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## ***Lactococcus lactis* strains from raw ewe's milk samples from the PDO Ossau-Iraty cheese area: levels, genotypic and technological diversity**

**Fabienne Feutry · Paloma Torre · Ines Arana · Susana Garcia · Nathalie Desmasures · Erick Casalta**

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**Abstract** The aims of this work were to assess the levels of *Lactococcus lactis* (*L. lactis*) in ewe's milk produced in three Ossau-Iraty cheese sub-areas and to investigate the genotypic and technological diversity of isolated wild strains of *L. lactis* in order to assess their suitability for use as components of starter formulations. Thirty-two milk samples from 32 farms were collected. Strains of *L. lactis* were identified and quantified using a combination of species and subspecies-specific polymerase chain reaction (PCR) and PCR amplification of repetitive bacterial DNA elements (Rep-PCR). The genotypic and technological diversity of the indigenous strains was compared to that of 12 commercial strains. *L. lactis* was detected in milk samples from only 20 farms. The levels detected were below  $4 \log_{10} \text{ cfu.mL}^{-1}$  in 75% of the milks. *L. lactis* subsp. *lactis* dominated in 66% of the samples. Forty-three genotypic profiles of wild *L. lactis* strains were detected and showed greater diversity than those of the commercial strains. Milks containing *L. lactis* contained one to four distinct strains. With the exception of two strains, each strain was found in milk from only one farm. The Prt<sup>+</sup> strains were the most acidifying. Sensitivity to phages collected from wheys differed widely between the commercial (60%) and indigenous strains (5%). Wild strains of *L. lactis* displayed a wide genotypic and technological diversity. Genotypic diversity seemed to be linked to the farm of origin. This study addresses questions regarding the environmental factors which influence such natural diversity. A deeper knowledge of the strain-dependent technological properties would be useful in selecting strains for use in starter blends.

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## Ossau-Iraty 原产地名号保护干酪生产地区生鲜羊乳中乳酸乳球菌的含量, 基因型和特性

**摘要:** 本文目的是评价Ossau-Iraty原产地名号保护 (PDO) 干酪生产地区生鲜羊乳乳酸乳球菌的含量以及分离的野生乳酸乳球菌的基因型和多样性, 以及这些乳酸菌作为发酵剂的可行性. 32个乳样分别来自32个农场. 采用物种和亚种特异性聚合酶链反应(PCR)和重复细菌DNA元素PCR扩增相结合的方法来分析和鉴定乳酸乳球菌. 将分离菌株的基因型和技术特性与12株商业菌株进行了比较, 仅从20个农场的羊乳样本中检测到乳酸乳球菌. 75%的乳样中乳酸乳球菌的含量低于 $4 \log \text{ cfu mL}^{-1}$ , 乳酸乳球菌乳酸亚种在样品中占66%. 野生乳酸乳球菌中检测出43种基因型, 与商业菌株相比, 野生乳酸乳球菌更具有多样性. 乳酸乳球菌中含有1–4株特殊的菌株. 除2株菌外, 每个农场的羊奶样品中都能够检测到不同于其他农场的特有菌株. 从乳清中收集的噬菌体与商业菌株和本土菌株比较存在较大的差异. 蛋白酶阳性 (Prt +) 菌株的酸化能力最强. 野生乳酸乳球菌显示了较广的基因型和多样性. 基因多样性似乎与农场有关. 本研究进一步说明了环境因素是影响菌株固有多样性的主要因素. 对菌株技术特性的深入研究将有助于混合发酵剂菌株的筛选.

**Keywords** *Lactococcus lactis* · Wild strain · Diversity · Technological · Genotypic · Ewe's milk

**关键词** 乳酸乳球菌 · 野生菌株 · 多样性 · 技术的 · 基因型 · 羊奶

### 1 Introduction

The species *Lactococcus lactis* has been classified as having the generally recognized as safe (GRAS) status (Casalta and Montel 2008). The ability to rapidly produce L(+) lactic acid is probably the most important property of this species, and explains their widespread use in the manufacture of many cheese varieties. Two main phenotypically defined subspecies of *L. lactis* are used intensively as dairy starters, namely *Lactococcus lactis* subsp. *lactis* (plus a diacetyl-forming biovariety, *L. lactis* subsp. *lactis* biovar *diacetyllactis*) and *Lactococcus lactis* subsp. *cremoris*. The distinction between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* is based on a set of phenotypic characteristics (ability to grow at 40 °C, in 4% NaCl and at pH 9.2, ability to ferment maltose and capacity to deaminate arginine) and is of great technological interest, but this distinction may be erroneous as it may vary among strains and hence is very strain dependent (Fernández et al. 2011). As a consequence, various DNA-based techniques such as random amplified polymorphic DNA, pulse-field gel electrophoresis, repetitive sequence-based PCR (Rep-PCR), amplified fragment length polymorphism, multilocus sequence analysis, and restriction fragment length polymorphism have been used to distinguish *L. lactis* at subspecies and strain levels (Taïbi et al. 2010).

Ossau-Iraty cheese is one of two French PDO ewe's milk cheeses. It is a ripened, uncooked pressed ewe's milk cheese produced in the southwestern region of France, in a limited geographic area which includes the Bearn and the Basque Country. It is made from raw or pasteurized milk and coagulated with rennet. Ossau-Iraty cheese-makers are allowed to use commercial lactic starters, mainly composed of strains of *L. lactis*, to ensure regular, efficient acidification.

