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Diversity of lactic acid bacteria isolated from AOC Salers cheese

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The objective of this work was to describe the diversity of lactic acid bacteria in traditional raw milk Salers cheeses at the species and strain levels. The characterization of 381 strains isolated during ripening and various strain collections was investigated using physiological analysis and molecular techniques: Rep-PCR, species and genus specific amplifications and the sequence analysis of 16S rDNA for strain typing and taxonomic identification. The strains belonged to *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactococcus lactis*, *Lactococcus garviae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Streptococcus salivarius*, *Streptococcus millieri*, *Streptococcus macedonicus* and *Pediococcus pentosaceus*. A wide phenotypic and genomic heterogeneity was observed within the different species (*Lactobacillus plantarum*, *Lactobacillus paracasei* and *Leuconostoc mesenteroides*) according to the origin and the time of ripening. The natural microflora was different from strain collection and each method must be combined to identify and characterize natural microflora. This study revealed the low selectivity of selective media used for the isolation of different groups of lactic acid bacteria except the Facultatively Heterofermentative lactobacilli medium selecting mesophile lactobacilli and SB medium selective for *Enterococcus*. The study reveals, for the first time, the microbial lactic acid bacteria community of Salers cheese and its diversity. A better knowledge of microbial flora will be useful to improve understanding of sensory quality of cheeses.

Keywords: Lactic acid bacteria, Salers cheeses, characterization, diversity.

The traditional cheeses manufactured with raw milk exhibit complex and varied sensory properties with regard to their complex microbial community (Cogan et al. 1997). The micro-organisms may be involved to a significant extent in the curd ripening process in order to be sure to obtain the typical taste and aroma of the final product and their hygienic properties. To better understand the role of microbial flora in the development of sensorial qualities of cheeses, the description of the microbial ecosystem at species and strain level and the biochemical properties must be determined. The conventional approach for describing microbial diversity consists in the enumeration of microbial groups on various culture media, followed by the identification of dominant micro-organisms by taxonomic and/or phylogenetic methods. The microbial community of raw milk cheeses can be constituted of lactic acid bacteria and other Gram positive belonging to *Staphylococcus*, *Corynebacteria*, *Arthrobacter* (Irlinger et al. 1999), Gram negative bacteria and yeasts. Lactic acid bacteria are the most widely studied and several methods have been developed to identify them. Identification by phenotypic features is time-consuming and not always accurate. As a result, molecular methods based on nucleic acid analysis are being more and more widely applied. Isolates can be identified at species level by DNA–DNA hybridization, species specific PCR, plasmid profiling and 16S rDNA sequencing. Species PCR with specific primers was used for the identification and comparison of lactobacilli (Ward et al. 1999; Berthier et al. 2001; Guarneri et al. 2001) or *Streptococcus salivarius* subsp. *thermophilus* (ex *Str. thermophilus*) (Giraffa et al. 2001; Moschetti et al. 2001). A DNA probe was used to discriminate *Str. salivarius* (Igarashi et al. 2001). rDNA sequence analysis has been used for the taxonomic identification of lactic acid bacteria from dairy products (Cocconcelli et al. 1997; Monstein et al. 1998). Some methods based on PCR have been developed to reveal intra-specific differences in bacterial genomes. Randomly amplified polymorphic DNA (RAPD) PCR is widely used as a genotypic typing technique. RAPD was used to characterize lactic acid bacteria isolated from Mozzarella (Morea et al. 1999), or Italian Ewe cheeses (De Angelis, 2001), *Enterococcus* in Pecorino Sardo cheese (Mannu et al. 1999), lactobacilli (Andrighetto et al.

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1998; Quiberoni et al. 1998) and lactic acid bacteria in Cheddar (Fitzsimons et al. 1999). The amplification of 16S rDNA fragments followed by restriction analysis (ARDRA) was used to discriminate lactobacilli (Roy et al. 2000) and lactococci (Desmasures et al. 1998). PCR amplification of repetitive bacterial DNA elements fingerprinting was proven to be useful for differentiating a wide range of lactobacilli at the species, subspecies and potentially up to the strain level (Berthier et al. 2001; Gevers et al. 2001).

The purpose of this study was to evaluate the diversity of lactic acid bacteria isolated from Salers cheese by a combination of different molecular methods such as Rep-PCR, species-specific PCR and sequencing 16S rDNA. Salers cheese is a non-cooked semi-hard cheese variety, ripened for 3 months or more, manufactured from raw cows' milk in a limited area in the centre of France, and labelled Appellation d'Origine Contrôlée (AOC). It must be made only from the milk of cows that graze on mountain pastures in summer. Salers cheese is produced exclusively with raw milk collected in a 'gerle' (wood container), which allows a natural microbial flora enrichment and may contribute a specific flora. The lactic acid bacteria of this cheese has never been studied unlike the exhaustive studies on Comté (Berthier et al. 2001), Mozzarella (Hatzikamari et al. 1999; Morea et al. 1999), Camembert (Desmasures et al. 1998) and goat milk cheese in Chile (Prado et al. 2001).

Materials and Methods

Cheese samples

The cheese samples used in this study were obtained from three farms (A, B, C) which produce AOC Salers cheese with traditional technologies. The farms were located in different areas of production and selected as their cheeses presented different sensorial qualities. Milk was collected in a wooden recipient and was neither heated nor refrigerated after the milking. No starter cultures were added. Raw milk was coagulated by adding calf rennet between 31·3 °C and 33·6 °C. Curd was cut into tiny pieces and pressed between 1 h 15 min and 1 h 50 min to remove whey. The dry curd was matured for between 18 h 15 min and 23 h 15 min. The curd was milled and salt at 2·2–2·3% was added. Further maturation occurred during storage for 3 h 30 min to 4 h 25 min at 16–18 °C. Then the salted curd was filled into the hoops for pressing. Cheeses were pressed at 12–15 °C for between 41 h 15 min and 48 h 50 min and turned several times. The cheeses were then ripened during 5 months in the right temperature conditions between 10 °C and 15 °C and a relative humidity of 97%. Variations of technological parameters according to the farm production are indicated in Table 1.

Samples were taken for microbiological analysis at different interval times during milking, cheese making and ripening.

Table 1. Variability of technical parameters during the production of Salers cheese by the three producers at farms A, B and C

	Farm A	Farm B	Farm C
Coagulation†	33·6 °C	31·3 °C	32·9 °C
Pressing‡	1 h 40 min	1 h 15 min	1 h 50 min
Maturation‡	20 h 35 min	18 h 15 min	23 h 15 min
Salt§	23 g/kg	22 g/kg	23 g/kg
Salt maturation‡	3 h 30 min	4 h 25 min	3 h 55 min
Pressing‡	48 h 15 min	48 h 50 min	41 h 15 min

† Temperature, ‡ Duration, § Rate of addition

Bacteriological analysis

Lactic acid bacterial counts were carried out in milk and in cheeses at different times of ripening (1, 8, 30 and 150 d) on the following media: M17 agar (Terzaghi & Sandine, 1975) incubated at 42 °C for 48 h; M17 agar containing 2 g lactose/l, 5 mg bromocresol/l and 40 mg nalidixic acid/l) incubated at 30 °C for 48 h; MRS agar, pH 6·5 (De Man et al. 1960) incubated at 30 °C under aerobic conditions for 48 h, Facultatively Heterofermentative (FH) lactobacilli or FH agar medium (Isolini et al. 1990) incubated at 37 °C for 3 d under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany); Mayeux, Sandine & Elliker (MSE) agar medium incubated at 30 °C for 48 h; Slanetz and Bartley medium (SB) (Slanetz & Bartley, 1957) incubated at 42 °C for 48 h. All media were purchased from Biokar Diagnostics (Pantin, France).

