions of the 16S rRNA genes in the clone library and in the isolates were analyzed by SSCP.

In the same way, the peaks in the yeast SSCP profiles of the milks were assigned according to coelution with isolates from milks and reference strains representing the yeast communities of a variety of cheeses.

Data analysis

Discriminant analysis (DA) was performed with Statistica software (Statsoft, version 6) with the four periods as classification variables, the relative areas of the 16S V3 SSCP and 18S V4 SSCP peaks as variables, and the milks as observations.

The counts on media and the relative areas of the bacterial and yeast SSCP peaks form a global profile for each milk. Therefore a Multivariate Analysis of Variance (MANOVA) was performed on these data with the four multivariate test statistics of Statistica software output (Wilks' lambda, Pillai's trace, Hotelling-Lawley and Roy's maximum root). This first analysis were followed by an Analysis of Variance (ANOVA) on data from counts on each media or relative peaks of global SSCP profiles, in order to describe those that are the best discriminated by the period. Tukey's test was performed to compare the means and significant differences were noted ($P < 0.05$).

Results

Bacterial diversity in goat milk according to clone and isolate identification

The results of the identification of 281 isolates and 341 clones, after screening by RFLP analysis (results not shown) and 16S rRNA gene sequencing of representative groups are detailed in Tables 1 and 2. As is it only based on 16S rRNA sequence comparison, the

Table 1. Phylogenetic affiliations of 281 isolates from two different milks (151 and 49): screening of 16S rRNA gene by RFLP analysis followed by 16S rRNA gene sequencing

RFLP ^a clusters	Number of isolates	Coelution ^b SSCP peaks	Number sequenced	Accession numbers	Closest 16S rDNA sequences ^d						
G6	28	25	5	EF627518–EF627522	Gram + catalase + Staphylococcus epidermidis						
G8	21	3	10	EF611783–EF611792	Kocuria rhizophila–kristinae- carniphila						
G31	12	21	3	EF611797–EF611799	<i>Microbacterium oxydans</i>						
G36	7	21	5	EF588266 EF611800–EF611803	<i>Exiguobacterium</i>						
G4	3	28	3	EF611804–EF611806	<i>Staphylococcus simulans</i>						
G32	2	5	1	EF611796	<i>Corynebacterium variabile</i>						
G15	3	5	1	EF611760	Brevibacterium stationis						
G35	2	1	1	EF588264	<i>Brachybacterium paraconglomeratum</i>						
G9	2	23	1	EF588265	<i>Salinicoccus</i> sp.						
G5	2	31	1	EF588263	<i>Jeotgalicoccus psychrophilus</i>						
G48	2	2	2	EF621471,EF621472	Micrococcus sp.						
G3	1	23	1	EF611758	Staphylococcus caprae						
G25	1	1	1	EF633693	<i>Arthrobacter</i> sp.						
G45	1	nd	1	EF611751	Bacillus cereus- thuringiensis						
Species PCR amplifications ^c											
		pa	Ln	dm	ddIE	Lhis	Lg				
G7	92	nd	nd	+	–	–	nd	23	8	EF621444–EF621451	Gram + catalase– Enterococcus faecalis
G22	19	+	nd	nd	nd	nd	nd	29	5	EF611813–EF611817	<i>Lactobacillus casei</i>
G26	4	nd	nd	–	+	–	–	33	1	EF611761	Lactococcus lactis lactis
G30	4	nd	+	nd	nd	nd	nd	33	4	EF611809–EF611812	<i>Leuconostoc mesenteroides</i>
G26	1	nd	nd	–	–	–	+	35	1	EF611807	<i>Lactococcus garvieae</i>
G27	1	nd	–	–	–	–	–	33	1	EF611808	<i>Streptococcus mitis</i>
						Gram–					
G10	24							32	5	EF621423–EF621427	Acinetobacter baumannii
G14	16							28	1	EF621473	Pseudomonas putida
G29	14							25	8	EF588267 EF621429–EF621435	<i>Stenotrophomonas maltophilia</i>
G1	7							19	4	EF588268 EF611793–EF611795	<i>Chryseobacterium indologenes</i>
G43	4							31	1	EF621439	Pseudomonas aeruginosa
G2	3							18–14	1	EF588271	Pantoea agglomerans
G33	2							25	1	EF588269	<i>Delftia acidovorans</i>
G46	2							nd	1	EF621469	Citrobacter freundii
Total 281											

^aThe RFLP patterns were analyzed with BioNumerics software using UPGMA analysis. Isolates with the same pattern were grouped together and one or several from each group were analyzed by 16S rDNA sequencing.

^bThe bacterial V3 region of the 16S rDNA gene was analyzed by SSCP and peaks were compared to the V3 16S rDNA SSCP profiles of milks.

^cThe description of different primers is given in section “Materials and methods”. nd: not determined.

^dSpecies indicated in bold are common to isolates and clones libraries.