Over the past two decades there has been an increasing interest in screening lactococci from natural dairy products in order to isolate strains with improved or novel properties for their potential application in the manufacture of traditional products. These studies have highlighted the wide diversity which exists among wild strains, much greater than among commercial or reference strains (Sánchez et al. 2000). Strain diversity can produce a wide range of genotypic profiles (Corroler et al. 1998; Fernández et al. 2011) which are not always consistent with phenotypic profiles at subspecies or strain levels (de la Plaza et al. 2006; Fernández et al. 2011). This diversity can lead to a wide range of technological abilities of individual strains including flavor formation, acidifying activity, production of antimicrobial compounds, and phagic resistance.

Compared to raw cow's milk (Franciosi, et al. 2009), to our knowledge, little data are available on strains of *L. lactis* isolated from raw ewe's milks (Feutry et al. 2011). Most investigations have focused on the genotypic diversity and/or technological properties of strains of *L. lactis* isolated only from fresh (Gaya et al. 1999; Nieto-Arribas et al. 2009) or ripened (Nieto-Arribas et al. 2009) ewe's milk cheese. Moreover, to our knowledge, the incidence of the area of production on the diversity of isolates found in raw ewe's milk has not been studied.

Our purpose was to assess the levels of *L. lactis* in ewe's milk produced in the Ossau-Iraty PDO cheese sub-areas and to investigate the genotypic and technological diversity of strains of isolated wild *L. lactis* for their potential for use in multi-strain starter formulations.

## 2 Materials and methods

### 2.1 Sampling

Thirty-two dairy farms in three dairy sub-areas A, B, and C (10, 10, and 12 farms, respectively) of the PDO Ossau-Iraty cheese region were selected on the basis of their high-quality raw milk according to the quality criteria set by the milk payment system ( $\leq 10^5$  cfu.mL<sup>-1</sup> total viable counts,  $\leq 500$  coliforms.mL<sup>-1</sup>,  $\leq 1,000$  butyric spores.L<sup>-1</sup>). The sub-areas were 25 to 70 km distant from each other.

One bulk raw milk sample (50 mL) was collected from each farm, in spring, immediately after first milking. All samples were transported within 4 h to the laboratory under refrigerated conditions and were stored at 4 °C until analysis.

The whey samples (20 mL) were collected from one cheese-making production at 21 farmhouses (W1–W21) located across the Ossau-Iraty cheese area. All samples were transported within 4 h to the laboratory under refrigerated conditions. They were prepared as described by de Godoy Oriani and Yokoya (2004) and stored at -80 °C with 30% glycerol until use.

### 2.2 Isolation of LAB

Raw milk sample preparation and decimal dilutions were carried out according to the IDF Standard 122C (1996) and samples were plated in duplicate. Standard plate counts were performed using Plate Count Agar medium (PCA, Difco, Spain). Lactococci were isolated from L-M17 agar medium (Callon et al. 2004): M17 agar

(Difco, France) (Terzaghi and Sandine 1975) containing 2 g.L<sup>-1</sup> lactose, 5 mg.L<sup>-1</sup> bromocresol purple, 20 mg.L<sup>-1</sup> amphotericin B, and 40 mg.L<sup>-1</sup> nalidixic acid. All media were incubated at 30 °C for 72 h.

After incubation, a representative number of colonies (at least the square root of the cfu count per plate) were randomly taken from each L-M17 plate and colonies were purified by successive subcultures on the same medium. All isolates were checked for Gram reaction and catalase activity. The Gram-positive and catalase-negative isolates were further studied. Twelve commercial strains of *L. lactis* from two starters (S1 (five) and S3 (seven)) commonly used in the Ossau-Iraty cheese-making were included in the study (Table 1). All LABs were stored frozen at -80 °C in cryotubes (AES, Combourg, France). These frozen stocks were used for further typing and identification.

### 2.3 *L. lactis* screening, genotypic typing, and quantification

After rapid DNA extraction (Gaya et al. 1999), a first screening of commercial and indigenous LAB isolates were performed by PCR amplification of repetitive bacterial DNA elements (Rep-PCR) as described by Berthier et al. (2001). REP1R-Dt/REP2-D primers were first used to screen *L. lactis* strains. ERIC1R/ERIC2 primers (Versalovic et al. 1991) were subsequently used to improve the genotypic fingerprinting discrimination of the *L. lactis* isolates. The reference strains *L. lactis* subsp. *lactis* CECT 185T; *L. lactis* subsp. *lactis* biovar *diacetylactis* DSMZ 20661T; and *L. lactis* subsp. *cremoris* ATCC 9596, DSMZ 20069T, and ATCC 11603 were used for comparison.

After electrophoresis and gel scan, GelCompar II<sup>®</sup> software version 4.0 (Applied Maths, Kortrijk, Belgium) was used to calculate the similarity between the band profiles, based on the Pearson correlation coefficient. A dendrogram was deduced from the matrix of similarities using an arithmetic average clustering algorithm (UPGMA: Unweighted Pair Group Method with Arithmetic Averages). Rep-PCR fingerprints with a similarity coefficient  $\geq 85\%$  and visually identical were deemed to be fingerprints of the same strain (Feutry et al. 2011) and permitted representative strains to be designated. Specific PCR reactions (Table 2) was performed with DNA from one representative strain of each Rep-PCR cluster in order to identify the presumed *L. lactis* subspecies and biovar. When isolates were grouped with a reference strain, specific primers amplifying DNA from that reference strain were first used. The levels of *L. lactis* in the milks were estimated by multiplying the

**Table 1** *Lactococcus lactis* strains isolated from two commercial starters, S1 and S3, used in Ossau-Iraty cheese manufacture

