

1 Monitoring Bacterial Communities in Raw Milk and Cheese by Culture-Dependent
2 and -Independent 16S rRNA Gene-Based Analyses
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5 **Running title :** Bacterial communities of raw milk cheese

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1 **ABSTRACT**

2 The diversity and dynamics of bacterial populations in Saint-Nectaire, a raw milk, semi-
3 hard cheese were investigated using a dual culture-dependent and direct molecular approach
4 combining Single-Strand Conformation Polymorphism (SSCP) fingerprinting and sequencing of
5 16S rRNA genes. The dominant clones, among 125 16S rRNA genes isolated from milk,
6 belonged to the *Firmicutes* (58 % of the total clones) affiliated mainly to the *Clostridiales* and the
7 *Lactobacillales*, followed by the *Proteobacteria* (21.6%), the *Actinobacteria* (16.8%) and the
8 *Bacteroidetes* (4%). Sequencing the 16S rRNA genes of 126 milk isolates collected from four
9 culture media revealed the presence of 36 different species showing a wider diversity in the
10 *Gammaproteobacteria* phylum and *Staphylococcus* genus than that found among clones. In
11 cheese, a total of 21 species were obtained from 170 isolates, with dominant species belonging to
12 the *Lactobacillales* and sub-dominant affiliated to *Actinobacteria*, *Bacteroidetes*
13 (*Chryseobacterium* sp.) or *Gammaproteobacteria* (*Stenotrophomonas* sp.). Fingerprinting DNA
14 isolated from milk by SSCP yielded complex patterns whereas analysing DNA isolated from
15 cheese resulted in patterns composed of a single peak which corresponded to lactic acid bacteria.
16 SSCP fingerprinting of mixtures of all colonies harvested from Plate Count Agar supplemented
17 with crystal violet and vancomycin showed good potential for monitoring the subdominant
18 *Proteobacteria* and *Bacteroidetes* (*Flavobacteria*) in milk and cheese. Likewise, analysing
19 culturable subcommunities from the Cheese Ripening Bacteria medium permitted assessment of
20 the diversity of halotolerant *Actinobacteria* and *Staphylococcus*. Direct and culture-dependent
21 approaches produced complementary information thus generating a more accurate view of milk
22 and cheese microbial ecology.

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1 INTRODUCTION

2
3 Microbial diversity in raw milk is considered essential to the sensory richness and variety of
4 traditional cheeses (3, 11, 13). However, some members of these complex communities may also
5 be responsible for cheese flavour defects (7) or may constitute a health risk (10). Being able to
6 characterize the microbial communities in raw milk and follow the dynamics of the entire
7 populations throughout the cheese-making and ripening processes is therefore critical. The
8 bacterial community in raw milk and cheese has been described by cultivation followed by
9 molecular identification of isolates (1, 2, 6, 28, 31). Culture-independent studies based on
10 molecular fingerprinting techniques such as DGGE, TTGE and SSCP have been carried out to
11 evaluate bacterial diversity in raw milk (23), the cheese core (9, 14, 15, 34, 39) or the cheese
12 surface (17, 38). The cheese-making process aims at favouring the growth and activity of lactic
13 acid bacteria (LAB). The addition of selected LAB starter cultures ensures safety through rapid
14 acidification. As expected, LAB were subsequently found to be predominant on bacterial
15 fingerprints from the cheese core. Hence, *Streptococcus thermophilus* and thermophilic
16 lactobacilli were dominant in the DGGE patterns of Ragusano cheese (39), and *S. bovis* and
17 *Lactococcus lactis* in patterns of Pecorino Siciliano cheese (40). Ogier *et al.* (33), using direct
18 TTGE and DGGE analyses also found that the microbial community in Saint-Nectaire cheese
19 cores was dominated by *S. thermophilus* and *L. lactis*. Indeed, the fingerprints produced by such
20 techniques as SSCP, DGGE or TTGE using general bacterial primers reveal only the most
21 dominant populations of a bacterial community. In the presence of one or several largely
22 dominant populations, these methods do not permit investigation of the diversity of less dominant
23 populations (37). In food fermentation microbial communities, which are generally believed to
24 harbour a large fraction of culturable species (30), this limitation may be partly overcome by
25 applying fingerprinting techniques to subcommunities capable of growing on different culture