Table 2. Phylogenetic affiliations of OTUs from milks (16S rDNA clone libraries from six different milks: screening of 16S rRNA genes by RFLP followed by 16S rRNA gene sequencing)

RFLP clusters ^a	Number of clones	Coelution ^b SSCP peaks	Number sequenced	Accession numbers	Phylogenetic affiliation after sequencing ^c
					Gram + cat +
G45	8	24	4	EF611752–EF611755	<i>Bacillus cereus-thuringiensis</i>
G6	6	25	3	EF621464–EF621466	<i>Staphylococcus epidermidis</i>
G3	4	23	1	EF611757	<i>Staphylococcus caprae</i>
G8	3	3	3	EF611780–EF611782	<i>Kocuria rhizophila –kristinae-carniphila</i>
G9	1	25	1	EF621462	<i>Staphylococcus equorum</i>
G42	1	1	1	EF621461	<i>Unidentified</i>
G16	1	24	1	EF611756	<i>Macrococcus caseolyticus</i>
G15	1	5	1	EF611759	<i>Brevibacterium stationis</i>
G40	1	3	1	EF588270	<i>Ornithinococcus</i> sp.
G19	1	4	1	EF588274	<i>Dietza maris</i>
G48	1	2	1	EF621470	<i>Micrococcus</i> sp.
G12	1	3	1	EF621467	<i>Rothia</i> sp.
G13	1	Nd	1	EF621463	<i>Clostridium</i>
G51	1	Nd	1	EF621443	<i>Firmicutes</i> sp.
					Gram + cat-
G7	54	23	9	EF621452–EF621460	<i>Enterococcus faecalis</i>
G26	48	33	18	EF611762–EF611779	<i>Lactococcus lactis lactis</i>
G49	1	35	1	EF627486	<i>Enterococcus saccharominimus</i>
					Gram-
G2	134	18–14	10	EF611741–EF611750	<i>Pantoea agglomerans</i>
G14	51	28	8	EF627478–EF627485	<i>Pseudomonas putida</i>
G10	7	32	2	EF6588272–EF621428	<i>Acinetobacter baumannii</i>
G44	1	25	1	EF588273	<i>Hahella chejuensis</i>
G43	5	31	3	EF621436–EF621438	<i>Pseudomonas aeruginosa</i>
G19	3	20	2	EF613318,EF613319	<i>Klebsiella milletis- oxytoca</i>
G46	2	Nd	1	EF621468	<i>Citrobacter freundii</i>
G	2	Nd	2	EF621440,EF621441	<i>Pseudomonas fulgida</i>
G50	1	23	1	EF621442	<i>Enterobacter absuriae</i>
	Total 340				

^aThe RFLP patterns were analyzed with BioNumerics software using UPGMA analysis. Isolates with the same pattern were grouped together and one or several from each group were selected and analyzed by 16S rDNA sequencing.

^bThe bacterial V3 region of the 16S rDNA gene was analyzed by SSCP and peaks were compared to V3 16S rDNA SSCP profiles of milks.

^cSpecies indicated in bold are common to isolates and clones libraries.

identifications are not strict in a taxonomic sense. Based on the closest affiliation of clones and isolates, the presence of 41 different species were suggested from 6 goat milks from the same herd at different periods of the year. Among the Gram-positive, non lactic acid bacteria, sequences affiliated to *Staphylococcus epidermidis* (10% of isolates and 2% of OTUs) and *Kocuria rhizophila-kristinae-carniphila* (8% of isolates and 1% of OTUs), *Bacillus thuringiensis-cereus*, *Staphylococcus caprae*, *Micrococcus* sp. and *Brevibacterium stationis* were found both among the clones and among the isolates. Nine species – *Microbacterium oxydans* and *Exiguobacterium* (4% and 2% of isolates), *Staphylococcus simulans* (1.5%), and *Corynebacterium variabile*, *Brachybacterium paraconglomeratum*, *Arthrobacter* sp., *Salinicoccus* sp., *Jeotgalicoccus psychrophilus* (less than 1%) were present only among the isolates. Seven sequences affiliated to *Staphylococcus*

equorum, *Macrococcus caseolyticus*, *Ornithinococcus* sp., *Dietza maris*, *Rothia* sp., *Clostridium* and *Firmicutes* sp. (less than 1%) were suggested only by 16S rRNA cloning.

Among the Gram-positive lactic acid bacteria, the isolates were more diverse than the clones. Sequences affiliated to *E. faecalis* (33% of isolates and 16% of OTUs) were the most commonly found in both isolates and clone libraries, whereas sequences affiliated to *Lc. lactis* (1.5% of isolates and 14% of OTUs) were the most commonly recovered among the clones. Four species were only found among the isolates. These were affiliated to *Lb. casei* (7%), *Ln. mesenteroides* (1.5%), *Lc. garvieae* and *Streptococcus mitis* (less than 1%). The sequence affiliated to *Enterococcus saccharominimus* (less than 1%) was only identified among the clones.