Strain origin

Representative colonies (approximately ten isolates per count Petri dish) were picked out from these media. After purification, they were stored frozen at −20 °C: 381 strains were isolated from cheeses.

Twenty type reference strains and 29 other strains from different culture collections of the species usually found in dairy products were included in the study for reference. Details of species, origin and strain numbers are given in Table 2.

Phenotypic characterization of isolates

All isolates were checked for Gram reaction and catalase activity and examined microscopically. Growth in MRS broth was tested at 10 °C and 45 °C and after heat treatment at 60 °C for 30 min. Growth in MRS broth with 65 g NaCl/l and MRS broth at pH 4·4 was recorded. Gas production in MRS was determined according to Gibson & Abd-el-Malek (1945). Production of ammonium from arginine was evaluated by Nessler reaction in Niven medium modified by Hitchener et al. (1982).

DNA extraction

Total chromosomal DNA was extracted from 5 ml overnight culture on MRS broth, according to the phenol-chloroform

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Table 2. List of strains of *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Weissella* used as references in the different experiments for the identification of isolates from Salers cheeses

Species	Reference strains (^{T} type strains)
Lactobacillus paracasei	ATCC 334^T, CNRZ 62^T, CNRZ 763, CNRZ 383, DSM 4905, DSM 20023, DSM 20012, DSM 20006, DSM 20207
Lactobacillus paracasei subsp. *paratolerans*	LMG 9191^T
Lactobacillus plantarum	ATCC 14917^T CNRZ 738, CNRZ 424, CNRZ 1228, CNRZ 184, CNRZ 1246
Lactobacillus paraplantarum	CNRZ 1885^T, CNRZ 1886
Lactobacillus rhamnosus	LMG 6400^T, CNRZ 442, DSM 20711
Lactobacillus pentosus	CNRZ 1555, CNRZ 1570, CNRZ 1547, CNRZ 1537
Lactobacillus zeae	DSM 20178^T, CNRZ 313
Lactobacillus delbrueckii	CNRZ 225^T, CNRZ 207
Lactobacillus helveticus	ATCC 15009^T, CNRZ 65
Lactococcus lactis lactis	CNRZ 142^T, CNRZ 124, CNRZ 301, CNRZ 125, CNRZ 258, CNRZ 1075
Lactococcus lactis cremoris	1L8
Lactococcus plantarum	CNRZ 1322^T
Lactococcus raffinolactis	CNRZ 1214^T
Enterococcus faecium	CNRZ 2021^T
Enterococcus durans	CNRZ 129^T
Pediococcus acidilactici	LMG 10636
Pediococcus pentosaceus	LMG 10488^T
Leuconostoc mesenteroides mesenteroides	CNRZ 749^T
Leuconostoc mesenteroides dextranicum	CNRZ 77^T
Leuconostoc mesenteroides cremoris	CNRZ 361^T
Leuconostoc amelibiosum	CNRZ 2001^T
Weissella paramesenteroides	CNRZ 2004^T

ATCC: American Type Culture Collection, Rockville, Md, USA; LMG: Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; DSMZ: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; CNRZ: Centre National de Recherches Zootechniques, INRA Jouy-en-Josas, France

protocol described by Berthier et al. (1999). DNA extracted was precipitated with ethanol (Merk, Darmstadt, Germany) and lithium chloride (Merk), and resuspended in 100 µl 10 mM-Tris–HCl, pH 8·0 (Interchim, Paris, France), 1 mM-EDTA (Q-Biogene S.A., Illkirch, France). After treatment with 2 mg/ml RNAse (Sigma–Aldrich, Saint Quentin Fallavier, France), the quality of DNA extraction was visualized on agarose gel (8 g/l) in 0·5 × TBE buffer (0·45 mM-Tris–HCl, 0·45 mM-boric acid, 1 mM-EDTA, pH 8·0). The gels were stained with ethidium bromide and photographed on a UV trans-illuminator.

Rep-PCR

Primer sets Rep–1R-Dt (5′-NCGNCGNCATCNGGC-3′)/REP2-D (5′-NCGNCTTATCNGGCCTAC-3′) (Versalovic et al. 1991) were used for repetitive DNA sequence amplifications. PCR amplification was performed in a final volume of 20 µl containing 1 × PCR buffer with $MgCl_2$, 2 µl phenol extracted DNA, 1·0 mM-$MgCl_2$, 0·25 µM-REP1 and 0·25 µM-REP2 (Eurogentec France S.A., Angers, France), 200 µM each dNTP (Roche Molecular Biochemicals, Meylan, France) and 1 unit Taq DNA polymerase (Appligene Oncor). PCR reactions were carried out in a thermal cycler Gene Amp PCR system 9700 apparatus (PE Applied Biosystems, Courtaboeuf, France) programmed for 5 min at 94 °C and 30 cycles of amplification of 1 min at 94 °C, 1 min at 40 °C, 6 min ramping to 72 °C and 1 min at 72 °C. Electrophoresis was performed as described by Berthier et al. (1999). The densitometric traces of the band profiles were analysed with BioNumerics software version 2.5 (Applied Maths, Kortrijk, Belgium) and molecular sizes of different DNA fragments were calculated. Calculation of similarity between the band profiles was based on the Pearson correlation coefficient (*r*). A dendogram was deduced from the matrix of similarities by using an arithmetic average clustering algorithm (UPGMA: Unweighted Pair Group Method with arithmetic Average).

Species-specific PCR

Specific amplification of *Lactobacillus* (*Lb. plantarum*, *Lb. paracasei*, *Lb. pentosus*, *Lb. rhamnosus* and *Lb. curvatus*) was performed with the primers listed in Table 3. They were provided by Genosys Biotechnologies (Cambridge, UK). The primer 16 reverse was paired with primers paracasei 16S and rhamnosus 16S. The primer 16 forward was paired with primers Lc, Lpapl and Lpe specific for respectively *Lb. curvatus*, *Lb. plantarum* and *Lb. pentosus*. Standard reaction mixture of PCR was as described in the paragraph above and PCR reactions were carried out in following conditions: a denaturation 5 min at 94 °C and 30 cycles of amplification of 1 min at 94 °C, 0 min at 53 °C (primers 16S pairs, 16/Lc, 16/Lpapl), or 1 min at 53 °C

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Table 3. Sequences of the oligonucleotide primers used for species and genus-specific PCR amplifications and their specificity