1 media. This approach was previously used to explore the diversity of culturable bacteria in cheese
2 (16, 35, 40) and other fermented foods such as capers (36) but was mainly focused on LAB. The
3 aim of the present study was to evaluate the relevance of direct versus culture-based approaches
4 combining SSCP fingerprinting and 16S rRNA genes sequencing to monitor the diversity and
5 dynamics of bacterial populations in Saint-Nectaire, a semi-hard cheese made from raw milk. To
6 identify the dominant bacterial populations of raw milk, the diversity of 16S rRNA genes in a
7 clone library constructed from DNA isolated from milk and in bacterial colonies isolated from
8 various culture media was analysed. In an attempt to reveal the fate of subdominant populations
9 in cheese, the diversity of bacterial cell suspensions harvested from culture media allowing
10 growth of non-LAB bacteria was investigated by SSCP fingerprinting.

11 12 **MATERIALS AND METHODS** 13

14 **Milk and cheese samples.** Farm productions of Saint-Nectaire, a non-cooked, Registered
15 Designation of Origin, semi-hard cheese variety made from raw cow's milk and produced in the
16 mid-mountainous area of Massif Central, France, were studied. Nine cheese-making batches from
17 three farm productions named F1, F2 and F3, which were sampled three times at 3-month
18 intervals, in winter, spring and summer were analysed. Milk protein content was approximately
19 31 g/l and fat content ranged between 27.6-39.5 g/l. The raw milk was inoculated immediately
20 after milking with a commercial starter culture (*Streptococcus thermophilus*, *Lactococcus lactis*).
21 The milk was coagulated by adding calf rennet (27.5-30 ml/100 litres). After coagulation, the
22 curd was cut into small pieces (around 0.5 cm) and gently stirred, then it was gathered, filled into
23 moulds and pressed under 3-bar pressure for 10 min to remove whey. Thirty to forty grammes of
24 salt per kg were added on the surface of each 1.7-kg cheese and pressing was completed at 23-26
25 °C for 6 h. The cheeses were ripened at 10-12°C for 28 days. Samples were taken aseptically

1 from the raw milk prior to the addition of the starter culture, and from the cheese on day 1 and
2 also after 28 days. At each sampling point, three cheeses from the same production batch were
3 sampled using a sterile cheese trier, the rind was discarded (5 mm thickness) and the three core
4 sub-samples (around 10 g each) were mixed together by grinding. All samples were stored at –
5 20°C for further bacteriological and molecular analyses.

6 **Microbiological analyses of milk and cheese samples and collection of isolates and**
7 **cell mixtures.** Cheese samples were emulsified in sterile phosphate buffer (20 mM KH_2PO_4 -
8 0.01M K_2HPO_4 pH 7.5) and blended in a Stomacher Lab Blender (Seward Medical, London,
9 UK) for 4 min. Both the milk and the cheese suspensions were diluted in Ringer's solution and
10 appropriate dilutions were spread in triplicate on M17 agar (42) incubated at 42°C for 48 h, on
11 Cheese Ripening Bacteria Medium (CRBM) described by Denis *et al.*(12), containing 50 g/l
12 NaCl, 9 mg/l natamycin and 40 mg/l nalidixic acid, incubated at 25°C for 5 days, on Plate Count
13 Agar supplemented with 1% milk (PCAM) (32) and on Plate Count Agar supplemented with 1%
14 milk, 5 mg/l vancomycin and 5 mg/l crystal violet as inhibitors of Gram-positive bacteria (PCAI).
15 Both PCA media were incubated at 30°C for 48 h. All media were purchased from Biokar
16 Diagnostics (Pantin, France). Within 24 h after counting, duplicate plates of each medium,
17 counting 30 to 100 colonies, were selected. One ml of 4 M guanidine thiocyanate– 0.1 M Tris
18 (pH 7.5)- 1% *N*-lauroylsarcosine solution was spread on the first plate, all the colonies present on
19 the surface of the agar were suspended with a rake and the resulting cell suspension was collected
20 and stored at –20°C for further molecular analyses. For F2 milk and cheese, individual colonies
21 were also picked from the second plate. One specimen of each colony morphotype was picked
22 and additional isolates were picked randomly among non-confluent colonies up to a total of 20 to
23 50 isolates per plate. Colonies were restreaked for purification prior to direct PCR amplification.
24 All isolates were further identified by 16S rRNA gene sequencing.