Among the Gram-negative bacteria, sequences affiliated to *Pantoea agglomerans* (1% of isolates and 40%

Table 3. Identification of yeast isolates from six different milks (72, 81, 168, 181, 209 and 280): phenotypic identification and phylogenetic affiliations identified by 26S rRNA gene sequencing

Number of isolates	Phenotypic identification ^a	Number sequenced	Closest 26S rDNA sequences	Accession numbers	Coelution SSCP ^b peaks of milk
10	<i>Rhodotorula glutinis</i> / <i>graminis</i>	8	<i>Rhodotorula glutinis</i> / <i>graminis</i>	EF627488–EF627495	s
7	<i>Trichosporon beigelii</i>	4	<i>Trichosporon</i> sp 9	EF627506–EF627509	r
6	<i>Debaryomyces hansenii</i>	4	<i>Debaryomyces hansenii</i>	EF627496–EF627499	s
5	<i>Cryptococcus ater</i> / <i>Cr.</i> <i>magnus</i>	4	<i>Cryptococcus magnus</i>	EF627513–EF627516	m
5	<i>Cryptococcus curvatus</i>	3	<i>Cryptococcus curvatus</i>	EF627500–EF627502	m
4	<i>Rhodotorula minuta</i>	1	<i>Rhodotorula minuta</i>	EF627517	f
4	<i>Candida intermedia</i>	4	<i>Candida intermedia</i>	EF627505–EF627510–EF627512	b
2	<i>Candida pararugosa</i>	1	<i>Candida pararugosa</i>	EF627503	t
1	<i>Candida inconspicua</i>	1	<i>Candida inconspicua</i>	EF627504	c
1	<i>Kluyveromyces lactis</i>	1	<i>Kluyveromyces lactis</i>	EF627487	k
Total 46					

^aPhenotypic identification using the morphological, biochemical and physiological characteristics of Kurtzman and Fell [20] as described by Callon et al. [8], and assignment to species with the dichotomous key of Barnett et al. [4].

^bThe V4 region of the 18S rDNA gene was analyzed by SSCP and peaks were compared to V4 18S rDNA SSCP profiles of milks.

of clones), *Pseudomonas putida* (6% of isolates and 15% of clones), *Acinetobacter baumannii* (9% of isolates and 3% of clones), *Citrobacter freundii* and *Pseudomonas aeruginosa* (less than 1%) were identified among both isolates and clones. The three species assigned to *Stenotrophomonas maltophilia* (6%), *Chryseobacterium indologenes* (3%) and *Delftia acidovorans* (less than 1%) were only encountered among the isolates. Sequences assigned to *Enterobacter absurdus* and *Enterobacter* sp, *Hahella chejuensis*, *Klebsiella milletis-oxytoca* and *Pseudomonas fulgida* (less than 1%) were identified among the clones.

Yeast diversity in goat milk by isolate identification

To characterize yeast populations in different milks, 46 isolates were retrieved from OGA medium from six goat milks and characterized phenotypically and genotypically. The results of both types of identification of yeast isolates are detailed in Table 3. Seven main species were distinguished: *Rhodotorula glutinis* (22%), *Trichosporon beigelii* (15%), *Debaryomyces hansenii* (15%), *Cryptococcus magnus* (11%), *Cryptococcus curvatus* (11%), *Rhodotorula minuta* (9%) and *Candida intermedia* (9%). Other species identified (totaling less than 5% of isolates) were *Kluyveromyces lactis*, *Candida inconspicua* and *Candida pararugosa*.

Discrimination between periods by cell counting on media

The microbial characteristics of the milks were evaluated by cell counting on different media. The

selectivity of the different media was assessed as shown in Table 4. The microbial community retrieved from CRBM agar plates consisted of Gram-positive catalase positive genera (*Staphylococcus*, *Kocuria*, *Corynebacterium*, *Arthrobacter*, *Salinicoccus*, *Jeotgalicoccus*, *Brevibacterium* and *Brachybacterium*), although some *Enterococcus* were also found. Isolates from PCAI agar medium belonged to the Gram-negative genera *Stenotrophomonas*, *Chryseobacterium*, *Delftia*, *Pseudomonas* and *Pantoea*. The bacteria retrieved from M17 agar at 30 °C were very diverse and belonged to various Gram-positive genera such as *Kocuria*, *Microbacterium*, *Micrococcus*, *Brachybacterium*, *Bacillus*, *Exiguobacterium*, *Enterococcus*, *Lactococcus* and to Gram-negative genera such as *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, *Chryseobacterium* and *Citrobacter*. The bacterial community identified on M17 agar at 42 °C was more selective, as *Enterococcus* and one strain of *Lactococcus* predominated and only one isolate of *Pantoea* was found. The FH medium was selective for mesophilic *Lactobacilli*, while the SB medium selected for *E. faecalis*. Isolates retrieved from the TA medium belonged to *E. faecalis* and *Lc. lactis* and the majority of isolates from the MSE agar medium were identified as *E. faecalis*, with a few *Ln. mesenteroides*. The bacterial community identified on the RPF medium was composed essentially of the genera *Staphylococcus*, *Microbacterium*, *Exiguobacterium*, *Brevibacterium* and sporadically of *Enterococcus*. The CFC agar medium mainly selected for *Pseudomonas*.