	Commercial starters	
Species and subspecies	S1	S3
<i>L. lactis</i> subsp. <i>lactis</i>	L1, L8, L14	L324, L326, L3210
<i>L. lactis</i> subsp. <i>cremoris</i>	C3	C322, C328, C3212
<i>L. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i>	D15	D5

Each strain corresponds to normalized Rep and Eric-PCR profiles analyzed using Pearson's product-moment correlation coefficient and clustered by UPGMA

**Table 2** Specific primer pairs, amplification cycles, and expected size of PCR products

PCR specificity	Primers	Primer sequence (5'-3')	Amplification cycle	PCR product size (pb)	Reference
<i>L. lactis</i>	Lla	CAGTCGGTACAAG TACCAAC	35×[30 s/94 °C, 45 s/55 °C, 1 min/72 °C]	87	Barakat et al. (2000)
	27f	AGAGTTTGATCMTG GCTCAG			
<i>L. lactis</i> subsp. <i>lactis</i>	LLhis3F	AAAGAATTTTCAGA GAAA	30×[1 min/94 °C, 1 min/58 °C, 90 s/72 °C]	343	Beimfohr et al. (1997)
	LLhis4R	ATTTAGAATTGGTT CAAC			
<i>L. lactis</i> subsp. <i>cremoris</i>	LLchis1F	GCGCTGAATTTAC CTGAC	30×[30 s/94 °C, 1 min/54 °C, 2 min/72 °C]	556	Beimfohr et al. (1997)
	LLchis2R	TTCGCGCACCGC CGTC			
<i>L. lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i>	Lhis5F	CTTCGTTATGATT TTACA	30×[30 s/94 °C, 1 min/46 °C, 2 min/72 °C]	934	Beimfohr et al. (1997)
	Lhis6R	AATATCAACAATT CCATG			

percentage of L-M17 isolates identified as *L. lactis* by the number of bacteria counted from L-M17 as described by Depouilly et al. (2004). The proportion of each *L. lactis* subspecies in each sub-area was calculated by dividing the number of isolates of that subspecies by the total number of identified *L. lactis* isolates. The intraspecies diversity of *L. lactis* was assessed by dividing the number of genotypic profiles by the total number of identified *L. lactis* isolates.

## 2.4 Technological characterization

Technological properties were evaluated for one representative autochthonous *L. lactis* strain of each main cluster (see Section 2.3) and for the 12 commercial *L. lactis* strains (see Section 2.2).

### 2.4.1 Proteolytic ability assessment

Although no proteolytic ability was measured, proteolytic ability was assessed by studying the presence of the gene coding for the cell envelope proteinase (Prt). It was detected by specific PCR using the primer set P6: 5'-AACACGGCATGCATGTTGC-3'/P7: 5'-CTGGCGTTCACCATTCA-3' as described by Klijn et al. (1995). The expected size of the PCR product was 393 bp. The reference strain, *L. lactis* subsp. *cremoris* DSMZ 4367, was used as positive control.

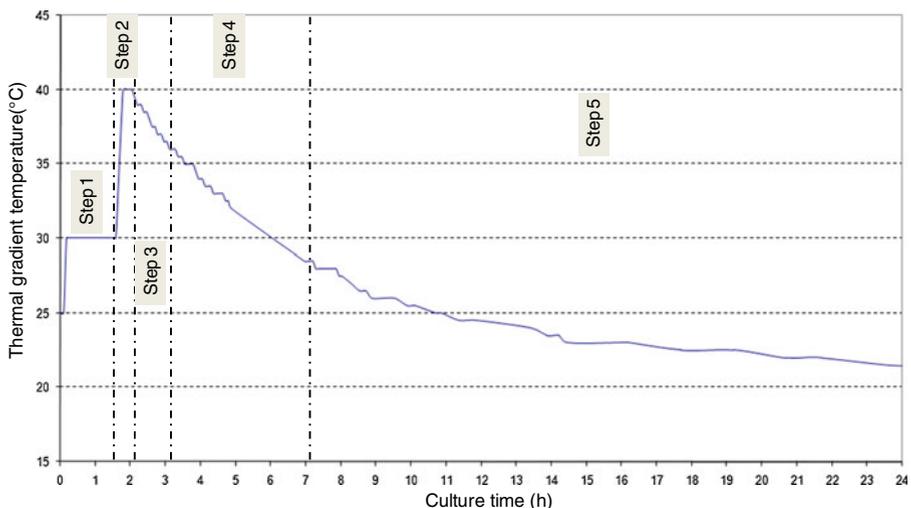
### 2.4.2 Acidifying activity

The acidifying activity of the strains was evaluated twice by pH measurements on reconstituted ewe's milk powder (Udipal, Onet-le-Château, France). The milk was

reconstituted at 10% (w/v) with sterile water and then pasteurized at 63 °C for 30 min. The protocol described by Casalta et al. (1995) was used. Two subcultures of each strain were carried out in pasteurized (118 °C, 18 min) skim cow's milk (Difco, Spain) at 30 °C, first for 24 h and then for 16 h to reach  $10^9$  cfu.mL<sup>-1</sup>. Two milliliters of this final culture were inoculated into 198 mL of ewe's milk at 25 °C. The thermal gradient commonly used for making Ossau-Iraty cheese was used to evaluate the behavior of all the tested strains (Fig. 1): the temperature was increased to 30 °C (in 5 min) and kept constant for 1 h 30 min (step 1). Then it was increased progressively (1 °C/2 min) to 40 °C and kept constant for 20 min (step 2). Heating was then stopped. The temperature decreased gradually to 20 °C over 24 h, simulating curd settling, molding (step 3), pressing (step 4), and draining (step 5). The pH and temperature were then measured by electrodes (FC210B, Hanna Instruments, Eibar, Spain) previously washed with a pepsine/HCl solution (HI387, Hanna Instruments) and disinfected (2 min in sodium hypochlorite 1%). The electrodes were linked to a transmitter/receptor system (Labguard, AES, Combours, France). The Temperanet 4.1.d Com2 software (AES, Combours, France) recorded pH and temperature data at 1-min intervals for 24 h. The pH differences ( $\Delta$  pH) at each step (Fig. 1) were calculated for the presumptive Prt<sup>+</sup> and Prt<sup>-</sup> autochthonous strains and for the commercial strains.