1 **Extraction of total bacterial DNA from milk, cheese and plate count cell suspensions.**

2 One-ml milk samples or 1-g cheese samples were first mixed with 1ml of 4M guanidine
3 thiocyanate-0.1M Tris (pH 7.5) and 125µl of 10% N-Lauroyl sarcosine. The cheese suspension
4 was homogenized by grinding with a stainless steel-bead (1 cm diam.) in a stainless steel-
5 container (Kurt Retsch GmbH, Haan, Germany), using a reciprocating shaker (MM200, Kurt
6 Retsch GmbH) for 2 min at 30 Hz. DNA extraction from cell suspensions harvested from agar
7 plates started directly at this stage. A 2ml tube containing 250µl of the suspension was filled with
8 200mg of zirconium beads (0.1 mm diam.), 100µl of 20% SDS solution, 400µl of 0.1M
9 phosphate buffer, pH8.0, 400µl of 50mM sodium acetate containing 10mM EDTA pH6.0, 400µl
10 of phenol-chloroform-isoamyl alcohol (25:24:1), pH8.0. The tube was heated at 80°C for 2 min
11 in a water bath, chilled on ice for 2 min and shaken for 2 min at room temperature in the
12 reciprocating shaker. Both the shaking and heating steps were performed a second time. Total
13 bacterial DNA was then purified by a phenol-based method as previously described (14).

14 **Bacterial-targeted SSCP-PCR.** DNA isolated from milk, cheese or plate count cell
15 suspension was subjected to SSCP-PCR. The target DNA amplified was the variable region V3
16 of the 16S rRNA gene, which corresponds to a-200 bp fragment (*E. coli* 16S rRNA gene
17 positions 330 to 533) (5). The primers, corresponding to conserved sequences bordering the
18 variable regions, were w49 (5'-ACGGTCCAGACTCCTACGGG-3') and w34 (5'-
19 TTACCGCGGCGTGCTGGCAC-3'). Primer w34 was labelled with 5'-fluorescein
20 phosphoramidite: NED. All primers were synthesized by Applied Biosystems. The amplification
21 reaction contained : 1 µl of DNA, 1X PCR reaction buffer, 200 µM of each dNTP, 2 mmol l⁻¹
22 MgCl², 0.5 µmol l⁻¹ of each primer and 1.25 U *Pfu* Ultra DNA polymerase (Stratagene, La Jolla,
23 Ca., USA). The PCR amplification was performed under the following conditions: 3 min initial

1 denaturation at 96°C; 25 cycles of denaturation (30 s at 96 °C), annealing (30 s at 61°C) and
2 extension (30 s at 72 °C) ; a final extension at 72 °C for 10 minutes. Amplification products were
3 analysed on 1.5 % (w/v) agarose gels.

