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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 of Herault 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.

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.

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 NH_3.

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 Hoerdt, 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).