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Phenotypic and genotypic characterization of lactic acid bacteria isolated from raw goat milk and effect of farming practices on the dominant species of lactic acid bacteria

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ABSTRACT

Lactic acid bacteria, in particular *Lactococcus lactis*, play a decisive role in the cheese making process and more particularly in lactic cheeses which are primarily produced on goat dairy farms. The objective of this study was therefore to identify the main lactic acid bacteria found in raw goats' milk from three different regions in France and evaluate if certain farming practices have an effect on the distribution of species of lactic acid bacteria in the various milk samples. Identification at genus or species level was carried out using phenotypic tests and genotypic methods including repetitive element REP-PCR, species-specific PCR and 16S rRNA gene sequencing. The distribution of the main bacterial species in the milk samples varied depending on farms and their characteristics. Out of the 146 strains identified, *L. lactis* was the dominant species (60% of strains), followed by *Enterococcus* (38%) of which *Enterococcus faecalis* and *Enterococcus faecium*. Within the species *L. lactis*, *L. lactis* subsp *lactis* was detected more frequently than *L. lactis* subsp *cremoris* (74% vs. 26%). The predominance of *L. lactis* subsp *cremoris* was linked to geographical area studied. It appears that the animals' environment plays a role in the balance between the dominance of *L. lactis* and enterococci in raw goats' milk. The separation between the milking parlor and the goat shed (vs no separation) and only straw in the bedding (vs straw and hay) seems to promote *L. lactis* in the milk (vs enterococci).

1. Introduction

The sensorial particularity of farmhouse goats' cheese is partly linked to the use of raw milk of which the properties vary according to farming practices. The physico-chemical characteristics of milk depend on the breed of goat and the feed, which in turn influence the technological and sensorial characteristics of the cheeses. The microbial flora in raw milk is also a key characteristic in cheese quality as it increases the diversity of flavors (Steele and Ünlu, 1992; Fox et al., 1996; Lynch et al., 1997; Monteil et al., 2014). Due to their acidifying capacity, lactic acid bacteria play a key role in the acidification of the curd essential to cheese making, but they also contribute to cheese aroma and texture as they possess endo and exopeptidases which are involved in the production of sapid molecules; they generate precursors of aromatic compounds (Mauriello et al., 2001; Herreros et al., 2003). Lactic acid bacteria are also very important in the manufacturing of farmhouse raw goats' milk cheese as the coagulation at low temperature (20 °C) lasts approximately 24 h. The whey, rich in *Lactococcus lactis* (Tormo

and Talliez, 2000), is used as a natural lactic starter. Raw milk is often described as a major source of lactic acid bacteria in the whey (Bachmann et al., 1996; Centeno et al., 1996; Manopoulou et al., 2003; Duthoit et al., 2005) and so it is important, particularly for these cheeses, to control the microbiological quality of the milk as the success of the whey and the cheese depends on it. The dominance of *L. lactis* in the whey is a factor of success (Demarigny et al., 2006). Raw milk, rich in *L. lactis* may therefore be very interesting, particularly for this type of cheese making. Certain studies have shown that the microbiological characteristics of milk depend on the farm and the farming practices (Michel et al., 2001; Verdier Metz et al., 2009; Tormo et al., 2011; Mallet, 2012; Mallet et al., 2012). However, to date, no studies have been undertaken which look at the relationships between the species of lactic acid bacteria found in raw goat milk and the farming practices.

The objective of this study was to (i) identify the major lactic acid bacteria in raw goats' milk that potentially have the capacity to acidify raw milk. The bacteria were identified using phenotypic tests, analyses and genotypic methods including repetitive element REP-PCR, species-specific PCR techniques, and 16S rDNA sequencing; and (ii) to evaluate the relationship between farm practices and the distribution of dominant species of lactic acid bacteria in raw goats' milk. These practices concerned: general management, monitoring of flock, bedding management practices, environmental conditions during and after

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milking, cleaning of teats and milking machine, handling and characteristics of the milking machine.

2. Materials and methods

2.1. Choice and monitoring of farms

The 21 farms selected were all farms producing farmhouse goats' cheese from the three French geographical areas: PDO Rocamadour (11 farms from department of Lot), Pélardon (6 farms from departments

Table 1
Groups of variables describing the management practices and number of farms per practice.