The counts on the different media were used as dependant variables for MANOVA analysis, with the

Table 4. Frequency of each species identified on the different cell counting media

Media	Total isolates	Species as percentage of isolates
Cheese ripening bacteria media (CRBM). Incubation: 5 days at 37 °C	40	<i>Kocuria</i> sp. 30%; <i>Enterococcus faecalis</i> 30%; <i>Staphylococcus epidermidis</i> 13%; <i>Corynebacterium variabile</i> 5%; <i>Salinicoccus</i> sp. 5%; <i>Jeotgalicoccus psychrophilus</i> 5%; <i>Brevibacterium stationis</i> 6%; <i>Brachybacterium paraconglomerans</i> 3%; <i>Arthrobacter</i> sp. 3%
Plate count agar + cristal violet inhibitor (PCAI). Incubation: 3 days at 30 °C	18	<i>Stenotrophomonas maltophilia</i> 56%; <i>Chryseobacterium indologenes</i> 17%; <i>Delftia acidovorans</i> 11%; <i>Pseudomonas aeruginosa</i> 11%; <i>Pantoea agglomerans</i> 5%
Terzaghi and Sandine M17. Incubation: 2 days at 30 °C	67	<i>Acinetobacter baumannii</i> 35%; <i>Kocuria</i> sp. 13%; <i>Microbacterium oxydans</i> 7%; <i>E. faecalis</i> 7%; <i>Ps. aeruginosa</i> 7%; <i>Ch. indologenes</i> 6%; <i>Ste. maltophilia</i> 6%; <i>Micrococcus</i> sp. 3%; <i>Citrobacter freundii</i> 3%; <i>Pseudomonas putida</i> 3%; <i>Br. paraconglomerans</i> 2%; <i>Exiguobacterium</i> 2%; <i>Bacillus cereus</i> 2%; <i>Lactococcus garvieae</i> 2%
Terzaghi and Sandine M17. Incubation: 2 days at 42 °C	10	<i>E. faecalis</i> 80%; <i>Streptococcus mitis</i> 10%; <i>P. agglomerans</i> 10%
Turner Sandine Elliker with acide nalidixique (30 mg/l) (TA). Incubation: 2 days at 30 °C	8	<i>E. faecalis</i> 50%; <i>Lactococcus lactis</i> 50%
Slanetz and Bartley (SB). Incubation: 2 days at 42 °C	11	<i>E. faecalis</i> 100%
Facultatively heterofermentative lactobacilli (FH). Incubation: 3 days at 30 °C in anaerobiose	19	<i>Lactobacillus casei</i> 100%
Mayeux Sandine Elliker (MSE). Incubation: 2 days at 30 °C	54	<i>E. faecalis</i> 95%; <i>Leuconostoc mesenteroides</i> 5%
Cetrimin-fucidin-cephalosporin (CFC). Incubation: 2 days at 25 °C	12	<i>Ps. putida</i> 92%; <i>Ln. mesenteroides</i> 8%
Rabbit plasma fibrinogen (RPF). Incubation: 2 days at 37 °C	42	<i>S. epidermidis</i> 53%; <i>Mic. oxydans</i> 16%; <i>Exiguobacterium</i> 14%; <i>Staphylococcus simulans</i> 8%; <i>Staphylococcus caprae</i> 3%; <i>Br. stationis</i> 3%; <i>E. faecalis</i> 3%

Table 5. Microbial counts (log₁₀ cfu/ml) of milks on different media

Counts on media	Significance	Period 1 (16 milks)		Period 2 (33 milks)		Period 3 (44 milks)		Period 4 (23 milks)	
		Mean ¹	SD	Mean	SD	Mean	SD	Mean	SD
FH	***	1.73 b	0.43	1.31 a	0.43	2.40 c	0.74	2.26 c	0.56
CFC	***	2.71 c	1.35	2.30 bc	0.93	1.77 b	1.41	0.86 a	1.10
RPF	***	2.76 a	0.51	2.92 ab	0.22	3.03 bc	0.23	3.15 c	0.27
PCAI	***	2.83 ab	0.77	2.74 a	0.60	3.30 bc	0.70	3.40 bc	0.60
VRBG	***	0.84 a	0.94	1.11 a	0.90	2.01 b	1.13	1.80 b	1.03
MSE	***	3.67 b	0.38	2.98 a	0.86	3.26 ab	0.64	3.21 a	0.33
CRBM	**	3.67 b	0.20	3.28 a	0.45	3.51 ab	0.50	3.35 a	0.31
OGA	**	1.37 a	0.99	1.40 a	0.88	1.92 ab	0.86	2.00 b	0.69
TA	*	4.22 b	0.75	3.93 ab	1.15	3.76 ab	1.07	3.35 a	1.23

¹Mean values of counts of all milks from the period.