Primer	Location/GenBank accession number	Oligonucleotide sequence (5′–3′)	Reference	Primer specificity
16 forward	16S rRNA gene, 5′ end, forward	GCTGGATCACCTCCTTTC	Berthier & Ehrlich (1999)	Universal
16 reverse	16S rRNA gene, 5′ end, reversed primer 16S	GAAGGAGGTGATCCAGC	Berthier & Ehrlich (1999)	Universal
Lc	16S/23S spacer region of *Lb. curvatus* DNA, reversed	TTGGTACTATTTAATTCTTAG	Berthier & Ehrlich (1999)	*Lb. curvatus*
Paracasei 16S	16S rRNA gene of *Lb. casei* ATCC 334, 5′ end, forward/d86517	CACCGAGATTCAACATGG	Ward et al. (1999)	*Lb. paracasei*
Lpapl	16S/23S spacer region of *Lb. paraplantarum* DNA, reversed	ATGAGGTATTCAACTTATT	Berthier & Ehrlich (1999)	*Lb. paraplantarum/plantarum*
Lpe	16S/23S spacer region of *Lb. pentosus* DNA, reversed	GTATTCAACTTATTAGAACG	Berthier & Ehrlich (1999)	*Lb. pentosus*
Rhamnosus 16S	16S rRNA gene of *Lac. rhamnosus* type strain, 5′ end, forward/d16552	TTGCATCTTGATTTAATTTTG	Berthier et al. (2001)	*Lb. rhamnosus*
Lnm1	*Esch. coli* 16S rRNA position 185 forward	TGTCGCATGACACAAAAGTTA	Brosius et al. (1978)	*Ln. mesenteroides*
Lnm2	*Esch. coli* 16S rRNA position 470 reversed	ATCATTTCCTATTCTAGCTG	″	*Ln. mesenteroides*
Lnl1	*Esch. coli* 16S rRNA position 457 forward	ATAGGGAATGATTCTAGTTC	″	*Leuconostoc lactis*
Lnl2	*Esch. coli* 16S rRNA position 1283 reversed	AGATTAGCTCACCCTCGCG	″	*Ln. lactis*
Lncit1	*Esch. coli* 16S rRNA position 183 forward	ACTTAGTATCGCATGATATC	″	*Leuconostoc citreum*
Lncit2	*Esch. coli* 16S rRNA position 1326 reversed	AGTCGAGTTGCAGACTGCAG	″	*Ln. citreum*
Wpm1	*Esch. coli* 16S rRNA position 163 forward	TACCGTATAATACCACAAC	″	*W. paramesenteroides*
Wpm2	*Esch. coli* 16S rRNA position 834 reversed	AACCCTCGAACATCTAGCAC	″	*W. paramesenteroides*
L1	*Esch. coli* 16S rRNA position 450 forward	AACTCTGTTGTTAGAG	″	*Lactococcus*
L2	*Esch. coli* 16S rRNA position 1021 reversed	ATCTCTAGGAATAGCAC	″	*Lactococcus*
E1	*Esch. coli* 16S rRNA position 632 forward	TCAACCGGGGAGGGT	″	*Enterococcus*
E2	*Esch. coli* 16S rRNA position 1353 reversed	ATTACTAGCGATTCCGG	″	*Enterococcus*

(16/Lpe) and 1 min at 72 °C, followed by 7 min at 72 °C. 10 µl were electrophoresed in agarose gel (10 g/l) in 0·5 × TBE and were visualized using UV illumination.

Specific amplification of different *Leuconostoc* species (*Ln. mesenteroides*, *Ln. lactis* and *Ln. citreum*), and *Weissella paramesenteroides* (Cibik et al. 2000) was performed with primers listed in Table 2. PCR amplification was carried out in 20 µl containing 1 µg DNA, 1·5 mM-$MgCl_2$, 0·6 µM each primer, 200 µM each dNTP and 2·5 U Taq DNA polymerase in 10 mM-Tris–HCl pH 9·0. After an initial denaturation at 94 °C for 5 min, 30 temperature cycles were performed at 94 °C for 30 s, either 58 °C for 30 s (primers Lnm1-Lnm2, Lncit1-Lncit2, Wpm1-Wpm2) or 60 °C for 30 s (primers Lnl1 and Lnl2), and 72 °C for 1.5 min, followed by a final extension step of 72 °C for 7 min.

Genus-specific amplification

Genus specific lactococcal (L1 and L2) and enterococcal (E1 and E2) primers used are described in Table 2 (Deasy et al. 2000). The DNA was amplified as for the leuconostoc specific amplification. DNA Thermal cycler was set to the following parameters: 1 cycle at 94 °C for 5 min and 30 cycles of amplification by denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s with enterococcal primers, or at 55 °C for 1 min with lactococcal primers, followed by polymerization at 72 °C for 1 min; the mixture was incubated at 72 °C for 10 min for final extension and kept at 4 °C until further processing.

Sequencing

At least 400 bp of the 5′ region of the 16S rDNA gene from strains were sequenced. 16S rDNA was amplified with the universal primers WO2 (5′-GNTACCTTGTTACGACTT-3′) and W18 (5′-GAGTTTGATCMTGGCTCAG-3′). PCR amplification was carried out in a final volume of 25 µl containing 1 × PCR buffer with $MgCl_2$, 1 µl phenol extracted DNA, 0·25 µM each primer, 200 µM each dNTP and 1 U Taq DNA polymerase. The thermal cycler apparatus was programmed for 5 min at 95 °C, 30 cycles of amplification of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C, followed by a final extension step of 72 °C for 10 min. The reaction sequences were obtained using an ABI PRISM™ dye terminator cycle sequencing kit (PE Applied Biosystems) with Ampli Taq DNA polymerase FS and the primer WO2. The pellet was resuspended in 20 µl Template Suppression Reagent (PE Applied Biosystems) and denatured 3 min at 95 °C. The reaction products were analysed using an automated DNA sequencer ABI PRISM 310 (PE Applied Biosystems) following manufacturer's instructions. To identify the partial 16S rDNA sequences obtained, a search of the GenBank DNA database was conducted by using the BLAST algorithm. The percentage of similarity with DNA sequences deposited in this bank was determined.

Results

Lactic acid bacteria identification

Phenotypic characterization. The results of phenotypic tests such as morphology, arginine production, CO_2 production, growth at 10 °C and 45 °C in MRS broth, in MRS broth with 65 g NaCl/l and in MRS broth pH 4·4 and heat treatment of 60 °C for 30 min are presented in Table 4. Among 381 isolates, 175 isolates belonged to homofermentative bacilli or coccobacilli arginine negative CO_2 negative, 92 isolates belonged to cocci or coccobacilli arginine negative and producing CO_2, nine isolates were cocci CO_2 negative and arginine negative and 105 isolates were cocci CO_2 negative and arginine positive. The growths at different temperature (10 and 45 °C), pH 4·4 and salt concentration were greatly variable among the isolates.

Classification by Rep-PCR. The 381 isolates were classified according to their Rep-PCR profiles. The discriminatory power of Rep-PCR was assessed. Rep-PCR analysis was applied to 49 type and reference strains. The different species studied were separated at 70% similarity level (Fig. 1). At this percentage, cheese isolate patterns were separated into 62 different groups. The description of each group is given in Table 3. Out of 49 reference strains, 13 were grouped with Salers isolates at 70% of similarity. 20 isolates were in the same group G15 as the type strain ATCC334T of *Lb. paracasei*, CNRZ 62T and other strains of this species CNRZ 383, LMG 9191, DSM 20006 and DSM 20012. Twenty isolates were placed in group G16 with the strain CNRZ 763 of *Lb. paracasei*. One strain (G21) had a similar profile to the reference strain DSM 4905 of *Lb. paracasei*. Fifty three isolates from AOC Salers were placed in the same group G36 as the type strain CNRZ 749^T of *Ln. mesenteroides* subsp. *mesenteroides*. One isolate (G38) had a similar profile to the type strain CNRZ 77^T of *Ln. mesenteroides* subsp. *dextranicum*. One isolate was grouped (G41) with the type strain CNRZ 142^T of *Lc. lactis* subsp. *lactis* and three isolates (G44) had a similar profile to the reference strain CNRZ 1075 of *Lc. lactis* subsp. *lactis*. Two strains of G62 had a similar profile to the type strain LMG 10488^T of *Pc. pentosaceus*. Nevertheless most of the isolates had less than 70% similitude between their Rep-PCR profiles and those of reference strains.