Variable label	Level	Number of farms
1. General management		
Size of flock	116 ± 83	21
Pasture in spring and summer	Yes	18
	No	3
Level of production (kg/goat/year)	639 ± 196	21
Area	PDO Rocamadour	11
	PDO Pélardon	6
	Franche-Comté	4
2. Monitoring of flock		
Milk testing	No milk testing	9
	Milk testing	12
Monitoring of somatic cells counts	No monitoring	15
	Monitoring	6
Antiparasitic treatment	No	4
	Yes	17
Antibiotic treatment during drying off	No	10
	Yes	11
Homeopathic treatment during drying off	No	10
	Yes	11
3. Bedding management practices		
Bedding	Straw	12
	Straw + hay	9
Additive in the bedding	Yes	3
	No	18
4. Environmental conditions during and after milking		
Mulching during milking	Yes	4
	No	17
Frequency of cleaning milking platforms	Frequently: after each milking	11
	Not frequently: less frequently than after each milking	10
Position of milking parlor	No separation with the bedding area	9
	Physical separation with the bedding area	12
Method of cleaning milking platform	Dry method	17
	With water	4
5. Practices concerning the teats		
Disposal of premilking	Yes	16
	No	5
Desinfecting of teats after milking	Yes	3
	No	18
6. Cleaning of milking machine		
Maximal temperature (°C) of cleaning of milking machine	60,5 ± 12	21
Intercleaning with alkaline and acid products	Frequently: change of product every day	13
	Not frequently: change less frequently than every day	8
Residue of water in the MM ^a	Yes	13
	No	8
Washable sanitary trap	Yes	5
	No	16
7. Handling and characteristics of the MM^a		
Length of pipeline	<120 m	15
	≥120 m	6
Number of elbows and fittings	<4	9
	≥4	12

^a MM: Milking Machine.

of Hérault and Gard) and Franche-Comté (4 farms). They were chosen on the basis of their diverse farming practices and methods of milk production and were representative of their region. The management and the farming practices were reflected in Table 1. The practices were monitored in May and June 2006. For each farm, milk samples were collected after milking; the samples (once sample of milk from one milking per farm) were cooled to 10 °C and frozen without cryoprotectant at -25 °C for a maximum of one month.

2.2. Numeration, isolation and purification of lactic acid bacteria

Elliker medium modified according to Chamba et al. (1981) was chosen for numeration, isolation and culture of lactic acid bacteria. This selective medium is used to count acidifying bacteria of which the majority is lactic acid bacteria.

After inoculation in the mass of dilutions of milk in sterile buffered peptone water (Biomérieux, France) and incubation of 72 h at 20 °C, approximately ten acidifying bacterial colonies (colonies with yellow halo) were isolated from the suitable dilution and incubated in Elliker broth overnight at 30 °C. The isolates (50 µL of the culture) were purified by subculture on Elliker agar (48 h, 30 °C) and one colony was incubated overnight at 30 °C in Elliker broth. After centrifugation (5000 rpm during 5 min at 4 °C), the bacterial pellets were dispersed in skim milk, frozen and stored at -80 °C in reconstituted sterile semi-skimmed milk (150 g/L) with 20% glycerol (500 µL of pellet in 500 µL of broth).

2.3. General procedure for identification of lactic acid bacteria

Firstly the bacterial isolates were characterized using phenotypic tests in order to verify that the isolates were lactic acid bacteria and to have phenotypic profiles. Strains belonging to groups of lactic acid bacteria with different phenotypic profiles and from milk samples from different farms were selected for the continuation of the characterization: PCR to confirm the *Lactococcus lactis* subspecies and the genus of the other lactic acid bacteria completed by REP-PCR, a molecular tool which is useful for elucidating relationships within and between bacterial species (Mancuso et al., 2007). Finally, the strains with different profiles (phenotypic, Rep-PCR) were sequenced and subsequently assigned at species or subspecies level.

2.4. Phenotypic characterization of the isolates

The Gram positive, catalase negative isolates were analyzed at genus level. The growth of the isolates in Elliker broth (DIFCO, France) at 10 °C for a week, at 45 °C, pH 9.6 and 6.5% (P/V) salt for 96 h as well as growth in "litmus milk" (Becton Dickinson and Company, USA) were tested. Subsequently, the isolates were characterized using the following tests: growth in Elliker broth (DIFCO, France) with 4% salt and at 40 °C, growth at pH 9.2, ability to ferment maltose, ribose, sorbitol and raffinose in MRS broth (DIFCO, France). The presence of an arginine dihydrolase was investigated in BHI broth with 0.3% L-arginine (SIGMA, France). After incubation 24 h at 30 °C, 2 to 3 drops of Nessler reagent were added. An orange precipitate indicates the presence of the NH₃.

2.5. PCR-based method

2.5.1. DNA extraction

Strains were incubated at 30 °C for 24 h in MRS broth and genomic DNA was extracted using the NucleoSpin tissue kit (Macherey Nagel, 67 722 Hoerd, France).

2.5.2. PCR amplification

The strains were confirmed to belong to *Lactococcus lactis* subsp *lactis*, *Lactococcus lactis* subsp *cremoris* or enterococci by means of a PCR-based method. *Lactococcus lactis* subsp *lactis* or subsp *cremoris* were identified using primers His 1 and His 2 (Corroler et al., 1998).