4 **SSCP electrophoresis and fingerprint data analysis.** The SSCP-PCR products were
5 analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, Ca., USA) as
6 previously described (14). The non-denaturing polymer matrix used was 5.6% GeneScan
7 Polymer (Applied Biosystems)-10% glycerol- 1X buffer with EDTA (Applied Biosystems). The
8 buffer was 1X buffer with EDTA (Applied Biosystems)-10% glycerol. The fluorescence signal
9 was analyzed using the GeneScan Analysis software (Applied Biosystems). The patterns to be
10 compared were aligned using the internal standard Genescan 400-ROX (Applied Biosystems). To
11 analyse the different profiles, relative peak areas were calculated as $P_i = a_i / \sum a_i$, where a_i was the
12 area of one peak and $\sum a_i$ is the sum of the peak areas from the whole SSCP pattern. Only peaks
13 with $a_i > 700$ and $P_i > 1\%$ of the total profile area were taken into account to estimate pattern
14 richness (number of peaks) and for subsequent multivariate statistical analyses. Tests to evaluate
15 the reproducibility of patterns had been conducted previously (14).

16 **Milk DNA clone library, sequencing of 16S rRNA genes of clones, individual isolates**
17 **obtained from culture media, and identification of SSCP peaks.** The dominant bacterial
18 populations of raw milk were identified both by a culture-independent approach based on
19 construction of a 16S rRNA gene clone library from DNA isolated from milk and plasmid insert
20 sequencing, and by a culture-dependent approach based on sequencing of the 16S rRNA genes of
21 individual isolates recovered from milk on the different culture media. To generate the clone
22 library, the complete 16S rRNA gene was amplified from total DNA directly obtained from F2
23 raw milk as previously described (14). The PCR product was ligated in pCR4Blunt-TOPO vector
24 and transformed into *Escherichia coli* TOP10 OneShot cells as specified by the manufacturer

1 (Invitrogen, Carlsbad, Ca., USA). Plasmid inserts were amplified by PCR with universal plasmid
2 primers T3 (5'-ATTAACCCTCACTAAAGGGA-3') and T7 (5'-
3 TAATACGACTCACTATAGGG-3') as specified by the manufacturer (Invitrogen). For both
4 amplified inserts and individual isolates, the 450 bases of the 5' end, of the 16S rRNA gene,
5 comprising the V3 region, were sequenced with the dye-terminator cycle sequencing ready
6 reaction kit with AmpliTaq DNA polymerase FS (Applied Biosystems) and an ABI Prism 310
7 Genetic Analyzer (Applied Biosystems). Each sequence obtained was identified by comparison
8 with the closest sequences available in databases (Genbank and RDP) (25).

9 To identify 16S rRNA genes liable to correspond to peaks on complex SSCP patterns
10 directly obtained from milk or cheese DNA, amplified plasmid inserts were subjected to a second
11 nested SSCP-PCR. Peaks of plasmid inserts were then compared with patterns obtained from
12 milk or cheese DNA. Similarly, to identify peaks on SSCP patterns of the bacterial cell mixtures
13 harvested from culture plates, SSCP patterns of individual isolates obtained from each culture
14 medium were compared with patterns obtained from cell mixtures collected on the same medium.

15 **Statistical analysis.** The differences in microbial counts (CFU/ml, log transformed) and
16 in richness of SSCP pattern between each farm were tested using analysis of variance (ANOVA)
17 and a subsequent Fisher's LSD test. Principal component analyses were performed from the
18 relative peak area data set. All statistical analyses were performed with Statistica software
19 (Statsoft, version 6, Tulsa, OK, USA).

20 21 **RESULTS** 22

23 **Monitoring bacterial communities from milk and cheese by direct molecular**
24 **methods.** To identify the dominant bacterial populations of raw milk, a clone library was
25 constructed from the complete 16S rRNA genes amplified from DNA isolated from the milk