#### 2.4.3 Lysogeny and phage sensitivity

Strain lysogeny was detected by P335 prophage investigation using specific primers (P335A: 5'-GAAGCTAGGCGAATCAGTAA-3'/P335B: 5'-GATTGC-CATTGCGCTCTGA-3') as described by Labrie and Moineau (2000). Expected fragment size was 682 bp. The strain *L. lactis* ssp. *lactis* CECT 916 and phage type P335 were used as positive controls.



**Fig. 1** Thermal gradient used to simulate the Ossau-Iraty cheese process in reconstituted and pasteurized ewe's milk culture. *Step 1* addition of starter–renneting–coagulation–cutting (1.5 h; end at 1.6 h). *Step 2* heating/stirring (40 min, 1 °C/2 min; end at 2.3 h). *Step 3* molding (50 min; end at 3.1 h). *Step 4* pressing (4 h; end at 7.1 h). *Step 5* draining (~17 h; end at 24 h)

Strains were tested twice for phage sensitivity against 23 collection phages (Christian Hansen Laboratorium, Horsholm, Denmark; Oviedo University, Spain) and 21 wheys (W1 to W21) collected from the Ossau-Iraty cheese area and potentially contaminated by phages. The double-layer method (Terzaghi and Sandine 1975) was used: individual strains were mixed with M17 semisolid agar containing 0.2% CaCl<sub>2</sub> and overlaid onto M17 agar plates. Phage and whey preparations were spotted onto the agar and examined for lysis plaques after overnight incubation at 30 °C.

#### 2.4.4 Compatibility of strains

To screen for antimicrobial activity, 43 wild strains (each one representative of a genotypic profile; see Section 2.3) which were non-lysogenic and resistant to phages from the collection and wheys (see 2.4.3) were assayed against each other by evaluating the resulting acidification after co-inoculation of two strains in similar proportions. The method described for individual strains was used (see 2.4.2).

### 2.5 Statistical analysis

Statistical analysis consisted of determination of the mean, minimum, maximum, and 75th percentile values. One-way analysis of variance (ANOVA) was applied to the results of *L. lactis* subspecies counts to evaluate the effect of sub-area. ANOVA was also applied to the  $\Delta \text{pH}_{24\text{h}}$  and  $\Delta \text{pH}$  (pH  $n$  step – pH  $n+1$  step) obtained after each step of the thermal gradient of Ossau-Iraty cheese-making as defined in Fig. 1, to differentiate the strains group according to strain origin and the presence or absence of the Prt gene. For this, the SPSS software package ver. 15.0 (SPSS Inc., Chicago, IL., USA) was used.

## 3 Results and discussion

### 3.1 *L. lactis* presence and levels in ewe's milks

Five hundred and ninety-three isolates were collected from L-M17 plates. Among them, 392 (66%) were identified as LAB. LABs were detected in all the milks and represented between 10 and 100% of the isolates collected. Species-specific PCR identified 180 of the 392 LAB as *L. lactis*. However, despite the systematic presence of LABs and total counts ranging from 4.4 to 5.5 log<sub>10</sub> cfu.mL<sup>-1</sup>, *L. lactis* was detected in milk samples from only 20 farms (62%; Table 3). Where detected, levels of *L. lactis* were 1 log<sub>10</sub> lower than the total viable counts. *L. lactis* levels varied between 1.9 and 5.2 log<sub>10</sub> cfu.mL<sup>-1</sup>, as observed in other ewe's milks (Casalta et al. 2001). In 75% of the milks levels did not exceed 3.9 log<sub>10</sub> cfu.mL<sup>-1</sup>.

Contrary to our results, Corroler et al. (1998) detected *L. lactis* in 100% of cow's milks. Few studies on ewe's milk allow a comparison with our results to be performed. Feutry et al. (2011) recently observed that *L. lactis* was only detected in a third of raw ewe's milks at levels of <2 log<sub>10</sub> cfu.mL<sup>-1</sup>. In that study, the authors suggested that cold storage of milk (4 °C for 48 h) might have favored a decrease in lactococci, as suggested by Lafarge et al. (2004). However, Medina et al. (2001)

**Table 3** Distribution of LAB and *Lactococcus lactis* subspecies in ewe's milks from three Ossau-Iraty cheese production sub-areas (A, B, and C)

	Sub-area		
	A	B	C
No. of farm	1–10	11–20	21–32
Number of milk samples	10	10	12
Number of milk samples containing LABs	10	10	12
Number of milk samples containing <i>L. lactis</i> <sup>a</sup>	7	9	4
Total number of isolates	203	208	182
Number of LABs isolates from M17L (%)	107 (52.7)	149 (71.6)	111 (61.0)
Number of <i>L. lactis</i> isolated from M17L (%)	49 (24.1)	82 (39.4)	49 (26.9)
Number of subsp. <i>lactis</i> isolates (% over total number of <i>L. lactis</i> )	43 (87.8)	57 (69.5)	19 (38.8)
Number of subsp. <i>cremoris</i> isolates (% over total number of <i>L. lactis</i> )	6 (12.2)	25 (30.5)	30 (61.2)
Total viable counts ( $\log_{10}$ cfu.mL <sup>-1</sup> ± SD)	4.4±0.3	5.5±0.7	5.2±0.6
<i>L. lactis</i> counts ( $\log_{10}$ cfu/mL <sup>-1</sup> ) <sup>a</sup>			
Mean	3.6	4.3	4.4
Minimum	1.9	2.0	3.0
Maximum	4.0	5.2	4.8
75th percentile	3.8	3.9	4.5
Number of <i>L. lactis</i> subsp. <i>lactis</i> genotypic profiles (see Fig. 2)	14	8	4
Number of <i>L. lactis</i> subsp. <i>cremoris</i> genotypic profiles (see Fig. 2)	5	8	5