Amplification by specific primers. DNA of isolates were tested with primers listed in Table 2. Only DNA from isolates with bacilli or coccobacilli morphology was tested with the specific primers of *Lb. paracasei* and *Lb. plantarum*. All the 103 strains belonging to groups G1–G13 amplified with the primers of *Lb. plantarum*. 72 strains classified into groups G14–G28 and the type strain ATCC334T, amplified with the primers of *Lb. paracasei*.

DNA from 'cocci isolates' producing CO_2 was tested with different specific primers for different species of *Leuconostoc* and *Weissella* described in Table 2. None of the DNA of these isolates amplified with specific primers of *Ln. lactis, Ln. citreum* and *W. paramesenteroides*. 82 isolates of G32, G33, G36, G37 and G38 amplified with primers for *Ln. mesenteroides*. Groups 29, 30, 31, 34 and 35 listed in Table 4 did not amplify with any of these primers.

All strains belonging to clusters 39–62 and presenting a coccal morphology, were tested with lactococcal and enterococcal specific primers. DNA from isolates belonging to G52–G57 gave a positive reaction with enterococcal primers, and none with lactococcal primers. DNA from isolates belonging to G39–G51 was amplified with lactococcal primers. On the other hand, DNA from strains from G58 to G62 did not amplify with any primer.

DNA sequencing. Partial rDNA from some representative strains of each Rep-PCR group were sequenced to confirm presumptive identification obtained by Rep-PCR and specific species amplifications but also to identify non identified isolates.

rDNA from representative strains identified with *Lb. plantarum* with specific primers showed more than 99% homology with *Lb. plantarum* and *Lb. paraplantarum*.

In the same way, the representative strains presumptively classified in groups of *Lb. paracasei* had 99% homology with *Lb. casei* and *Lb. paracasei*. Some strains had only 95% homology for their rDNA sequences.

The identification of strains of G32, G33 and G36–G38 which amplified with specific primers of *Ln. mesenteroides* was confirmed by sequencing. All the strains tested exhibited 98–100% homology with *Ln. mesenteroides* subsp. *mesenteroides*, then they could be identified as *Ln. mesenteroides* subsp. *mesenteroides*. Strains of G29–G31 and 34 which did not amplify with primers tested, exhibited, by sequencing, 100% homology with the two species: *Ln. pseudomesenteroides* and *Ln. mesenteroides*. As they did not amplify with specific primer for *Ln. mesenteroides* they may be identified as *Ln. pseudomesenteroides*. The two strains from G35 which did not amplify with specific primers tested exhibited, by sequencing, 98% homology with *Ln. mesenteroides*.

The isolates classified in group G52–G54 showed 100% rDNA sequence homology with *Ec. faecalis* and isolates from groups G55–G57 had 99% rDNA sequence homology with *Ec. faecium*. The two strains of group G58 exhibited 100% homology with the species *Str. millieri*. Three strains of groups G59 and G60 exhibited 100% homology with *Str. salivarius*. Two strains of group G61 were sequenced and showed 99% homology with *Str. macedonicus*. The two strains of group G62 were identified as *Pc. pentosaceus* with 99% homology. The strain from G39 exhibited 100% homology with *Lc. garviae*, and could therefore be presumed to identify with this species.

Level and diversity in cheese. The number of isolates of each species encountered on different media are

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Table 4. Description of 62 clusters obtained by comparison of 49 reference strains and 381 isolates using the unweight pair-group method with arithmetic average (Pearson correlation coefficient, tol 1·55%, opt 0%)

	Rep-PCR			Species amplification										
Number of strains	Clusters	CO_2	Arginine	10 °C	45 °C	6·5% NaCl	pH 4·4	30′ 60 °C	Lpapl	*Paracasei*	lnm1-2	E1-E2	L1-L2	Sequencing
4	G1	–	–	+	+	+	+	v	+	–	nd	nd	nd	
3	G2	–	–	+	+	+	v	+	+	–	nd	nd	nd	*Lb. plantarum* 98%
4	G3	–	–	+	+	v	+	–	+	–	nd	nd	nd	
2	G4	–	–	+	+	–	+	–	+	–	nd	nd	nd	
16	G5	–	–	v	v	+	+	+	+	–	nd	nd	nd	*Lb. plantarum*
8	G6	–	–	v	+	+	+	v	+	–	nd	nd	nd	
24	G7	–	–	+	v	v	+	v	+	–	nd	nd	nd	*Lb. plantarum* 91%
1	G8	–	–	+	+	+	+	+	+	–	nd	nd	nd	*Lb. plantarum*
1	G9	–	–	+	+	+	+	+	+	–	nd	nd	nd	
1	G10	–	–	+	+	+	+	+	+	–	nd	nd	nd	*Lb. plantarum* 99%
3	G11	–	–	+	+	V	+	–	+	–	nd	nd	nd	*Lb. plantarum* 93%
14	G12	–	–	+	V	V	+	V	+	–	nd	nd	nd	*Lb. plantarum* 99%
22	G13	–	–	V	+	+	+	–	+	–	nd	nd	nd	*Lb. plantarum* 100%
3	G14	–	–	+	+	+	V	+	–	+	nd	nd	nd	*Lb. paracasei* 93%
6 ref strains† 20	G15	–	–	+	v	+	+	v	–	+	nd	nd	nd	*Lb. paracasei* 95%
1 ref strain‡ 20	G16	–	–	+	v	v	+	v	–	+	nd	nd	nd	*Lb. paracasei*
3	G17	–	–	+	+	+	+	v	–	+	nd	nd	nd	
3	G18	–	–	–	V	–	V	–	–	+	nd	nd	nd	*Lb. paracasei*
2	G19	–	–	+	+	+	+	–	–	+	nd	nd	nd	
2	G20	–	–	+	+	+	+	+	–	+	nd	nd	nd	*Lb. paracasei* 100%
1 ref strain§1	G21	–	–	+	+	+	+	+	–	+	nd	nd	nd	*Lb. paracasei* 98%
2	G22	–	–	+	+	+	+	+	–	+	nd	nd	nd	*Lb. paracasei* 100%
1	G23	–	–	–	+	+	+	+	–	+	nd	nd	nd	*Lb. paracasei* 99%
3	G24	–	–	V	+	V	+	V	–	+	nd	nd	nd	
3	G25	–	–	+	+	v	+	v	–	+	nd	nd	nd	
3	G26	–	–	–	+	V	+	V	–	+	nd	nd	nd	
3	G27	–	–	v	+	+	+	v	–	+	nd	nd	nd	*Lb. paracasei* 95%
3	G28	–	–	+	–	–	+	–	nd	nd	+	nd	nd	*Lb. paracasei* 95%
3	G29	+	–	+	v	+	v	–	nd	nd	–	nd	nd	*Ln. pseudomes, Leuc. mes* 100%
2	G30	+	–	+	V	–	V	V	nd	nd	–	nd	nd	*Ln. pseudomes, Leuc. mes* 100%
2	G31	+	–	+	+	V	V	+	nd	nd	–	nd	nd	*Ln. pseudomes, Leuc. mes* 100%
6	G32	+	–	+	V	+	V	V	nd	nd	+	nd	nd	*Ln. mesenteroides* 99%
1	G33	+	–	+	–	+	+	–	nd	nd	+	nd	nd	*Ln. mesenteroides*
1	G34	+	–	+	+	+	+	+	nd	nd	–	nd	nd	*Ln. pseudomes, Leuc. mes* 99%
2	G35	+	–	–	V	–	+	V	nd	nd	–	nd	nd	*Ln. mesenteroides* 98%
1 ref strain¶ 53	G36	+	–	+	V	V	V	V	nd	nd	+	nd	nd	*Ln. mesenteroides*
21	G37	+	–	+	V	V	V	V	nd	nd	+	nd	nd	*Ln. mesenteroides* 98%
1 ref strain†† 1	G38	+	–	+	+	+	+	–	nd	nd	+	nd	nd	*Ln. mesenteroides* 99%
1	G39	–	+	+	+	+	+	+	–	–	–	–	+	*Lc. garviae* 100%
2	G40	–	+	+	–	–	–	–	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 99%