1 collected from farm F2 during the winter period. Among the 125 clones analyzed, we identified
2 61 Operational and Taxonomic Units (OTUs), as defined by 97 % sequence similarity or greater.
3 Table 1 shows the closest relative species names assigned to each clone by comparison with
4 sequences from the Genbank database. The *Firmicutes* (low G + C Gram-positive bacteria) were
5 distributed over 37 OTUs (57.6 % of the total clones). Among them, *Clostridiales* constituted the
6 most frequent bacterial order, representing 24 % of the total clones. Six of the *Clostridiales*
7 clones were affiliated to *Clostridium lituseburense* (97 % sequence similarity), two other clones
8 were identified as *C. glycolicum*, but the other 22 could not be assigned to a species. The other
9 dominant *Firmicutes* belonged to the *Lactobacillales* (16%) and *Bacillales* (7.2%) with most
10 frequent OTUs affiliated to *Turicibacter sanguinis*, *Lactococcus lactis*, *Streptococcus*
11 *dysgalactiae*, *Lactobacillus casei*, *Facklamia* sp. The *Proteobacteria* were distributed over 9
12 OTUs (21.6 % of the clones), with dominant sequences affiliated to *Ralstonia pickettii*. The
13 *Actinobacteria* (high-G + C Gram-positive bacteria) were distributed over 11 OTUs (16.8 % of
14 the clones) with *Arthrobacter arilaitensis* and *Corynebacterium confusum* being the dominant
15 species. Finally, the *Bacteroidetes* were represented by 4 OTUs (4 % of the clones).

16 The diversity and dynamics of bacterial communities from milk and cheese in the three
17 farms F1, F2 and F3 were investigated through SSCP analysis based on direct DNA extraction
18 followed by PCR amplification of the V3 region of 16S rRNA genes. Ribosomal RNA genes
19 liable to correspond to peaks on SSCP patterns were identified by comparison with SSCP peaks
20 of clones. Some peaks probably resulted from the co-elution of two or more different sequences
21 as can be seen from different species assigned to identical peak numbers (Table 1).

22 The total number of peaks on SSCP patterns from milks and cheeses is summarized in
23 Table 2. The data presented are the average of three batches sampled at 3-month intervals. Milks
24 from the three farms produced complex SSCP patterns with up to 21 peaks. The total number of

1 peaks was significantly higher in Farm 2 milk than in that in Farm 1 and Farm 3. Principal
2 Component analysis of peak ratio (Fig. 1) showed that F2 milks from the three sampling periods
3 clustered together and differed from F1 and F3 milks by their higher ratio for peaks
4 corresponding to *Clostridiales* (SSCP peak no.1-3, Table 1), *C. confusum* and *Nocardioides*
5 *dubius* (peak 7), *Arthrobacter psychrolactophilus* (peak 10), uncultured rumen bacterium (peak
6 10) and *Enterobacter agglomerans* (peak 10), and two peaks with which none of the clone inserts
7 co-migrated (peaks 9 and 12). In contrast, SSCP analysis of cheese samples on days 1 and 28
8 produced identical patterns composed of a single peak corresponding to *L. lactis* and *S.*
9 *thermophilus* sequences (peak 19).

10 **Monitoring bacterial communities from milk and cheese by culture-dependent**
11 **molecular analyses.** The diversity of the dominant culturable bacteria from F2 milk and cheese
12 on M17, PCAM, CRBM and PCAI media was investigated. Table 3 sums up the number of
13 individual colonies analyzed and the dilution from which they were recovered on each medium,
14 and shows the phylogenetic affiliations of the 126 isolates obtained from F2 milk and the 170
15 isolates obtained from F2 cheese on day 28. The 24 isolates retrieved from milk on M17 agar
16 were composed of *Firmicutes* belonging to the *Lactobacillales* and *Bacillales* and of two
17 *Actinobacteria* (*Streptomyces*, *Brachybacterium*). The 38 isolates retrieved from milk on PCAM
18 agar plates were composed of *Actinobacteria* (*Microbacterium oxydans*), *Firmicutes* (*L. lactis*),
19 α -*Proteobacteria* (*Sphingomonas* sp.), γ -*Proteobacteria* (*Stenotrophomonas maltophilia*,
20 *Luteibacter rhizovicina*, *Psychrobacter faecalis*, *Moraxella osloensis*) and *Flavobacteriaceae*
21 (*Chryseobacterium* sp., *Flavobacterium* sp.). When isolates retrieved from cheese on M17 and
22 PCAM agar were analyzed, only lactic acid bacteria (*S. thermophilus*, *L. lactis* and *L. casei*) were
23 found. In contrast, with PCAI agar, a large diversity was observed among milk isolates (44
24 isolates) as well as in cheese isolates (48 isolates). It was composed of *Flavobacteriaceae*