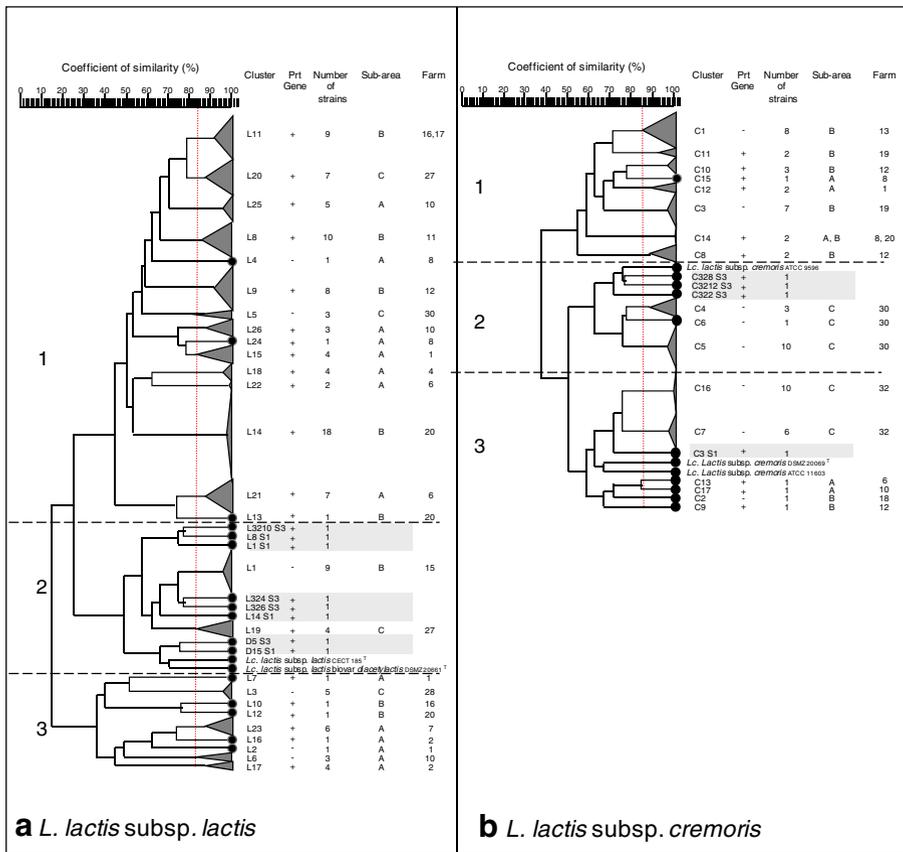
<sup>a</sup> Confirmed by specific PCR

showed, by phenotypic identification methods, that lactobacilli and enterococci dominated in Argentinean ewe's milks. Those studies also found that lactococci were not systematically present in ewe's milk.

Among the *L. lactis* identified, the genotype of subspecies *lactis* was represented twice as often (119 isolates, 66%) as the *cremoris* genotype (61 isolates, 34%). Moreover, in 10 milk samples, only the *lactis* genotype was detected. No isolate belonging to the diacetylactis biovar. was identified. Feutry et al. (2011) also found no diacetylactis biovar in ewe's milk samples. The dominance of the *L. lactis* subsp. *lactis* genotype has also been reported in raw cow's milk (Desmasures et al. 1998) and from fresh ewe's cheeses (Gaya et al. 1999). The *L. lactis* subsp. *lactis* biovar diacetylactis genotype has been identified in fresh ewe's cheeses (Gaya et al. 1999). Regarding the proportion of *cremoris* genotype in this study, it was comparable to that obtained by Desmasures et al. (1998) from cow's milk samples, with the *cremoris* genotype representing 37.5% of the isolates.

### 3.2 Genotypic diversity of strains of *L. lactis*

The combination of the two fingerprints generated by the REP1R-Dt/REP2-D and ERIC1R/ERIC2 primers grouped the 180 autochthonous *L. lactis* strains in 43 profiles of which 26 were *L. lactis* subsp. *lactis* (Fig. 2a) and 17 were *L. lactis* subsp. *cremoris* (Fig. 2b).



**Fig. 2** Clustering of 180 wild *L. lactis* strains from 32 from Ossau-Iraty ewe's milks samples collected in three sub-areas A (1–10), B (11–20), and C (21–32), and 12 commercial strains from two starters, S1 and S3 (square). Normalized Rep and Eric-PCR patterns were analyzed using Pearson's product–moment correlation coefficient and were clustered by UPGMA; **a** 119 wild *L. lactis* subsp. *lactis* strains, two reference strains, and eight commercial strains: four from starter S1 and four from starter S3 (see Table 1); **b** 61 wild *L. lactis* subsp. *cremoris* strains, three reference strains, and four commercial strains: one from starter S1 and three from starter S3 (see Table 1)

Among the 26 *L. lactis* subsp. *lactis* profiles (Fig. 2a), only eight strains were represented by one strain while 2 to 18 strains were grouped at a similarity level of between 85 and 98%. Three main clusters appeared with a similarity coefficient below 25%. Clusters 1 and 3 only grouped autochthonous strains. Cluster 2 was comprised of all the commercial and reference strains. Only two profiles (L1, L19), represented by 13 autochthonous *L. lactis* subsp. *lactis*, were present in this cluster. They grouped with some commercial strains at similarity coefficients of between 62 and 75%.