1	ref strain‡‡ 1	G41	–	+	+	–	–	–	–	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 100%
1		G42	–	+	+	–	–	–	–	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 98%
4		G43	–	+	+	v	v	v	v	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 98%
1	ref strain§§ 3	G44	–	+	+	+	v	–	–	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 99%
11		G45	–	+	+	v	v	v	v	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 99%
2		G46	–	+	+	v	v	+	v	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 99%
1		G47	–	+	+	+	+	–	–	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 99%
3		G48	–	+	+	V	+	+	V	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 99%
3		G49	–	+	+	V	V	V	V	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 98%
2		G50	–	+	+	v	+	+	v	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 98%
6		G51	–	+	+	v	v	+	v	nd	nd	nd	–	+	*Lc. lactis* subsp. *lactis* 98%
11		G52	–	+	+	+	+	+	v	–	–	–	+	–	*Ec. faecalis* 99%
12		G53	–	+	+	+	+	+	v	–	–	–	+	–	*Ec. faecalis* 98%
6		G54	–	+	+	+	+	+	+	–	–	–	+	–	*Ec. faecalis* 99%
27		G55	–	+	+	+	+	+	+	–	–	–	+	–	*Ec. faecium* 100%
5		G56	–	+	+	+	+	+	+	–	–	–	+	–	*Ec. faecium* 98%
3		G57	–	+	+	+	+	+	v	–	–	–	+	–	*Ec. Faecium* 95%
2		G58	–	–	–	+	–	–	+	–	–	–	–	–	*Str. millieri* 99%
1		G59	–	–	+	–	–	–	+	–	–	–	–	–	*Str. salivarius* 100%
2		G60	–	–	+	+	v	V	+	–	–	–	–	–	*Str. salivarius* 99%
3		G61	–	–	–	+	+	+	v	–	–	–	–	–	*Str. macedonicus* 100%
I	ref strain¶¶ 2	G62	–	–	+	+	+	+	+	–	–	–	–	–	*Pc. pentosaceus* 99%

†CNRZ383, ATCC334^T, CNRZ62^T, LMG9191, DSM20012, DSM20006
‡CNRZ763
§DSM4905
¶CNRZ749^T
††CNRZ77^T
‡‡CNRZ142^T
§§CNRZ2050
¶¶LMG10488^T

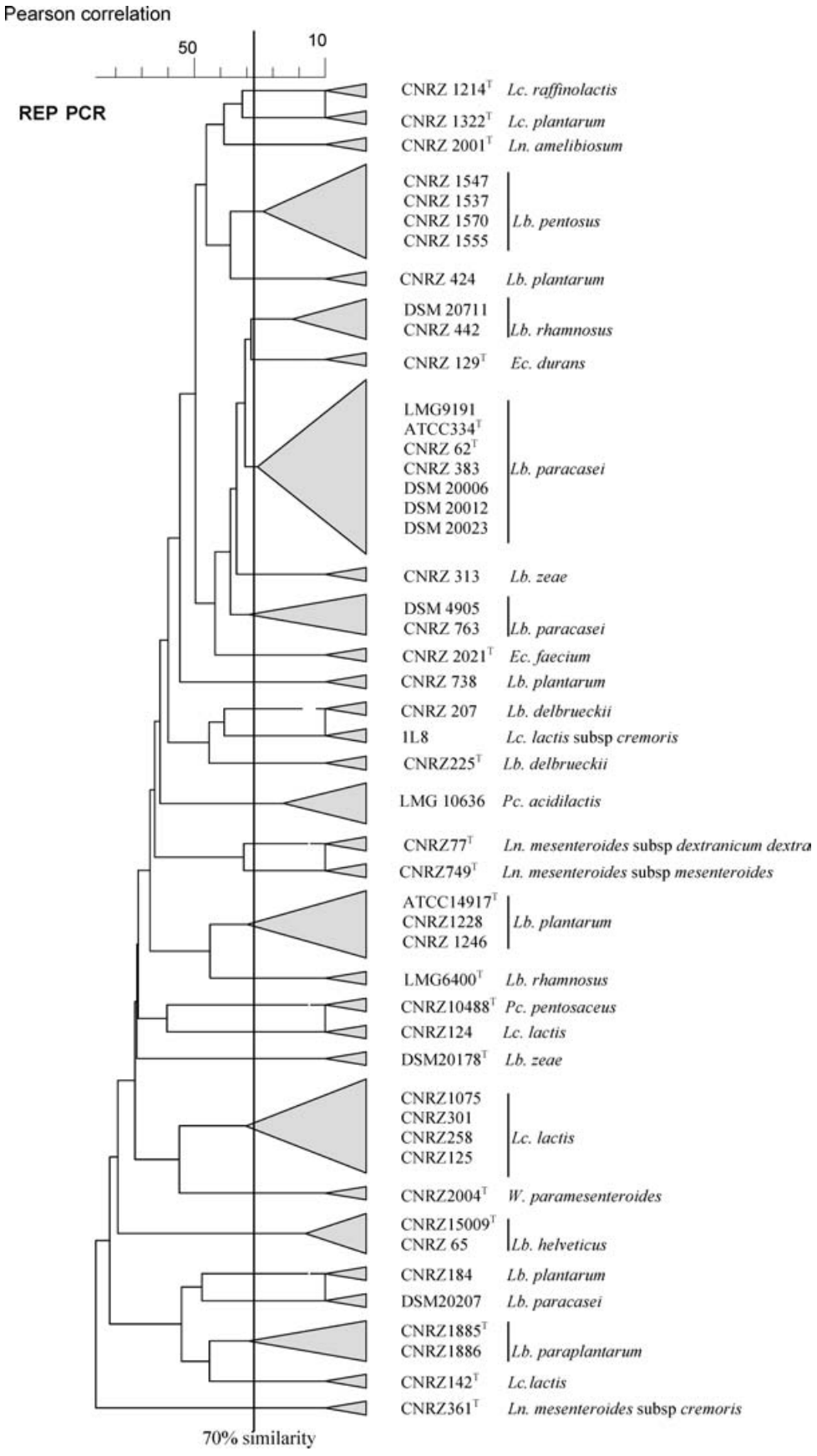

Fig. 1. For caption see opposite.