1 (*Chryseobacterium* sp., *Flavobacterium* sp.), *Firmicutes* belonging to the *Lactobacillales*
2 (*Enterococcus faecalis*, *L. lactis*, *S. dysgalactiae* and *S. parauberis*), α -*Proteobacteria* (clone
3 NOS7.108WL), γ -*Proteobacteria* (*Klebsiella oxytoca*, *K. terrigena*, *K. trevisani*, *S. maltophilia*,
4 *L. rhizovicina*, *P. faecalis*, *M. osloensis*, *Pseudomonas reactans*, *Enterobacter aerogenes*), whose
5 distributions were different in the milk and cheese. Finally, isolates retrieved from CRBM agar
6 plates (20 from milk and 38 from cheese) mainly belonged to *Staphylococcus* (*S. fleurettii*, *S.*
7 *saprophyticus*, *S. vitulinus*, *S. haemolyticus*, *S. epidermidis*, *S. equorum*) and *Actinobacteria*
8 (*Rothia* sp., *Brevibacterium linens*, *Brachybacterium* sp., *Kocuria rhizophila*, *K. carniphila*, *A.*
9 *arilaitensis*, *Corynebacterium flavescens*), also differently distributed between milk and cheese.
10 *E. faecalis*, *Marinilactibacillus psychrotolerans*, *Bacillus pumilus* and *Aerococcus viridans* were
11 also isolated on CRBM medium. In total, considering all culture media, 36 different OTUs were
12 obtained from F2 milk and 21 OTUs from F2 cheese.

13 The diversity of the culturable communities in milks and cheeses from the three farms
14 was investigated by SSCP-PCR analysis of DNA isolated from bacterial cell suspensions
15 collected from agar plates. The number of peaks on SSCP patterns of the culturable communities
16 from milks and cheeses and the corresponding microbial counts are summarized in Table 2. The
17 data presented are the average of three batches sampled at 3-month intervals. Milk samples
18 produced complex SSCP patterns comprising from 4 to 20 peaks depending on the culture
19 medium and the farm. Levels of the culturable populations from milk on the different media were
20 relatively similar with at most a one log difference between counts on the different media. For all
21 cheeses, an overall 2 to 5 log difference was observed between counts on PCAM or M17 media
22 and counts on CRBM or PCAI media. Counts on M17 and PCAM media increased markedly on
23 day 1 reaching values around 8 log CFU/g, while SSCP analysis of cell mixtures collected from
24 M17 and PCAM media resulted in single-peak patterns corresponding to *L. lactis* and *S.*

1 *thermophilus* sequences. In contrast, cell suspensions harvested from CRBM and PCAI plates
2 produced multiple-peak patterns. Counts on CRBM medium gradually increased between days 0
3 and 28 reaching values around 5 log. At the same time, the SSCP pattern richness of communities
4 culturable on CRBM medium did not change significantly from day 0 to day 28. Counts on
5 PCAI slightly increased between days 0 and 1 and reached around 4.2 log at day 28, while the
6 PCAI medium pattern richness significantly decreased from day 1 (Table 2).

7 Sequences corresponding to peaks on SSCP patterns of the cell mixtures harvested from
8 each culture medium were identified by comparison with SSCP patterns of individual isolates
9 from F2 milk and cheese collected from the same medium. Again, some peaks likely resulted
10 from the co-elution of two or more different sequences (Table 3).