Enterococcal DNA was amplified using primers Conrev 23 and Genter according to Frahm et al. (1998). In all cases, amplification reactions were performed in a final volume of 25 μ L containing 1 \times reaction PCR buffer (Qiagen, France), 0.3 μ M of each opposing primer, 2.5 mM of MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 0.5 U Taq polymerase and 5 μ L of DNA.

Inter-repetitive extragenic sequences were amplified by means of two 18-mer primers in combination (REP 1R-Dt, REP 2-D) (Invitrogen, Cergy Pontoise, France) as described in other publications (Versalovic et al., 1991; Berthier et al., 2001). The final PCR reaction mixture was 25 μ L: 2 μ M of each of the PCR primers REP-1 and REP-2, 200 μ M of each of the desoxyribonucleotides (dNTP) (Invitrogen, Cergy Pontoise, France), 0.4 units of Taq DNA Polymerase (Invitrogen, Cergy Pontoise, France), PCR buffer 1 \times with MgCl₂ (1/10th of total volume) (SIGMA, France) and 5 μ L of extracted DNA.

The primer sequences and the PCR amplification conditions are recapitulated in Table 2. The amplification cycles were performed with a thermal cycler (Gene Amp PCR System 9700, Perkin Elmer), then 25 μ L of PCR product was electrophoresed in a 10 g·l⁻¹ Seakem GTG agarose gel (Sigma) in TBE (Tris-Borate-EDTA pH 8) at 100 V for 3 h. The 123-pb DNA ladder (Invitrogen, 95613 Cergy Pontoise, France) was used as a size standard. The DNA fragments were stained with ethidium bromide (Sigma), examined under UV light (312 nm) and photographed (G-Box, SYNGENE). Digitized gel pictures of Rep-PCR were normalized by comparison with reference bands (G-Box, SYNGENE). The similarities among profiles were calculated using the Pearson correlation. Dendrograms were constructed using the unweighted pair group method with Arithmetic Mean (UPGMA).

2.5.3. Partial 16S rDNA sequencing

Fifty-one strains with different profiles (phenotypic, Rep-PCR) were sequenced and subsequently assigned at species or subspecies level. The REP-PCR profiles of the non-sequenced strains were compared to the profiles of the strains for which the DNA fragments have been sequenced in order to assign these strains at species or subspecies level. The 16S rRNA gene (V1–V4) was amplified by PCR using primers E8F and E807R (Baker et al., 2003). DNA was amplified in 50 μ L volumes containing 50 ng of template, 500 μ M dNTPs, 5 μ M of the respective primers, 2.5 U of Taq DNA polymerase (Invitrogen, Cergy Pontoise, France) and Thermopol buffer 10 \times . PCR products were cleaned using QUIAquick columns (Qiagen, France) according to the manufacturer's instructions and subsequently commercially sequenced (Eurofins MWG biotech, 91967 Les Ulis, France) using primer E8F. The analysis of the chromatograms and the multiple alignments were carried out using MEGA 4.1 software (Tamura et al., 2007). Subspecies identification was carried out by construction of a phylogenetic tree (using MEGA 4.1, Neighbor Joining method, bootstrap 1000) using the sequences obtained from the sequencing and the sequences of Firmicutes species acquired on the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

The primer sequences and the PCR amplification conditions are recapitulated in Table 2.

Table 2

Primer sequences, amplification and application of PCR reactions.

Primer sequences	Amplification conditions	Application
His1: 5'-CITCGTTATGATTTTACA-3'	5 min at 94 °C, 30 cycles of: 1 min 94 °C, 2 min at 45 °C, 2 min at 72 °C, final step 5 min at 72 °C	Detection of <i>Lactococcus lactis lactis</i> and <i>Lactococcus lactis cremoris</i>
His2: 5'-AATATCAACAATTCATG-3'		
Conrev 23: 5'-GGTGGATGCCTTGGCACT-3'	5 min at 94 °C, 30 cycles of: 30 s 94 °C, 30 s at 52 °C, 30 s at 72 °C, final step at 5 min at 72 °C	Detection of the genus <i>Enterococcus</i>
Genter: 5'-CTCTACTCCATCATTCT-3'	5 min at 94 °C, 30 cycles of: 1 min at 94 °C, 1 min at 40 °C, 6 min ramping to 72 °C and 1 min at 72 °C	Rep-PCR fingerprinting
REP1R-Dt: 5'-IIINCNGCNGCATCNGGC-3'		
REP2-D: 5'-NCGNTTATCNGGCCTAC-3'	3 min at 94 °C, 30 cycles of: 45 s 94 °C, 2 min at 55 °C, 1 min at 72 °C, final step 5 min at 72 °C	Amplification of the V1–V4 region of 16s rDNA The primer used for the sequencing was E8F
E8F: 5' –AGAGTTTGATCCTGGCTCAG-3'		
E807R: 5'TGGACTACCAGGTATCTAATC-3'		

2.6. Statistical analysis

For each farm, a dominant species or genus was assigned (70% of the isolates for each farm) with a dominant species or genus. The relationships between the dominant species or genus on the farm and the farming practices were studied using Pearson's chi-square test (SPAD version 5.5, Pantin, France).