a, b, c: Letters indicate homogeneous statistical processing groups within row that were significantly different according to the Tukey statistical test ($P < 0.05$), with $a < b < c$.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Abbreviations of media are given in Table 4.

period as categorical predictor. The four multivariate test statistics of Statistica output (Wilks' lambda, Pillai's trace, Hotelling–Lawley and Roy's maximum root) showed a significant effect of the period on global

profiles of milks, with a P -value < 0.001 . Univariate tests have been realized to describe the populations that are the best implied in this effect. The mean values (expressed as log₁₀ cfu/ml) and standard deviation of

each medium for the four periods are indicated in Table 5. The counts on M17 at 30 °C, M17 at 42 °C and SB media did not differ significantly according to the periods. The variables that best discriminated between periods were the counts on the FH, CFC, RPF, PCAI, VRBG and MSE ($P < 0.001$) media, followed by counts on the CRBM, OGA and TA ($P < 0.05$) media.

Taking into account the media's selectivity, the counts of *Enterococcus* were stable throughout the lactation year. The counts of mesophilic *Lactobacillus*, Gram-negative bacteria, *Staphylococcus* and coryneform bacteria increased during the year and reached their highest counts in milks from P3 and P4. The counts of the *Pseudomonas* and *Lactococcus* were highest in milks from P1, decreasing steadily to reach their lowest levels in milks from P4.

Discrimination between periods by 16S rDNA SSCP analysis

The SSCP patterns of the bacterial 16S rDNA V3 region varied quite widely between different milks, as illustrated in Fig. 1.

A one-way discriminant analysis was first performed (results not shown) to check the classification of the milks in the four defined periods according to the

relative peak areas of the V3 16S rDNA profiles. The results showed that some milks were not classified in the period defined as described in Material and methods section. This concerned two milks from P1, four from P2, five from P3 and four from P4 which were not taken into account in the following analysis.

The relative areas of the 39 peaks from the V3 16S rDNA profiles of 103 milks were used as dependent variables in MANOVA, with the period as categorial predictor. It was observed that the period had a significant effect on global profiles of milks with a P -value < 0.01 for the four multivariate test statistics of Statistica output. Univariate tests have been realized to evaluate the significance of this effect on the different populations. Eighteen peaks had a significant effect in differentiating milks from the four periods, as shown in Table 6A. Peaks 19, 32, 13, 7, 20, 9, 35, 3 and 22 were the most discriminating ($P < 0.001$) followed by peaks 36, 8, 37, 39, 2, 12, 21, 27 and 17. The different peaks were assigned to one or several species as shown in Tables 2 and 3. In the V3 16S rDNA profiles of milks from P1, the highest relative areas were those of the peak corresponding to the sequence of *Lc. garvieae*, as shown by the evolution of peak 35. These relative areas slightly decreased in the SSCP milk profiles of P2 and were low in the V3 16S rDNA profiles of milks from P3 and P4. All peaks in the V3 16S rDNA profiles of milks

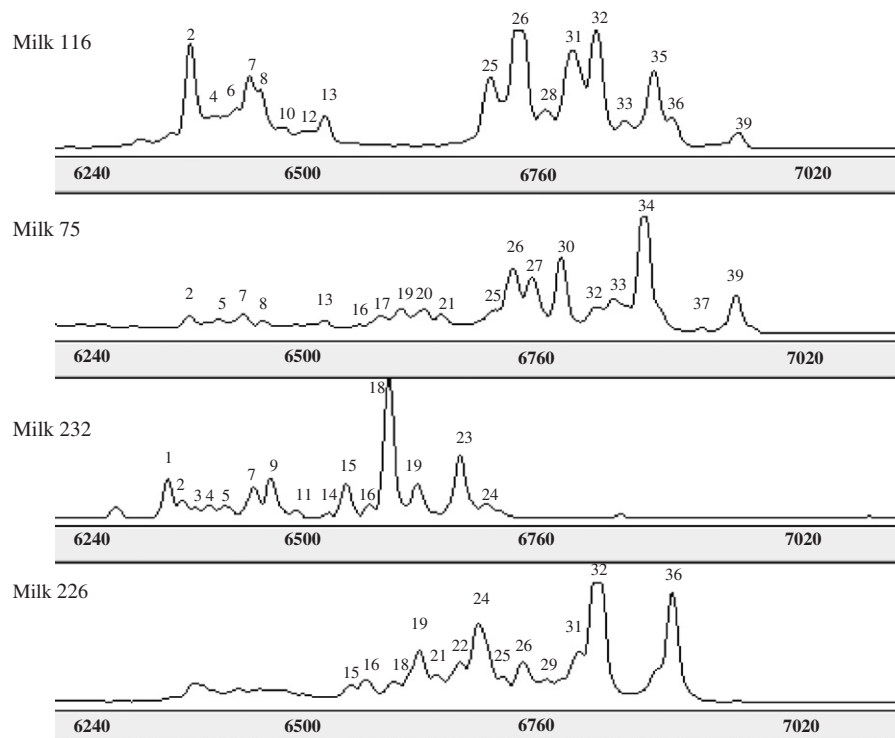


Fig. 1. Diversity of bacterial 16S rDNA region V3 SSCP profiles of four different milks: milk 75 (period 1), milk 116 (period 2), milks 226 and 232 (period 3). Thirty-nine different peaks were observed among the 118 V3 16S rDNA profiles of the milks. The profiles of milks have been lined up according to internal and external standards. Y-axis: fluorescens. X-axis: elution time expressed “in scans” (arbitrary unit of analysis software). The assignment of peaks to species is shown in Tables 2 and 3.