Among the 17 *L. lactis* subsp. *cremoris* profiles (Fig. 2b), six were represented by only one strain while the others included two to 10 isolates exhibiting similarity coefficients between 85 and 98%. The dendrogram shows three main clusters with a similarity coefficient of about 35%. Nine profiles represented by 34 autochthonous *L. lactis* subsp. *cremoris* were present in clusters 2 and 3. They grouped with some commercial strains with similarity coefficients below 65%. Cluster 1 only grouped autochthonous strains. With the exception of strain C3 from

commercial starter S1 which was in cluster 3, the rest of the commercial strains were located in cluster 2.

Genotypic profile diversity was higher for the autochthonous wild strains than for the commercial ones, as observed in others studies (Corroler et al. 1998; Sánchez et al. 2000). Intra-subspecies diversity calculated from number of genotypes/number of isolates belonging to each subspecies was slightly greater for the *cremoris* genotype (28%) than for the *lactis* genotype (22%), as observed by Gaya et al. (1999) in fresh ewe's cheeses. Conversely, Corroler et al. (1998) showed a greater diversity for the *lactis* genotype isolated from raw cow's milk.

### 3.3 Geographic distribution of *L. lactis*

*L. lactis* subsp. *lactis* strains were scarce in the C sub-area (38.8%) compared to the A (87.8%) and B (69.5%) sub-areas while the proportion of *cremoris* genotype was two and three times greater than in the A and B sub-areas, respectively (Table 3). However, ANOVA did not reveal any significant relationship between the percentage of the two subspecies and the origin of the milk samples. This finding agrees with the observations published by Oneca et al. (2003) on wild *Lactobacillus plantarum* populations of raw milks produced in the Roncal PDO area but disagrees with those of Corroler et al. (1998) who reported that 80% of isolates from the Bocage Falaisien area in Normandy were members of subsp. *lactis* genotype, while 70% of the Bessin isolates were members of subsp. *cremoris* genotype, farm of origin being probably more significant than area.

Although *L. lactis* mean levels were similar in the B and C sub-areas, the C sub-area had the fewest milks containing *L. lactis* strains, though it is also the sub-area where 75% of the milk samples had the highest levels of *L. lactis*. Indeed, 75% of the milk samples contained between 3.0 and 4.5  $\log_{10}$  cfu.mL<sup>-1</sup>, whereas levels were about 1  $\log_{10}$  lower in the other two sub-areas (Table 3).

Each of the 20 milk samples containing *L. lactis* included one to four distinct strains (Fig. 2a, b). Each profile corresponded to one farm except in the clusters L11 and C14. Greater strain diversity (number of *L. lactis* profiles/total number of *L. lactis* isolates) was found in sub-area A (38.8%) than in B (19.5%) or C (18.4%; Table 3).

### 3.4 Technological characterization

#### 3.4.1 Proteolytic ability assessment

The cell envelope proteinase gene (Prt) was detected in 29 (67%) out of the 43 representative autochthonous strains: 20 *L. lactis* subsp. *lactis* and nine *L. lactis* subsp. *cremoris*. The proportion of Prt<sup>-</sup> strains belonging to the *cremoris* genotype (8/17) was twice as high (47%) as for the *lactis* genotype (6/26; 23%). All the *L. lactis* commercial strains were Prt<sup>+</sup>.

#### 3.4.2 Acidifying activity

The initial mean pH value of the ewe's milk was 6.70±0.02 U. Table 4 indicates the  $\Delta$  pH<sub>24h</sub> and the  $\Delta$  pH (pH *n* step - pH *n*+1 step) obtained after each step of the thermal gradient of Ossau-Iraty cheese-making as defined in Fig. 1 for the 43 indigenous *L.*

**Table 4** Mean  $\pm$  standard deviation (2 measurements for each strain) of the  $\Delta$  pH caused by indigenous  $\text{Prt}^+$  and  $\text{Prt}^-$  *L. lactis* strains (OI) and by the S1 and S3 commercial strains at the end of each step (see Fig. 1) of the 24-hours incubation simulating the thermal gradient of Ossau-Iraty cheese manufacture

	Strains origin				P
	OI		S1	S3	
Prt gene	$\text{Prt}^-$ (n=14)	$\text{Prt}^+$ (n=29)	$\text{Prt}^+$ (n=5)	$\text{Prt}^+$ (n=7)	
Step 1 ( $\Delta$ pH <sub>0h-1.6h</sub> )	0.02 $\pm$ 0.02	0.01 $\pm$ 0.02	0.01 $\pm$ 0.02	0.02 $\pm$ 0.00	NS
Step 2 ( $\Delta$ pH <sub>1.6h-2.3h</sub> )	0.02 $\pm$ 0.02 <sup>a</sup>	0.10 $\pm$ 0.06 <sup>b</sup>	0.09 $\pm$ 0.05 <sup>b</sup>	0.09 $\pm$ 0.08 <sup>b</sup>	***
Step 3 ( $\Delta$ pH <sub>2.3h-3.1h</sub> )	0.07 $\pm$ 0.00 <sup>a</sup>	0.16 $\pm$ 0.00 <sup>b</sup>	0.22 $\pm$ 0.00 <sup>b</sup>	0.14 $\pm$ 0.01 <sup>b</sup>	***
Step 4 ( $\Delta$ pH <sub>3.1h-7.1h</sub> )	0.28 $\pm$ 0.12 <sup>a</sup>	1.11 $\pm$ 0.43 <sup>b</sup>	0.88 $\pm$ 0.56 <sup>b</sup>	0.84 $\pm$ 0.67 <sup>b</sup>	***
Step 5 ( $\Delta$ pH <sub>7.1h-24h</sub> )	0.51 $\pm$ 0.15	0.54 $\pm$ 0.31	0.60 $\pm$ 0.73	0.57 $\pm$ 0.44	NS
$\Delta$ pH <sub>0h-24h</sub>	1.06 $\pm$ 0.19 <sup>a</sup>	2.18 $\pm$ 0.42 <sup>b</sup>	1.94 $\pm$ 0.67 <sup>b</sup>	1.89 $\pm$ 0.86 <sup>b</sup>	***