3. Results

3.1. Phenotypic characterization of isolates

There was a high variability of the level of acidifying bacteria according to the farm: the size of the populations ranged from 44 to 54 000 CFU per mL of raw milk and the average was 640 CFU·mL⁻¹.

A total of 204 out of the 206 isolates had a *Lactococcus* or *Enterococcus* phenotype. For these 204 isolates, 6 phenotypic profiles were distinguished (Table 3). Profiles A and B correspond to the phenotype *Enterococcus* (47.5% of the strains). These two profiles differ in their capacity to coagulate milk: the isolates in group A do not coagulate milk, whereas those in group B do. Profiles C, D, E and F correspond to the phenotype *Lactococcus lactis* (52.5% of the strains). The strains belonging to profiles D, E and F have the phenotype *L. lactis* subsp *lactis* as they can grow in the presence of 4% NaCl, at 40 °C, hydrolyze arginine and produce acid in the presence of maltose. The strains in groups E and F can grow at pH 9.2. The strains in group E can grow in the presence of 6.5% NaCl. The strains in group C have an unusual phenotypic profile as they do not grow at 40 °C and do not hydrolyze arginine. They have a phenotype similar to that of *L. lactis* subsp *cremoris* even though they grow in the presence of 4% salt and most grow at pH 9.2. A total 146 strains out of the 206 were selected according to their different phenotypic profiles (belonging to the different groups A, B, C, D, E and F) and genotypic characterized.

3.2. Genus, species and subspecies specific PCR

Genus and species specific PCR confirmed that *L. lactis* and *Enterococcus* subsp were the dominant lactic acid bacteria isolated from Elliker agar modified according Chamba et al. (1981). So, 73 strains were identified as *L. lactis* subsp *lactis* (50% of strains tested), 17 strains as *L. lactis* subsp *cremoris* (12%) and 56 strains as enterococci (38%).

3.3. REP-PCR

Fig. 1 shows the profiles obtained from the 146 isolates. Four main groups can be distinguished. Isolates representative of the diversity of each group were sequenced (see Section 3.4). Then, profiles were successfully assigned to bacteria species. Profile 1 was assigned to *Enterococcus faecium*, 2 to *L. lactis* subsp *lactis*, 3 to *Enterococcus* subsp *faecalis* and 4 to *L. lactis* subsp *cremoris*. Only two of the 51 sequenced strains were not *Lactococcus* and *Enterococcus* and had similar fingerprint to those cited species (14H, 37G).

For each group, we were able to distinguish different clusters (85% similarity to the obtained profiles). Except for the farm numbers

Table 3

Phenotypic characteristics of strains isolated from milks. +: all strains positive; -: all strains négative; (././): number of positive/negative strains. All strains grew at 10 °C.

Clusters	Number of strains	Number of farm	Growth at 4% of salt	Growth at 6.5% of salt	Growth at pH 9,2	Growth at pH 9,6	Growth at 40 °C	Growth at 45 °C	Coagulation of litmus milk	NH3 from arginine	Maltose	Ribose	Phenotype
A	13	5	+	+	+	+	+	+	-	-	+	+	<i>Enterococcus</i> subsp
B	84	17	+	+	+	+	+	+	+	+	+	+	<i>Enterococcus</i> subsp
C	12	6	+	-	9/3	-	-	-	+	-	10/2	+	<i>Lactococcus Lactis</i>
D	28	10	+	-	-	-	+	-	+	+	+	+	<i>Lactococcus Lactis</i>
E	30	11	+	+	+	17/13	+	-	+	+	+	+	<i>Lactococcus Lactis</i>
F	37	17	+	-	+	11/26	+	-	+	+	+	-	<i>Lactococcus Lactis</i>

26 and 53, there was a different Rep-PCR fingerprint inside the same farm and, in an obvious way, between farms.