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Table 5. Number of isolates of *Lactobacillus, Enterococcus, Leuconostoc, Lactococcus, Pediococcus* and *Streptococcus* species (a) grown on the different media (the number is the total of all strains isolated from the 3 cheeses A, B and C and all time of ripening at once) and (b) found in different cheeses (the number is the total of strains isolated from several media all time of ripening at once)

	Growth media						Cheeses		
	M17L	MRS	MSE	FH	SB	M17	A	B	C
Lb. paracasei	17	9	9	29	4	4	35	20	17
Lb. plantarum	16	20	20	30	7	10	28	36	39
Ec. faecalis	5	1	0	1	21	1	10	14	5
Ec. faecium	9	4	0	1	15	6	2	4	29
Ln. mesenteroides	41	23	11	0	6	4	18	33	34
Ln. pseudomesenteroides	1	4	0	0	1	1	1	0	6
Lc. lactis lactis	24	2	0	2	1	10	11	6	22
Lc. garviae	1	0	0	0	0	0	1	0	0
Pc. pentosaceus	0	0	0	0	0	2	0	0	2
Str. millieri	1	1	0	0	0	0	1	1	0
Str. macedonicus	0	0	0	0	3	0	3	0	0
Str. Salivarius	3	0	0	0	0	0	2	1	0

summarized in Table 5. All the isolation media enabled growth for all the bacterial groups found and this low selectivity meant that it was not possible to determine the level of each group and their dynamics during the ripening. The evolution of the viable lactic acid bacteria counts on the different media throughout ripening and for the three cheeses A, B, C is shown in Table 6. The count of the MRS medium, which gives a good estimation of total lactic acid bacteria in this cheese variety, ranged between 2×10^4 and $1{\cdot}1 \times 10^6$ cfu/ml in the milk with the highest level in milk A. It ranged between $1{\cdot}1 \times 10^9$ and $1{\cdot}5 \times 10^9$ cfu/g in cheese at one day of ripening. Only two media appeared more selective and gave a real image of these populations in cheese and their evolution during the ripening. The FH medium appeared selective for the count of mesophilic lactobacilli. Accordingly, the level of lactobacilli varied between $1{\cdot}3 \times 10^3$ and $1{\cdot}7 \times 10^4$ cfu/ml in milk with the highest level in milk B and the lowest level in milk A. It increased to between $6{\cdot}5 \times 10^5$ and $6{\cdot}1 \times 10^6$ cfu/g at 1 d. The population was stable or increased slightly until 150 d of ripening. Slanetz and Bartley medium seemed suitable for the numeration of the genus *Enterococcus*. The evolution of this group distinguished cheese C from the others. Indeed, the level of *Enterococcus* was the highest in milk B with $8{\cdot}7 \times 10^2$ cfu/ml and it was the highest at 1 d in cheese C reaching $1{\cdot}3 \times 10^9$ cfu/g while the others were around 1×10^6 cfu/g. Then it decreased slightly until the end of ripening.

The different species found in each cheese are summarized in Table 5. The species *Lb. plantarum*, *Lb. paracasei*, *Ec. faecalis*, *Ec. faecium*, *Ln. mesenteroides* and *Lc. lactis* were systematically isolated from all the samples. Cheese from farm A was different by the isolation of *Ln. pseudomesenteroides*, mesophilic *Streptococcus* such as *Str.macedonicus* and *Lc. garviae*. Cheese from farm C was distinguished by the presence, in two samples, of the species *Pc. pentosaceus*.

Intra-species diversity. The Rep-pattern of strains identified as *Lb. plantarum*, *Lb. paracasei* and *Ln. mesenteroides* were intra-species compared. The reproducibility of Rep-PCR at strain level was evaluated (results not shown), and strains were considered to have the same Rep profile when they shared between 85 and 100% similarity. The dendrogram, after treatment by UPGMA and Pearson correlation of the 102 strains identified as *Lb. plantarum* and the reference strains, is shown in Fig. 2a. At 85% similarity, 44 groups were defined. Two groups showed only 25% similarity with the other groups. At the beginning of the ripening, the strains isolated from cheeses C were quite different from those isolated from cheeses A and B because they were never grouped in the same cluster. Some strains from cheeses A and B had similar profiles but others were classified in different clusters and have unique profiles. At the end of the ripening (150 d), some strains from A and C had particular profiles and few strains from cheeses A, B and C were grouped in same cluster.

The Rep profile dendrogram of strains identified as *Lb. paracasei* and reference strains is represented in Fig. 2b. At 85% similarity, 36 clusters were defined for only 72 strains, illustrating the diversity of strains among this species. Nevertheless the percentage of similarity was higher

Fig. 1. Generated dendrogram from combined Rep-PCR fingerprints of collection strains of lactic acid bacteria. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentage.

Values of the Pearson correlation coefficient (Opt 0%, tol 1·55%)

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Table 6. Bacterial count in milks (cfu/ml) and in three Salers cheeses (cfu/g) A, B, C produced at separate farms at different stages of ripening (1, 8, 30 and 150 d of ripening). Growth and specific conditions of each medium are described in Materials and Methods section

		Time of ripening			
Medium	Milk	1 d	8 d	30 d	150 d
A-cheese					
M17l	$3{\cdot}9\times10^{3}$	$3{\cdot}2\times10^{8}$	$1{\cdot}9\times10^{8}$	$4{\cdot}9\times10^{8}$	$7{\cdot}2\times10^{7}$
MRS	$1{\cdot}1\times10^{6}$	$1{\cdot}5\times10^{9}$	$7{\cdot}2\times10^{8}$	$4{\cdot}4\times10^{8}$	$1{\cdot}8\times10^{7}$
FH	$1{\cdot}3\times10^{3}$	$6{\cdot}5\times10^{5}$	$1{\cdot}0\times10^{6}$	$2{\cdot}2\times10^{7}$	$5{\cdot}7\times10^{7}$
M17 42 °C	$1{\cdot}2\times10^{6}$	$1{\cdot}0\times10^{9}$	$2{\cdot}4\times10^{8}$	$1{\cdot}9\times10^{8}$	$1{\cdot}2\times10^{8}$
SB	$5{\cdot}9\times10^{2}$	$1{\cdot}1\times10^{6}$	$3{\cdot}3\times10^{5}$	$2{\cdot}7\times10^{5}$	$3{\cdot}8\times10^{4}$
MSE	$8{\cdot}7\times10^{2}$	$2{\cdot}8\times10^{6}$	$2{\cdot}0\times10^{7}$	$1{\cdot}6\times10^{7}$	$1{\cdot}7\times10^{7}$
B-cheese					
M17l	$4{\cdot}2\times10^{4}$	$3{\cdot}1\times10^{8}$	$1{\cdot}6\times10^{9}$	$2{\cdot}5\times10^{8}$	$2{\cdot}6\times10^{8}$
MRS	$5{\cdot}0\times10^{4}$	$1{\cdot}1\times10^{9}$	$2{\cdot}3\times10^{9}$	$1{\cdot}5\times10^{9}$	$1{\cdot}8\times10^{8}$
FH	$1{\cdot}7\times10^{4}$	$6{\cdot}1\times10^{6}$	$2{\cdot}6\times10^{7}$	$3{\cdot}0\times10^{7}$	$3{\cdot}1\times10^{7}$
M17 42 °C	$3{\cdot}4\times10^{4}$	$7{\cdot}5\times10^{8}$	$6{\cdot}0\times10^{8}$	$3{\cdot}0\times10^{8}$	$1{\cdot}4\times10^{7}$
SB	$8{\cdot}7\times10^{2}$	$1{\cdot}0\times10^{6}$	$3{\cdot}7\times10^{5}$	$4{\cdot}2\times10^{5}$	$9{\cdot}5\times10^{4}$
MSE	$2{\cdot}6\times10^{4}$	$2{\cdot}0\times10^{8}$	$2{\cdot}0\times10^{8}$	$5{\cdot}6\times10^{7}$	$3{\cdot}3\times10^{7}$
C-cheese					
M17l	$6{\cdot}0\times10^{3}$	$5{\cdot}3\times10^{8}$	$1{\cdot}2\times10^{9}$	$4{\cdot}7\times10^{7}$	$6{\cdot}0\times10^{8}$
MRS	$2{\cdot}4\times10^{4}$	$1{\cdot}3\times10^{9}$	$1{\cdot}3\times10^{9}$	$9{\cdot}6\times10^{8}$	$9{\cdot}1\times10^{7}$
FH	$5{\cdot}2\times10^{3}$	$1{\cdot}1\times10^{6}$	$7{\cdot}7\times10^{6}$	$1{\cdot}0\times10^{7}$	$1{\cdot}0\times10^{7}$
M17 42 °C	$1{\cdot}3\times10^{4}$	$6{\cdot}1\times10^{5}$	$9{\cdot}0\times10^{6}$	$8{\cdot}2\times10^{6}$	$2{\cdot}9\times10^{6}$
SB	$2{\cdot}2\times10^{2}$	$1{\cdot}3\times10^{9}$	$7{\cdot}0\times10^{8}$	$6{\cdot}7\times10^{8}$	$2{\cdot}4\times10^{7}$
MSE	$1{\cdot}9\times10^{3}$	$8{\cdot}1\times10^{7}$	$2{\cdot}0\times10^{8}$	$2{\cdot}7\times10^{7}$	$1{\cdot}4\times10^{7}$