Table 6. Ratio of 16S V3 SSCP (A) and 18S V4 SSCP (B) peaks having a significant effect in differentiating the milks from the four periods by univariate tests

(A) 16S V3 SSCP									
V3 SSCP peaks ¹	Significance	Period 1 (14 milks)		Period 2 (29 milks)		Period 3 (39 milks)		Period 4 (20 milks)	
		Mean ²	SD	Mean	SD	Mean	SD	Mean	SD
2	**	1.40 a	1.52	3.81 b	3.38	3.39 ab	2.92	1.81 ab	1.57
3	***	1.5 b	1.9	0.005 a	0.02	0.31 a	0.82	0.35 a	1.08
7	***	1.05 ab	0.79	209 b	2.28	0.46 a	1.24	0.29 a	0.44
8	**	0.37 a	0.58	1.62 b	2.09	0.89 ab	1.00	0.16 a	0.50
9	***	0.22 a	0.44	0.37 a	0.85	1.77 b	2.45	2.50 b	1.80
12	**	0.32 a	0.50	1.21 b	1.39	0.59 ab	0.65	0.59 ab	0.65
13	***	0.36 ab	0.62	1.25 b	2.19	0.04 a	0.17	0.00 a	0.00
17	*	0.29 a	0.76	1.19 ab	1.55	3.36 b	6.02	1.27 ab	4.19
19	***	0.55 a	1.03	1.50 a	2.85	1.37 a	2.63	11.73 b	4.65
20	***	0.48 a	0.79	0.77 a	1.04	5.68 b	5.91	0.30 a	1.12
21	*	0.61 a	0.97	0.63 a	1.54	1.75 ab	3.09	2.44 b	1.43
22	***	1.90 a	2.66	2.21 b	3.34	4.49 c	2.48	4.38 bc	2.27
27	*	2.32 b	3.93	0.17 a	0.69	1.87 ab	3.66	1.20 ab	1.24
32	***	10.42 a	10.22	9.66 a	9.65	14.58 a	8.03	27.70 b	9.17
35	***	13.09 bc	11.12	14.28 c	12.68	3.65 a	5.61	6.09 ab	7.52
36	**	8.26 b	7.48	2.92 a	5.20	8.01 b	5.95	8.83 b	3.52
37	**	0.37 a	0.49	2.04 b	3.33	0.35 a	0.69	0.39 a	0.97
39	**	1.46 a	2.40	5.47 b	6.72	1.44 a	3.28	0.98 a	1.94
(B) 18S V4 SSCP									
V4 SSCP peaks ¹	Significance	Period 1 (8 milks)		Period 2 (8 milks)		Period 3 (20 milks)		Period 4 (13 milks)	
		Mean ²	SD	Mean	SD	Mean	SD	Mean	SD
c	***	4.01 a	4.72	0.37 a	0.98	3.08 a	0.8	27.3 b	13.28
f	***	0.00 a	0.7	0 a	0	0 a	0	4.01 b	2.56
d	***	1.36 a	2.73	0 a	0	1.87 a	2.5	17.4 b	10.14
k	*	2.88 ab	3.96	4.29 ab	6.64	11.1 c	10.41	1.71 a	3.82
m	*	36.1 ab	17.17	44.68 c	38.28	18.7 ab	16.16	16.17 a	16.19
s	*	23.65 b	16.43	2.44 a	5.99	7.99 a	7.66	3.7 a	7.84

¹All the peaks are named in Figs. 1 and 2.

²Mean values of 16S V3 (A) or 18S V4 SSCP (B) peak ratio of all milks from the period.

a, b, c: Letters indicate homogeneous statistical processing groups within row that were significantly different according to the Tukey statistical test ($P < 0.05$), with a < b < c.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

from P2 had globally low relative areas, with no peak predominating. However, peak 2 (corresponding to the *Micrococcus* sp. sequence) and peak 39 had the highest relative areas and peak 36 (unidentified) the lowest. In the V3 16S rDNA profiles of milks from P3, peak 20 (corresponding to the *Klebsiella* sequence) and peak 18 (assigned to *P. agglomerans*) had the highest relative areas. In the V3 16S rDNA profiles of the milks from P4, peak 19 (assigned to *Ch. indologenes*), peak 32 (corresponding to the *Ac. baumannii* sequence) and peaks 9 (*C. casei*) and 21 (*Microbacterium*, *Exiguobacterium*) had the highest relative areas.