3.4. Partial 16s rDna sequencing

Fifty-one strains representative of the diversity of the phenotypic, genotypic and protein profiles were classified genetically into 5 species

by partial 16s rDNA sequencing (Fig. 2). The two main groups contain the genus *Enterococcus* (group II) divided into 2 sub-groups containing the species *E. subsp faecalis* (II.1) and *E. subsp faecium* (II.2) and the species *L. lactis* subsp *cremoris* (I.2). Two strains did not belong to these groups and were identified as *Aerococcus viridans* (14H) and *Streptococcus parauberis* (37G). The correlation between the results of the phenotypic tests, the PCR tests and the sequencing is reported in Table 4.

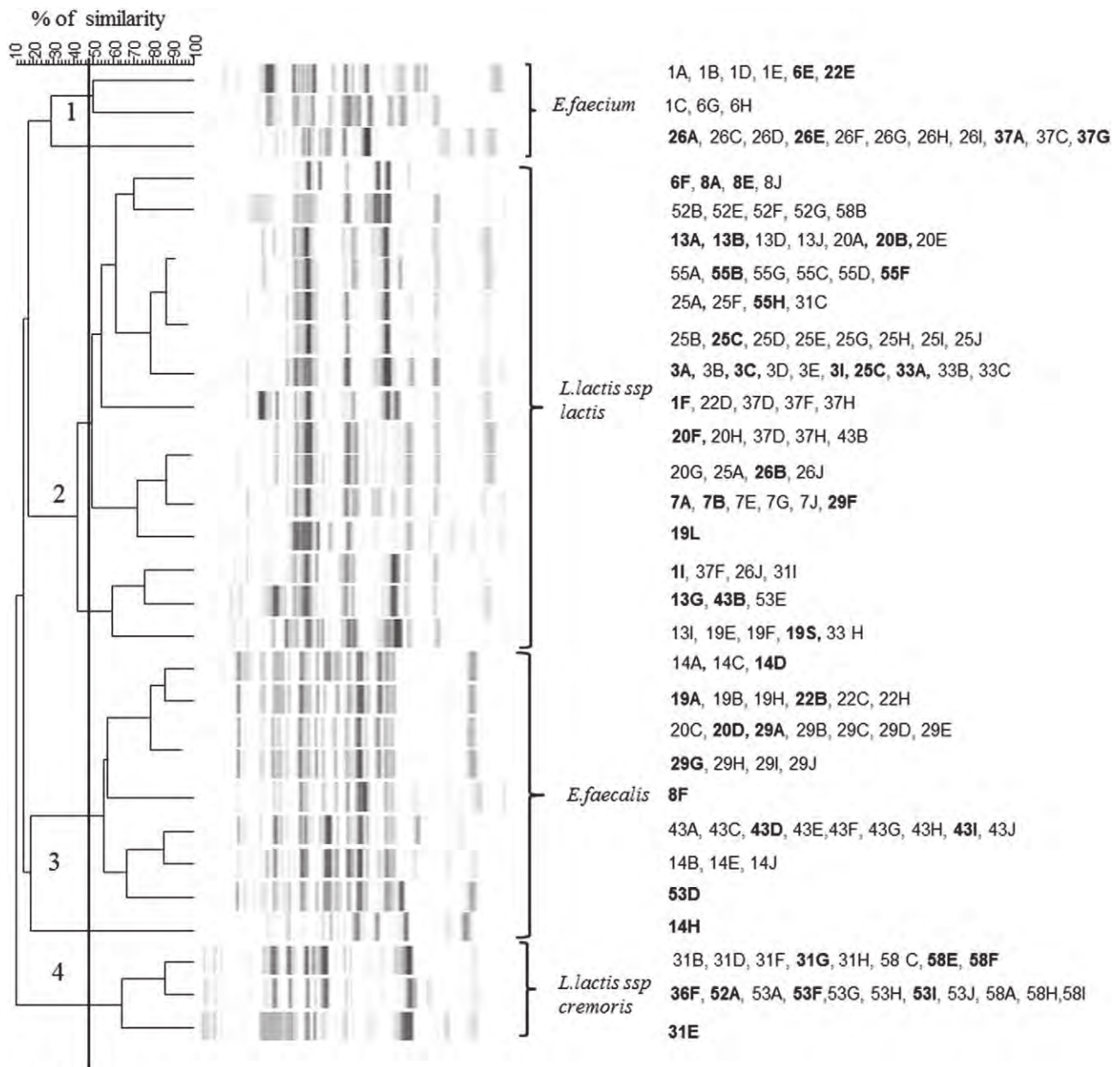


Fig. 1. Rep-PCR patterns showing the representative fingerprints of the different clusters. The numbers followed by letters to the right of the figure are the codes of the strains analyzed. The number corresponds to the farm, the letter to the clone isolated. The numbers in bold represent the sequenced isolates.