than for *Lb. plantarum* as it was above 48%. Above 85% similarity, most strain groups on the same cluster had the same origin. But a few strains from different cheeses at different times of ripening were located in the same clusters. Strains from the same cheese at different times of ripening were not located in the same group. The genomic diversity was higher at 150 d than at 1 d.

At 85% the 80 strains of *Ln. mesenteroides* were put into 36 groups as shown in Fig. 2c. Five strains showed only 30% similarity with the other strains. Strain groups depended more on their time of ripening than origin. For example, 22 strains from the 3 different cheeses at 1 and 8 d of ripening were in the same group G1. Most of the strains from group G3 were isolated at 30 d of ripening.

Discussion

Restricting counting to more or less selective media gave poor information on lactic acid bacteria in the three Salers cheeses studied. As observed by other authors, the culture media MRS, M17, M17l and MSE were not selective (Tornadijo et al. 1995; Centeno et al. 1996; Freitas et al. 1996). The count on these media was dependent on the composition of the microbial ecosystem; the more complex the ecosystem, the less selective the media. The total lactic acid bacteria count on MRS was similar to that found in other cheese technologies. The level of lactobacilli encountered on FH medium, the most selective medium, was the same as those reported for Comté type cheese (Demarigny et al. 1996), Gouda (Kleter, 1976), Greek cheeses (Hatzikamari et al. 1999), Spanish traditional cheeses (Lopez & Mayot, 1997), hard, semi-hard and blue-veined varieties and Italian cheeses (De Angelis et al. 2001). It was higher than that found in Cheddar: 10^{7} cfu/ml (Fitzsimons et al. 1999). The counting on SB medium gave a good view of enterococci level. The level observed in our cheeses was important especially in cheese C. Enterococci occur and grow in a variety of cheeses, especially artisanal cheeses (Centeno et al. 1996; Freitas et al. 1996; Beresford et al. 2001) and in some cases they can predominate over lactobacilli and lactococci. Levels of enterococci in different cheese curd range from 10^{4} to 10^{6} cfu/g, and in the fully ripened cheeses from 10^{5} to 10^{7} cfu/g (Franz et al. 1999). The dominance or persistence of enterococci in some cheeses during ripening can be attributed to their wide range of growth temperature, their high tolerance of salt (Tzanetakis & Litopoulou-Tzanetaki, 1992; Garcia et al. 2002). In our study, variable counts between the three cheeses could be explained by the differences of environmental contamination during milking and cheese manufacturing and variations in processing parameters in the three farms (Table 1). Especially, times of maturation of curd, quantity of salt and times of maturation in salt could influence the growth of species present. Salers technology favours rather salt resistant bacteria than thermophilic bacteria. Molecular methods were more suitable than phenotypic methods to obtain a qualitative picture of

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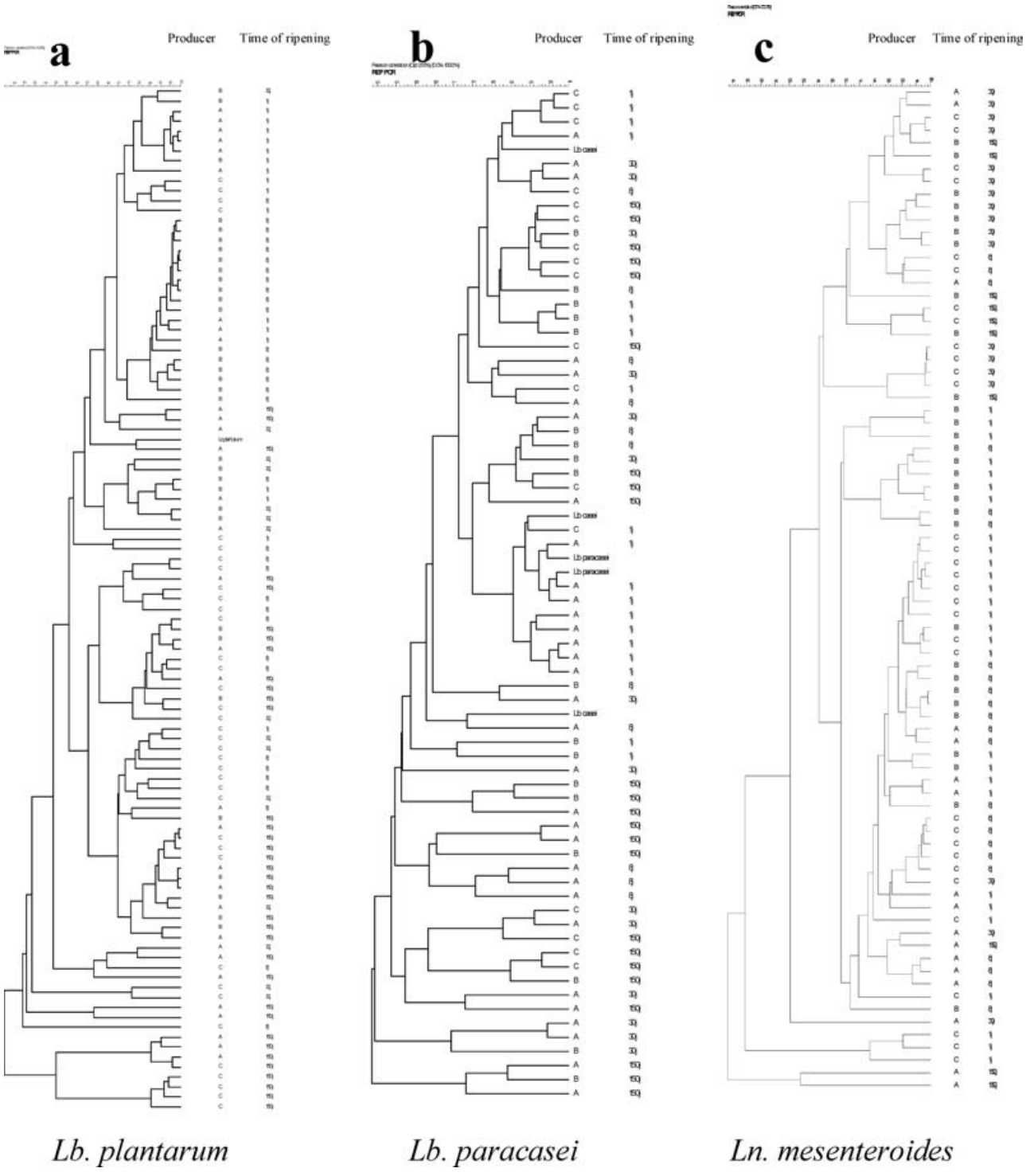