Prt : Protease gene;  $\text{Prt}^-$  : absence;  $\text{Prt}^+$  : presence

Different letters in the same row indicate significant differences between the average values

NS not significant; \*\*\* $P$ <0.001

*lactis* strains grouped according the presence of Prt gene and the 12 commercial strains according to the starter type (S1 or S3).

Regarding  $\Delta$  pH<sub>24h</sub> values, the  $\text{Prt}^+$  strains were significantly more acidifying ( $p$ < 0.001) than  $\text{Prt}^-$  strains (Table 4). The indigenous  $\text{Prt}^+$  strains led to a  $\Delta$  pH<sub>24h</sub> twofold higher than the  $\text{Prt}^-$  strains, probably because there is a good correlation between acidifying and proteolytic activities as observed by Bruinenberg et al. (1992). In our study, only the presence of the cell envelope proteinase gene was investigated, not its expression or its expression level.

The highest  $\Delta$  pH was observed at the end of the step 4 (Table 4), after around 7 h of incubation, for all the  $\text{Prt}^+$  strains, when the temperature was favorable (below 37 °C) to the growth of lactococci, as observed by Pérez-Elortondo et al. (1993) during Idiazábal cheese-making, which is technologically similar to Ossau-Iraty cheese. The  $\Delta$  pH caused by the  $\text{Prt}^-$  strains group at the end of the step 5 was twice as high than at the end of the step 4 (Table 4). In our study, the  $\text{Prt}^+$  strains were significantly ( $p$ <0.001) differentiated from  $\text{Prt}^-$  strains at the end of the steps 2, 3, and 4. At the end of the step 4, the  $\Delta$  pH caused by the  $\text{Prt}^+$  strains was three to four times greater than the decrease due to  $\text{Prt}^-$  strains. Moreover, the decrease in pH during this thermal gradient cycle showed that a temperature of 40 °C (step 2) did not prevent expression of the acidifying activity of the tested *L. lactis* strains, as also observed by Jeanson et al. (2003). No significant differentiation was observed between the indigenous and commercial  $\text{Prt}^+$  strains. The increase in standard deviation throughout the cheese-making steps demonstrates the diversity existing within each strain group, especially when the temperature decreased from 37 to 28 °C (step 4). Under temperature conditions similar to those of non-cooked (Casalta et al. 1995) and cooked (Jeanson et al. 2003) cheese, the wild *L. lactis* strains also exhibited a wide diversity of acidifying activity, showing that they differed in their temperature sensitivity. The most acidifying strains belonged to the *L. lactis* subsp. *lactis* genotype (data not

shown) but some strains belonging to the *cremoris* genotype were also good acidification agents ( $\Delta \text{pH}_{24\text{h}} > 2$ ). Acidifying capacity is more strain dependent than subspecies dependent, as shown by Sánchez et al. (2000). Unlike the results of Mannu et al. (2000), in this study no correlation was established between strain genotypic profiles and acidifying activity. However, those authors did not use the same genotypic methods as in this study to distinguish between strains.

### 3.4.3 Lysogeny and phage sensitivity

Lysogeny and phage sensitivity as employed by Ward et al. (2004) to characterize a group of closely related lactococcal strains, and proved to be the best criteria for distinguishing the commercial and autochthonous strains and to characterize a group of closely related lactococcal strains.

**Table 5** Pattern of lysogeny and phage sensitivity among wild (OI) and commercial (S1, S3) *Lactococcus lactis* strains

		Strain origin		
		OI	S1	S3
Collection phages	c2	C12, L25		
	P335	L26		<u>C328</u>
	971	L18, L20	L14	
	973	C2		L324
	974	L2, L5, L17		L324, L326
	122	C3, C5, C12, L2, L5, L6, L9, L10, L11, L13, L18, L21, L23		
	134	C2, C5, C12, C13, L5		
Wheys	W1	L26	<u>D15</u>	<u>C328</u>
	W2			<u>C328</u>
	W4			<u>D5</u>
	W5			<u>D5</u>
	W6			<u>C328</u> , C3212
	W7			<u>C328</u> , C3212
	W8			<u>D5</u>
	W9			<u>C328</u>
	W11			<u>C328</u>
	W14			<u>D5</u>
	W16	L11		
	W20			C322, <u>C328</u> , C3212, L3210, <u>D5</u>
	W21		<u>D15</u>	C3212, L3210