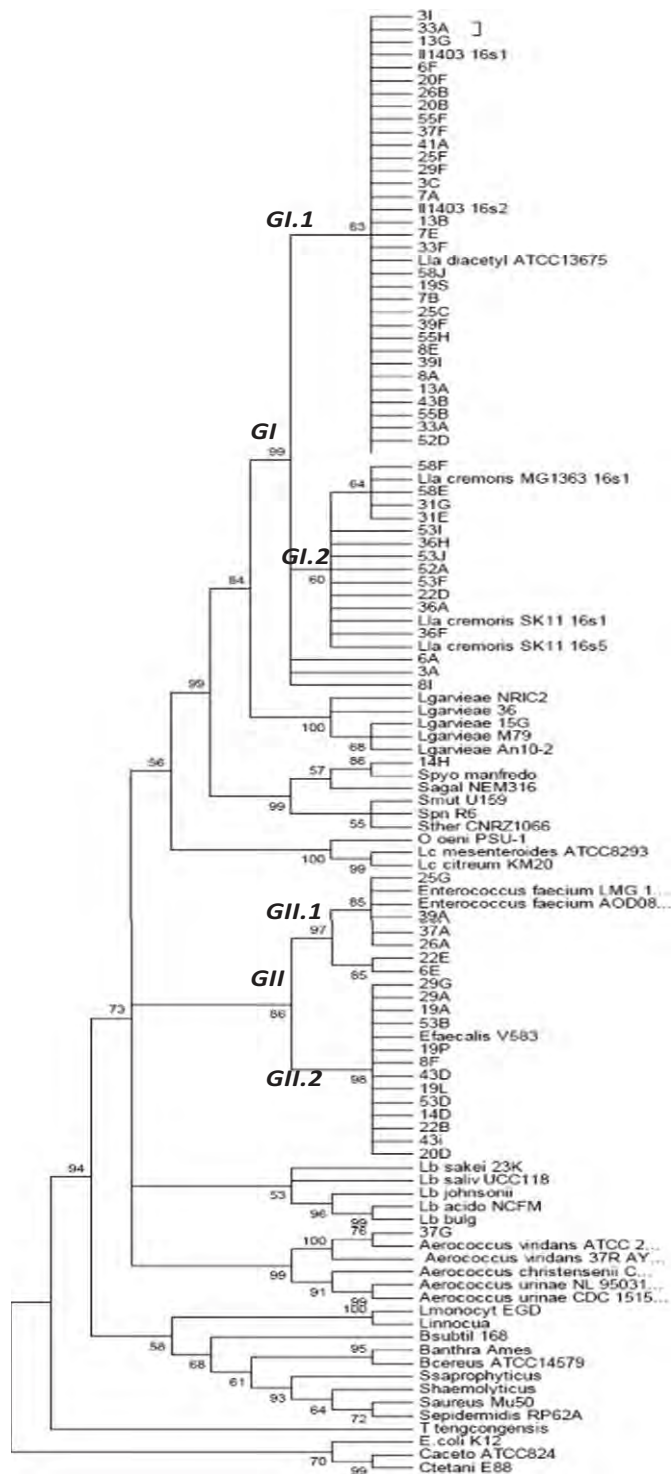


Fig. 2. Phylogenetic tree obtained by partial 16s rDNA sequencing of the strains isolated from goat milk and the reference strains obtained by the Neighbour-Joining (bootstrap 1000). Goat milk strains' codification: Number, letter (ex: 3A).

From the phenotypic tests it was possible to predict the species *L. lactis* as 26 strains out of the 33 were identified as *L. lactis*. The 7 remaining strains had the phenotypic characteristics of enterococci (grow at 45 °C, 6.5% salt, pH = 9.6).

Similar results were also observed for the genus *Enterococcus*: 13 strains out of the 15 identified had an *Enterococcus* phenotype.

A good correlation between the results of the PCR and the DNA sequencing was observed for *Enterococcus* subsp and *L. lactis* subsp *lactis* (Table 4). So, 13 strains out of 15 (87%) identified as enterococci by

DNA sequencing were characterized as enterococci by PCR and 22 strains out of 25 (82%) identified as *L. lactis* subsp *lactis* were also identified as such by PCR. However, the correlation is not as good concerning *L. lactis* subsp *cremoris* as only 6 strains out of 9 (67%) identified as *L. lactis* subsp *cremoris* were also identified as such by PCR.