Fig. 2. Rep-PCR fingerprinting patterns of all the strains of three species (*Lb. plantarum*, *Lb. paracasei* and *Ln. mesenteroides*) and corresponding dendrogram based on the unweighted pair group method with arithmetic average (UPGMA) of Pearson correlation coefficient (expressed as a percentage value).

lactic acid bacteria flora from Salers cheese. Indeed, phenotypic determination was inadequate for identification. The characteristics of isolates did not correspond with those generally described for the main lactic acid bacteria species found in dairy products. For example resistance to 60 °C for 30 min, described as characteristic of *Enterococcus*, was also noticed for other strains not belonging to *Enterococcus*. Lactococci and enterococci share

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many similar phenotypic characteristics which make their separation difficult when employing biochemical methods. The growth of species *Lb. plantarum*, *Lb. casei* and *Ln. mesenteroides* at 45 °C was surprising according to official taxonomy (Kandler & Weiss, 1986). Nevertheless such growth was described in the study of Fitzsimons et al. (1999).

Identification by molecular methods was confirmed by combining several methods. Rep-PCR alone was not sufficient for accurate identification especially for *Enterococcus*, *Lactococcus* and *Streptococcus*. The Rep-PCR profiles of isolates from Salers cheeses had a low percentage of similarity (less than 70%) with the corresponding reference type strain profiles. Consequently, it was impossible to assign them to a species. PCR with specific primers for *Lb. plantarum* and *Lb. casei* or for *Ln. mesenteroides* was very relevant for the identification of these species, just as was described by Berthier et al. (2001) and Cibik et al. (2000). Moreover the results corresponded to those obtained by sequencing. Partial rDNA sequencing was a helpful tool for identifying strains but longer sequences had to be determined when the percentage of homology was below 98%. Nevertheless the information provided by sequencing was limited when species showed a high degree of similarity in their rDNA sequences, for example, *Lb. plantarum* and *Lb. pentosus*.

Some species as *Lb. plantarum*, *Lb. paracasei*, *Ln. mesenteroides*, *Lc. lactis* subsp. *lactis*, *Ec. faecium*, *Ec. faecalis* were identified in all the three AOC Salers cheeses. The occurrence of *Lb. casei* and to a lesser extent *Lb. plantarum* in Salers cheese is not surprising as these species were frequently encountered in different kinds of cheese technologies, Greek cheeses (Tzanetakis & Litopoulou-Tzanenaki, 1992), Cabrales cheeses (Nunez, 1978), Majorero cheeses (Fontecha et al. 1990), Arzua cows' milk cheeses (Centeno et al. 1996), Armada cheeses (Tornadijo et al. 1995), Cheddar (Fitzsimons et al. 2001). They were associated with *Lb. rhamnosus* in Comté (Berthier et al. 2001). *Ln. mesenteroides* and *Ln.* p*seudomesenteroides* seem to be more characteristic of Salers cheese as these species were not often isolated from cooked cheeses. They have been isolated from cheeses made using traditional technology in France (Cibik et al. 2000), from traditional Greek cheeses (Tzanetakis & Litopoulou-Tzanetaki, 1992; Hatzikamari et al. 1999), from traditional Mozzarella cheeses (Morea et al. 1999) and from Cebreiro (Centeno et al. 1996). The principal natural acidifying flora in the milk and Salers cheeses was represented by the group of lactococci. *Lc. lactis* subsp. *lactis* was the species most often encountered in all cheeses. *Ec. faecalis* and *Ec. faecium* were also the two species of enterococci isolated in these cheeses. *Ec. faecalis* and *Ec. faecium* were the dominant enterococcal isolates in few type of raw milk cheeses. But *Ec. durans* or *Ec. gallinarum* were also found in San Simon artisanal cheese (Garcia et al. 2002), in Cebreiro (Centeno et al. 1996) and in Armada (Tornadijo et al. 1995). Thermophilic lactobacilli were often isolated in 'cooked cheeses' such as Comté or Cheddar. They were not found in this study. They were favoured by cooking the curd which is not practiced in Salers technology. Moreover, thermophilic lactobacilli in cooked cheese can result from starter culture inoculated. Such starters were not used in the Salers cheeses studied.

Other species such as *Str. macedonicus*, *Str. millieri*, *Str. salivarius*, *Lc. garviae* and *Pc. pentosaceus* found in Salers cheese have been rarely encountered. Thus it should be interesting to evaluate the real occurrence of these species in other Salers cheese farm productions. *Str. macedonicus* has been isolated in Italian cheeses (Andrighetto et al. 2002) and Greek Kasseri cheeses (Georgalaki et al. 2000). The species *Lc. garviae* was isolated from Mozzarella cheese (Morea et al. 1999). The species *Pc. pentosaceus* found in one production is most frequently encountered pediococci in raw goat milk cheese, Feta and Kaseri (Tzanetakis & Litopoulou-Tzanetaki, 1992).

The study reveals the intra-species genomic diversity in the natural microbial population through their Rep profiles varying according to origin and time of ripening. The percentage of similarity of strains belonging to the same species was often low. Such diversity can be linked with the diversity of farm environments and conditions during milking. The collect 'in gerle' which are specific of each farm in terms of nature of wood and cleaning, can also be the origin of the diversity. Selection of some strains common in all producers may be linked with particular biochemical characteristics in relation to the selective effect of technology (salting the curd). The link between Rep profiles and origin was less obvious than that observed for *Lactobacillus* strains found in Comté cheese (Berthier et al. 2001). As noticed by Fitzsimons et al. (2001), the greatest care must be taken before establishing a relationship between the origin and the diversity. The highest diversity observed at the end of ripening could be attributed to the fact that sub-dominant population initially present could develop during ripening with respect of Salers technology.

The qualitative microbial picture such as was obtained in the study raises the question 'what is the reality of microbial community diversity in cheese?' Indeed, as in many studies, it was dependent on culture media, isolation, procedure and methods of identification and typing. More and more a picture of the microbial community is obtained by independent culture methods. Various PCR-based molecular typing methods have been developed for the analysis of cheese communities: Denaturating Gradient Gel Electrophoresis (Coppola et al. 2001; Diez et al. 2001), Temperature Gradient Gel Electrophoresis (Felske et al. 1996; Zoetendal et al. 1998), Terminal-Restriction Fragment Length Polymorphism (Osborn et al. 2002), Single Strand Conformation Polymorphism (SSCP). Lactic acid species in the cheeses studied were also inventoried by molecular approach applying DNA clone sequencing and SSCP method (Duthoit et al. 2003) The molecular approach confirmed our results in term of species but

revealed also the presence of *Lb. pentosus*, but do not allow the evaluation of the intra-species diversity.

This study reveals the diversity of lactic acid bacteria flora from AOC Salers cheese. As the number of cheese was low, it could be assumed that by analysing more cheese the level of diversity will be greater. In the future, it will be interesting to establish the link between microbial diversity measured by different approaches and the sensorial qualities of cheese. In this purpose, the biochemical activities of the bacterial community will be also evaluated.

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