Discrimination between periods by 18S rDNA SSCP analysis

Forty-nine milks were characterized by determining their 18S rDNA region V4 SSCP profiles. Examples of the V4 18S rDNA profiles of different milks are shown in Fig. 2. Considering the profiles from all the milks, 26 different peaks were identified. As with the 16S rDNA analysis, a one-way discriminant analysis was performed to check the classification of the milks in the four

periods according to the relative areas of the different peaks in the profiles. The results revealed some milks were not classified in defined periods (results not shown). This concerned four milks from P1, two from P2, one from P3 and one from P4 which were not taken into account in the following analysis.

The relative areas of the peaks from the V4 18S rDNA profiles of 41 milks were used as dependent variables in a MANOVA to determine the effect of the period factor. It was observed that the period had a significant effect on global profiles of milks with a *P*-value <0.01 for the four multivariate test statistics of Statistica output. Univariate tests were performed to describe the effect of period on specific peaks; there was a significant effect on six peaks. The results are detailed in Table 6B. Peaks were identified by coelution with milk isolates, as shown in Table 3. Regardless of these results, the peak *s*, corresponding to the *Rh. glutinis* and *D. hanseni* sequences, had the highest relative areas in the V4 18S rDNA profiles of milks from P1. The relative areas of this peak barely decreased in the V4 18S rDNA profiles of milks from P2 and then increased gently in those from P3. In the V4 18S rDNA profiles of milks from P2 two peaks, *f* (assigned to *Rh. minuta*) and *d* (unidentified) were absent and peaks *c* (assigned to *C. inconspicua*) and *s* had the lowest relative areas. Peak *m* (assigned to *Cr. magnus* or *Cr. curvatus*) had the highest relative areas. In the V4 18S rDNA profiles of milks from P3 no peak corresponding to *Rh. minuta* appeared and peak *k* corresponding to *Kl. lactis* had the highest relative areas. In the V4 18S rDNA profiles of the milks from P4, peaks *c* (*C. inconspicua*), *f* (*Rh. minuta*) and *d* had the highest relative areas.

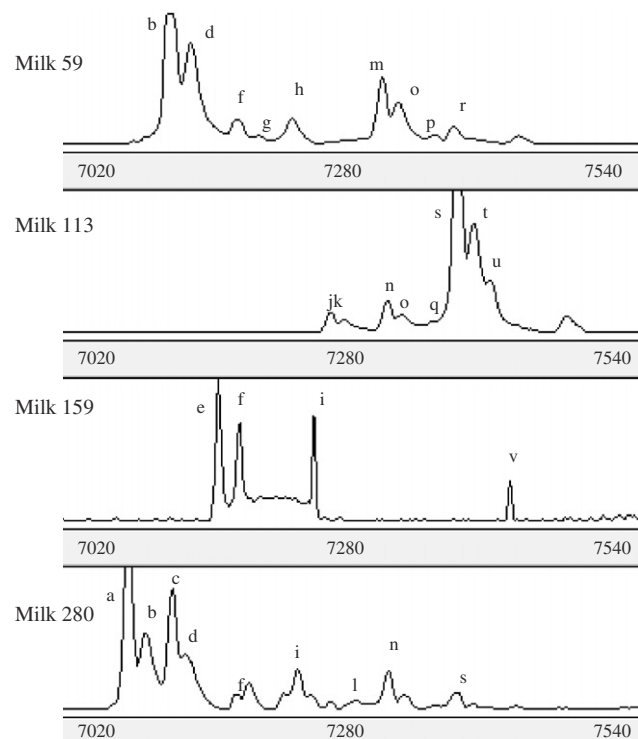


Fig. 2. Diversity of 18S rDNA region V4 SSCP profiles of four different milks: milk 59 (period 1), milks 113 and 159 (period 2), 280 (period 4). Twenty-six different peaks were observed among the 49 V4 18S rDNA profiles of the milks. The profiles of milks have been lined up according to internal and external standards. Y-axis: fluorescens. X-axis: elution time expressed “in scans” (arbitrary unit of analysis software). The assignment of peaks to species is shown in Table 4.

Discussion

This study gives the first overall analysis of microbial communities in raw goat milks.

The analysis was conducted on milk taken from the same herd at different periods. The combination of direct and culture-dependent methods enabled us to identify 42 different species of bacteria, reflecting the bacterial complexity of raw goat milk.

The diversity of the lactic acid bacteria was not very great. The species *E. faecalis*, *Lc. lactis*, *Lb. casei* and *Ln. mesenteroides*, extensively described in the literature, were also detected, with a predominance of *E. faecalis* as has often been reported in goat milks [15,42,48]. The presence of *Lc. garvieae* in goat milk was mentioned by Morea et al. [31]. 16S rRNA sequence comparison suggests presence of *St. mitis* and *E. saccharominimus* based on sequence similarities of 98%. These have never been described in goat milk.