3.5. Dominant species in milk and relationship with farming practices

Out of the 146 strains identified isolated from the medium modified according to Chamba et al. (1981), *L. lactis* was the dominant species (87 strains). The subspecies *L. lactis* subsp *lactis* was the most common (68 strains) in comparison with *L. lactis* subsp *cremoris* (19 strains). *E. faecalis* (37 strains) and *E. faecium* (20 strains) were the two species of *Enterococcus* isolated. Table 5 presents the distribution of the species according to the farms. A farm was removed from the analysis as only one isolate out of the five isolates could be cultured. When more than 2/3 of the strains identified from each of the farms belonged to the same species or genus, this species or genus was considered as dominant. The distribution of the species varied according to the farms. Twelve farms were characterized by the dominance of *L. lactis* and eight by a dominance of *Enterococcus*. For one farm (no. 37), the distribution between *L. lactis* and *Enterococcus* was similar; no dominant group was allocated. The groups of dominant species were significantly discriminated (p -value $\leq 5\%$) by certain farming practices (Table 6). Most of the farms putting hay in the bedding were associated with enterococci group (6 farms among 8) and most farms with only straw on the bedding were associated with lactococci group (10 farms among 12). Most of the farms which have no separation between bedding area and milking parlor was associated with enterococci group (6 farms among 9) and farms with separation between bedding area and milking parlor was rather associated with lactococci group (9 farms among 11).

The presence of *L. lactis* subsp *cremoris* seems to be linked to the geographic area. Indeed, 2 farms out of the 4 in Franche-Comté were characterized by a dominance of *L. lactis* subsp *cremoris*, while in the other two regions, *L. lactis* subsp *lactis* was largely dominant. Only one farm out of the 6 farms in the Pélardon PDO had milk in which *L. lactis* subsp *cremoris* was dominant. No *L. lactis* subsp *cremoris* were detected in the Rocamadour PDO area.

4. Discussion

L. lactis, *E. faecalis* and *E. faecium* are the three species most commonly isolated. These species were identified using a combination of phenotypic and genotypic methods. Subspecies specific PCR using primers targeting the Histidine operon biosynthesis region carried out according to the protocol developed by Corrolier et al. (1998) correctly discriminated *L. lactis* subsp *lactis* but seems to be less effective for *L. lactis* subsp *cremoris* (3 false positives out of 9 strains in total). REP-PCR profiles enabled the *Enterococcus* species and the *L. lactis* subspecies studied to be discriminated correctly, as previously reported by Jurkovic et al. (2006) and Jan et al. (2007).

For the species *L. lactis*, all the phenotypes corresponded to the species *L. lactis* subsp *lactis*, no specific phenotype was associated with *L. lactis* subsp *cremoris*. This result is not surprising as the strains of phenotype *L. lactis* subsp *cremoris* are isolated in a dairy environment where lactic starters are regularly used in the manufacture of fermented products (Klijn et al., 1995). The majority of *L. lactis* strains had a *L. lactis* subsp *lactis* genotype (68 strains out of the 87 belonging to the species *L. lactis*). The 19 remaining strains corresponded to the genotype *L. lactis* subsp *cremoris*. The dominance of *L. lactis* and *Enterococcus* in milk has already been underlined by numerous authors including Zamfir et al. (2006) in cows' milk and Badis et al. (2004) in goats' milk. Certain strains from different farms belonging to the *L. lactis* phenotype and genotype can grow in the presence of 6.5% salt and at pH 9.6. This result is not surprising as these environmental microorganisms

Table 4

Correlation between the sequencing results of the 16s rDNA V1–V4 region and the results of the phenotypic tests and PCR. The numbers correspond to the number of strains.

Analyses	Number of strains sequenced	Sequencing				
		<i>L. lactis lactis</i>	<i>L. lactis cremoris</i>	<i>Enterococcus</i> subsp	<i>Aerococcus viridans</i>	<i>Streptococcus parauberis</i>
Typology		25	9	15	1	1
Phenotype	<i>L. lactis lactis</i>	14	8	2	1	0
	<i>L. lactis cremoris</i>	3	0	0	0	1
	<i>Enterococcus</i> subsp	7	1	13	0	0
	n.i	1	0	0		
PCR	<i>L. lactis lactis</i>	19	1	2	1	1
	<i>L. lactis cremoris</i>	3	6	0	0	0
	<i>Enterococcus</i> subsp	2	2	13	0	0

undergo significant stress. These phenotypic particularities have already been underlined by Corrolier et al. (1998) for strains isolated from cows' milk.

The distribution of the species in the milk depends on the farms. This farm-specific characteristic has already been underlined by Corrolier et al (1998) in cows' milk from Normandy. Certain farms are characterized by a dominance of *L. lactis* subsp *lactis* (9 farms out of the 21 farms studied), others by *E. faecalis* (5 farms out of the 21) or *E. faecium* (3 farms out of the 21). The presence of a majority of *L. lactis* subsp *cremoris* in the milk (3 farms out of the 21) seems to depend on the geographic area as it is found preferentially on farms located in Franche-Comté. It is important to underline that the farms in Franche-Comté are equipped with bucket milking systems contrary to the other regions where milk is recovered via a milk line. The methods of cleaning and the materials being different, it can be assumed that the bacteria are transported in different ways (Laithier et al., 2004; Marchand et al., 2012).