11 Results of Principal Component analysis of the peak ratio of PCAI patterns are shown on
12 Figure 2. Axis 1 and axis 2 only explained 41.35 % of the variance but showed milk patterns of
13 the three farms clustered apart from cheese patterns. PCAI culturable communities from milk
14 were characterized by higher peak ratios for peaks corresponding to *M. osloensis* and
15 *Staphylococcus pasteurii* (SSCP peak no. p12, Table 3), *S. maltophilia* (p13), *P. reactans* (p14)
16 and *S. dysgalactiae* (p15). Cheese PCAI patterns were distributed according to their ratio for
17 peaks corresponding to *Flavobacterium* sp. (p3 and p8), *Chryseobacterium* sp. (p7 and p10), *K.*
18 *terrigena* (p9), *K. trevisani* (p11), *E. faecalis* (p11), *L. rhizovicina* (p11) and *L. lactis* (p16),
19 depending on time and farm.

20 Principal Component analysis of the CRBM pattern peak ratio (Fig. 3) showed that milk
21 patterns were characterized by higher ratios for peaks 8 and 13, corresponding to four
22 *Staphylococcus* species (*S. fleuretti*, *S. saprophyticus*, *S. vitulinus* and *S. epidermidis*). Patterns of
23 1- and 28-day cheeses were distributed according to their ratios for peaks corresponding to

1 various *Actinobacteria* (SSCP peaks no. c2', c3', c5', Table 3), *E. faecalis* (c11) and *M.*
2 *psychrotolerans* (c16).

3 **Comparison of direct and culture-dependent methods.** The distribution of the different
4 OTUs recovered from the clone library obtained from direct isolation of DNA from the milk F2
5 and from the individual colonies obtained from the various culture media according to
6 phylogenetic groups is shown on Fig. 4. The diversity of *Gammaproteobacteria* phylum and
7 *Staphylococcus* genus was greater in the isolates than in the clone library, whereas the diversity
8 of *Clostridiales* and other unclassified gram-positive bacteria, and *Betaproteobacteria* was
9 greater in the clone library. Culturable species such as *C. lituseburensis* or *R. pickettii* frequently
10 found among clones were not isolated. Only nine species, comprising LAB species (*E. faecalis*,
11 *L. lactis*, *Lb. casei*) as well as *S. dysgalactiae*, *A. arilaitensis*, *K. rhizophila*, *S. equorum*,
12 *Enterobacter cloacae* and *Chryseobacterium* sp. were recovered by both direct and culture-
13 dependent methods. Other species with population levels similar to these species on culture
14 media, especially *Actinobacteria* and staphylococci, were not detected among clones.

15 On the basis of peak counts (Table 2), SSCP patterns obtained after direct amplification of
16 milk DNA showed the greatest diversity, followed by patterns of milk communities culturable on
17 PCAM, CRBM, M17 and PCAI media. As regards cheeses, only the culture-dependent approach
18 permitted investigation of the diversity of some subdominant populations beside dominant LAB
19 through the analysis of subcommunities culturable on PCAI and CRBM media.

20

21 **DISCUSSION**

22 In an attempt to monitor the diversity of non-LAB bacteria in the cheese core by a culture-
23 dependent fingerprinting technique, we adopted a strategy that takes advantage of some culture
24 media which allow growth of broad microbial groups.

1 The cultural approach is dependent on the ability of bacteria to grow on the media under
2 the culturing conditions applied, and on the number of isolates and selection methodology. In
3 addition, in our study, the viability of bacteria may also have been affected by the storage of milk
4 and cheese samples at -20°C. The advantage of SSCP analysis of cells harvested from culture
5 plates is that it generates an image of the diversity of all the colonies growing on the plate. On the
6 other hand, it is subject to bias due to differences in growth rates between the different species on
7 the culture medium. The extent of the diversity that can be revealed on a given medium may be
8 affected by the presence of a dominant population growing on that medium (15, 16,30). It may
9 also vary with the stage of the cheese-making process. Indeed, while there was no predominant
10 group of bacteria in the raw milk, marked differences in population levels were observed in
11 cheese, thus precluding detection of sub-dominant populations. Discrepancies between results of
12 direct and culture-dependent inventories of raw milk bacterial populations may also result from a
13 number of biases like differences in the efficiency of cell lysis during DNA isolation from food
14 matrix, preferential PCR amplification prior to cloning or interspecies 16S rRNA operon copy
15 number heterogeneity (8). Hence, the prevalence of *Clostridiales* among clones may partly result
16 from the high ribosomal operon copy number encountered in species from this order (21), while
17 the aerobic conditions used in culturing were not suitable for their isolation.