Our study shows a wider diversity of Gram-positive non-lactic acid bacteria than is described in the

literature. Our results confirm the presence of different species of *Staphylococcus* (*S. epidermidis*, *S. simulans*, *S. caprae* and *S. equorum*) and *Micrococcus* sp. already reported in goat milks [21,34,39]. The species *S. epidermidis* and *S. caprae* and *S. simulans* are classically implicated in goat mammary infections [16,24,33]. *S. epidermidis* is known to be more chronically persistent [33], which explains why it was the most frequently isolated from the milks. Other species such as *K. rhizophila* and *K. carniphila*, *Mic. oxydans*, *Exiguobacterium*, *B. thuringiensis* have been described in raw cow milk, whereas *C. variabile*, *A. arilaitensis*, *Br. paraconglomerans*, *Clostridium*, *Rothia* have only been described in cheeses [14]. The numerous corynebacteria we isolated from the milks could come, like *Staphylococcaceae*, from the skin of the goats' udders, as shown by Contreras et al. [11]. However, preliminary isolate identification based on 16S rRNA sequences also suggests presence of two species, *Salinicoccus* sp. and *J. psychrophilus*, which have not previously been identified in milk. *J. psychrophilus* is a halophilic bacterium which has only been isolated from the traditional Korean fermented seafood jeotgal [47]. These species have also been described in association with *Arthrobacter* and *D. maris* from incoming process water in the leather industry [35]. The genus *Salinicoccus* has been isolated from various hypersaline environments [40]. All these halophilic species may have several origins: feed with high NaCl content, cleaning agent for tank and milking machine. Another atypical genus among dairy bacteria found in this study was *Ornithinococcus* sp., which has never been mentioned in milk but is described as a garden soil actinomycete [17]. It is not surprising as soil can contaminate teats and further milk.

Our study also highlights the presence of numerous Gram-negative bacteria. *P. agglomerans* was the predominant species isolated from raw milk, confirming the results observed by Pisano et al. [36] in raw ewes' milks. The association of *Pantoea* with the halophilic species *Exiguobacterium* mentioned above has been observed in marine water pipeline biofilm by Lopez et al. [26]. *Pseudomonas* was another predominant Gram-negative flora, certainly due to its frequent presence on the skins of goats' udders [11] and possible contamination by water [25]. *Pseudomonas* may also be selected for by the storage of milk at 4 °C. The presence of *Chryseobacterium* in dairy products was reported by Jooste et al. [19]. However, the presence of *H. chejuensis* is atypical for milk; it has only been described in the literature as a halophilic bacteria of marine environments [23].

The diversity of the yeast community was also evaluated, and confirmed the presence of *D. hansenii*, *Kl. lactis*, *Tr. beigeli* and *Rh. glutinis* in goat milks [38,44]. The species *C. pararugosa* and *C. intermedia* have been isolated in cow milks [8] whereas *Cr. magnus*

and *Rh. minuta* have only rarely been described in milk or cheese.

Culture-dependent and direct methods were complementary for evaluating the diversity and both showed instability in the microbial composition of the milk over the lactation year. We observed changes in the microbial balance between milks from different periods defined according to breeding conditions. These changes may be linked to several factors. Firstly, the results show a seasonal effect due to the combined effects of feeding regime, physiological state and weather on the milk microbial communities. Secondly, counts of *Lactococcus* on TA medium and *Pseudomonas* on CFC medium were the highest in milks during the winter period (period P1) and decreased in spring and summer. This tendency was confirmed by SSCP analysis, in which the peaks corresponding to sequences of *Lc. garvieae* and *Lc. lactis* showed higher ratios in milk profiles in winter than in the other periods. The summer and autumn milks (periods P3 and P4) on the other hand showed higher counts of Gram-negative bacteria on PCAI and VRBG media, and of *Staphylococcus* and *Corynebacteria* on RPF medium, than in other periods. The SSCP analysis confirmed these results. In the milks from P3 the sequences of *Klebsiella* and *P. agglomerans* had the highest peak ratios, while in the milks from P4 the sequences of *Ch. indologenes*, *Ac. baumannii*, *Corynebacterium*, *Microbacterium* and *Exiguobacterium* had the highest peak ratios. These seasons were also associated with changes in the goats' feeding regime and location. In winter and summer, the goats were fed indoors with dried alfalfa whereas in autumn, they were outdoors and fed on *Graminaceae*. Volume of milk also varied, being lowest in summer and autumn. However, the microbial community was quite stable within a given period, even if a few milks in each period were classed in the wrong period. This may be due to particular events such as weather conditions, rain particularly. For example, the misclassification of milks from periods 2 or 3, classed in period 4 because of the high relative areas of the *Ch. indologenes* peaks, may be linked to rainy days. The health of the animals may be also involved in abnormal profile changes because mastitis and diarrhea were also observed some days before the milk sample was taken. All these hypothesis should be checked in further experimental studies.

In conclusion, the dual approach applied in this study provides new tools for studying the microbial ecology of raw milk by taking account the sources of contamination and the factors affecting their composition.

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