The dominance of *Enterococcus* (*E. faecalis*, *E. faecium*) or *L. lactis* seems to be due in part to certain farm-specific characteristics. The direct contact between the milking parlor and the bedding area or the presence of hay in the bedding seems to promote inoculation of milk with *E. faecalis* or *E. faecium*. The natural habitat of *Enterococcus* is the intestines of humans and animals (Facklam et al., 2002). Gelsomino et al. (2001) showed that enterococci were present in the cow feces, human feces and milk. In goat farm, in the same areas of our study, Detomi (2009) showed that enterococci were

found as a dominant lactic acid bacteria in goat bedding (50 from 51 strains were enterococci). In the same study, in which 20 farms were monitored, enterococci were improved in the air of the milking parlor when there was no separation between milking parlor and bedding area ($p \leq 5\%$). Then, we could supposed that aerosol in the goat shed was contaminated by the enterococci of the bedding (probably contaminated by feces). When the milking parlor was not separated to the goat shed, the air was contaminated by the air of the goat shed. During the milking, the milk was more contaminated by enterococci than in a milking parlor separated to goat bedding. Concerning the relationship between the dominance of *Enterococcus* in milk and the presence of hay in the straw-based bedding, no scientific studies have been undertaken on this subject as far as we know. However, we can assume that the hay from fodder is less absorbent than straw, therefore favoring the development of bacteria on the surface of the bedding. This waste food may also be more contaminated as it is put in the troughs before being used for bedding.

These first results concerning the possible association between certain farming practices and the main bacteria isolated are worth confirming by analyzing the sources of contamination of *Enterococcus* and *Lactococcus*, *L. lactis* in particular. Continuing this study in this direction would permit to identify the farming operations that would enable a decrease in the contamination of *Enterococcus* and promote the development of *L. lactis* in milk. The technological and sensorial quality of lactic cheeses could therefore be improved.

Table 5

Distribution of number of strains per farm identified as being *L. lactis* or *Enterococcus* and allocation to a dominant group. 1 : total number of strains, 3 : Dominant species group. E: *Enterococcus* dominant, L: *L. lactis* dominant.

No. farms	Area	<i>L. lactis lactis</i>	<i>L. lactis cremoris</i>	<i>L. lactis</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>Enterococcus</i> subsp	Other species	nT ¹	%lactis	Groups ³
1	PDO Rocamadour	2	–	2	–	5	5	–	7	29	E
3	PDO Rocamadour	6	–	6	–	–	0	–	6	100	L
6	PDO Rocamadour	1	–	1	–	3	3	–	4	25	E
7	PDO Rocamadour	5	–	5	–	–	0	–	5	100	L
8	PDO Rocamadour	3	–	3	1	–	1	–	4	75	L
13	PDO Rocamadour	6	–	6	–	–	0	–	6	100	L
14	PDO Rocamadour	–	–	0	6	–	6	<i>S. parauberis</i>	7	0	E
19	PDO Rocamadour	3	–	3	6	–	6	–	9	33	E
20	PDO Rocamadour	6	–	6	2	–	2	–	8	75	L
22	PDO Rocamadour	1	–	1	3	1	4	–	5	20	E
25	PDO Rocamadour	10	–	10	–	–	0	–	10	100	L
26	PDO Pélardon	2	–	2	–	8	8	–	10	20	E
29	PDO Pélardon	1	–	1	9	–	9	–	10	10	E
31	PDO Pélardon	1	6	7	–	–	0	–	7	100	L
33	PDO Pélardon	4	–	4	–	–	0	–	4	100	L
37	PDO Pélardon	3	–	3	–	3	3	<i>A. viridans</i>	7	43	–
43	PDO Pélardon	1	–	1	9	–	9	–	10	10	E
52	Franche-Comté	4	1	5	–	–	0	–	5	100	L
53	Franche-Comté	1	6	7	1	–	1	–	8	88	L
55	Franche-Comté	7	–	7	–	–	0	–	7	100	L
58	Franche-Comté	1	6	7	–	–	0	–	7	100	L
Total		68	19	87	37	20	57		146		

Table 6

Farming practices discriminating the dominant groups of species.

Practices	Number of farms per practice	Number of farms according to groups of dominant specie		p value (%)
		E (n ^a = 8)	L (n ^a = 12)	
Addition of reject hay in straw-based bedding	8	6	2	1,5
No addition of reject hay in straw base bedding	12	2	10	
No separation of the milking parlor with the bedding area	9	6	3	2,8
Physical separation of the milking parlor with the bedding area	11	2	9	

^a Total number of farms per group of dominant species.

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