Lysogenic strains were underlined

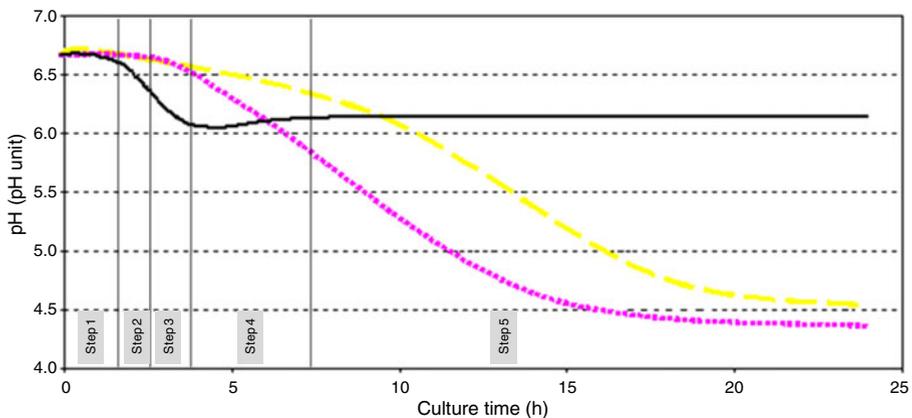
OI: Ossau-Iraty, S1 and S3: commercial starters, C: *L. lactis* subsp. *cremoris* genotype, L: *L. lactis* subsp. *lactis* genotype, D: *L. lactis* subsp. *lactis* biovar. *diacetylactis* genotype

The lysogenic strains were: among the indigenous strains, one *L. lactis* subsp. *lactis* strain (OI L19; Fig. 2a) and four *L. lactis* subsp. *cremoris* strains (OI C4, C7, C11, and C16; Fig. 2b) and among the commercial strains, one *L. lactis* subsp. *lactis* genotype (S1 L1), one *L. lactis* subsp. *cremoris* genotype (S3 C328), and two *L. lactis* subsp. *lactis* biovar. *diacetylactis* (S1 D15 and S3 D5; Table 5). The proportion of positive PCR P335 phage amplification was greater (16.4%) in wild *L. lactis* from Cabrales cheese (Martín et al. 2006). The presence of lysogenic strains in commercial starters has been shown a long time ago (Reiter and Kirikova 1976). Ayad et al. (2000) also showed that most commercial strains were lysogenic, in contrast to indigenous strains.

Seven collection phages out of the 23 tested and 13 wheys out of the 21 tested caused lysis plaques (Table 5). The phagic sensitivity of the commercial and autochthonous strains was inversely correlated with the origin of the tested phages. Indeed, 60% of the commercial strains were sensitive to the phages from wheys collected from dairies using these commercial strains, compared to only 5% of the autochthonous strains. Conversely, around 50% of the autochthonous strains were sensitive to one or several collection phages compared to 33% for the commercial strains. These results agree with those obtained by Ayad et al. (2000) who showed that autochthonous strains of *L. lactis* were particularly resistant to the phages from local wheys and therefore of great interest for manufacturing a specific starter for cheese-making. The sensitivity of commercial strains to phages from dairy wheys has also been noted by De Godoy Oriani and Yokoya (2004).

### 3.4.4 Compatibility of strains

Among the 171 tested combinations, those implicating the OI L12 strain involved an abrupt stop of the pH decrease after approximately 2 h of incubation (Fig. 3), compared to the acidification curve observed with each individual strain. The other combinations (data not shown) did not show any incompatibility. The reported percentage of



**Fig. 3** pH kinetics of some *L. lactis* strains in reconstituted and pasteurized ewe's milk culture under the Ossau-Iraty cheese thermal gradient (see Fig. 1). (■ ■ ■) pH kinetics of OI L12 strain; (— — —) pH kinetics of OI L7 strain; (· · ·) pH kinetics of the two strains in combination. The curves are averaged from the duplicates of each culture

strains (2.3% in this study) expressing antimicrobial activity varies between studies: Ayad et al. (2004) observed that 47% of wild strains of *L. lactis* expressed antimicrobial activity against strains belonging to the same species whereas Sánchez et al. (2000) found no incompatibility. It is noteworthy that if an antimicrobial compound was produced, it caused the L12 acidifying activity to stop at the same time.

## 4 Conclusions

This study gives a first overview of the quantitative, genotypic, and technological variability of indigenous strains of *L. lactis* from ewes' milk samples produced in the Ossau-Iraty cheese area. Strains of *L. lactis* were not always detected in the ewe's milk samples. However, when they were present, they displayed a wide genotypic diversity which seemed to be strongly dependent on the farm of origin. This suggests that it would be useful to study how the farm dairy environment and stock husbandry practices and conditions favor the presence and conservation of this microbiota.

The results also show that the *L. lactis* populations present in Ossau-Iraty ewe's milk have a high technological potential. Since the discrepancy between the phenotypic and the genotypic characteristics of the two subspecies *lactis* and *cremoris* has been reported (Corroler et al. 1998), identification based on the classical distinction criteria will be checked in a forthcoming study to ascribe the "*cremoris*" or "*lactis*" phenotype to the genotypic wild strains of *L. lactis*. Indeed, a phenotypic *L. lactis* subsp. *lactis* showing a *L. lactis* subsp. *cremoris* genotype and inversely a phenotypic *L. lactis* subsp. *cremoris* showing a *L. lactis* subsp. *lactis* genotype have been reported (Fernández et al. 2011). Phenotypic differences between genotypically similar strains of *L. lactis* have also been found, probably linked to differences in enzymatic expression (de la Plaza et al. 2006).

This preliminary study of *L. lactis* microbiota revealed its complexity and suggests that a deeper knowledge is needed to select strains with the best properties for use in starter cultures in the manufacture of Ossau-Iraty cheese and to understand the impact of this microbiota on cheese characteristics.

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