18 Caution should be taken when identifying bacterial populations using SSCP peak analyses
19 due to possible co-elution of different sequences. Co-elutions were observed within genus
20 (*Staphylococcus*) and across bacterial phyla (*Klebsiella trevisani* / *Enterococcus faecalis*). This
21 has been noticed in previous studies with SSCP (17) and with different fingerprinting techniques
22 (TTGE and DGGE (23, 34)). Therefore, it can be difficult to identify species reliably in complex
23 and relatively balanced bacterial communities such as those of raw milk on the sole basis of a
24 pattern database. This limitation can be partly overcome by using different sets of primers

1 targeting various variable regions (V2, V3) or specific groups of bacteria (high-GC% gram-
2 positive bacteria, (14)), or by focusing on culturable subcommunities, thus reducing the overall
3 diversity and at the same time providing isolates that can be identified.

4 The appropriateness of four culture media to monitor populations besides LAB in raw
5 cow's milks and cheeses from three farms was assessed. In this context, on the basis of 71
6 isolates, M17 agar incubated at 42°C offered an insight into the diversity of culturable aerobic
7 Gram-positive bacteria (mainly with low G + C %), when starter LAB were not highly
8 predominant, as in milk. However, growth of many additional species, among which Gram-
9 negative bacteria, may be observed on this general purpose medium if incubated at a lower
10 temperature. From the analysis of 58 isolates, CRBM appeared to be useful for assessing the
11 diversity of halotolerant *Actinobacteria* and *Staphylococcus* in milk and cheese. However, *E.*
12 *faecalis* can grow on CRBM medium, as observed by Denis *et al.* (12), and this medium also
13 revealed the presence of *Marinilactibacillus psychrotolerans* in cheese. Although the addition of
14 5 mg/l crystal violet (20) and 5 mg/l vancomycin in PCAI medium was not sufficient to
15 completely inhibit growth of Gram-positive bacteria, results from the analysis of 92 isolates
16 suggest that this medium has a good potential for investigating the diversity of the subdominant
17 *Alpha-* and *Gammaproteobacteria* and *Flavobacteriaceae* in milk and cheese.

18 Our results clearly confirmed the necessity to combine culture-dependent and –
19 independent methods to generate a more accurate view of the microbial ecology of foodstuffs, as
20 suggested by Ercolini *et al.* (16), Feurer *et al.* (17) and Miambi *et al.* (30). Direct and culture-
21 dependent analyses showed that milk bacterial communities were highly diversified. Milk from
22 Farm 2 was chosen for a detailed analysis of 16S rRNA gene sequence diversity on the basis of
23 its higher peak count on SSCP patterns, which suggested it may harbour the greatest diversity.
24 This analysis was only performed during the winter period. However, as can be seen from the

1 Principal Component analysis of SSCP patterns of DNA isolated from the milk, the dominant
2 bacterial communities of raw milk from the three farms seemed to be rather stable whatever the
3 sampling period. Hence, although the balance between bacterial populations likely varied with
4 the seasons, the diversity of the dominant populations does not seem to have changed markedly.

5 *Chryseobacterium* and *Flavobacterium* occur frequently in dairy products (20). New
6 species were recently isolated from raw milk (*C. joostei*, (18)) and from a lactic acid beverage (*C.*
7 *shigense*, (41)). Clone library analysis highlighted the occurrence of *Clostridiales*. They were not
8 affiliated to *Clostridium* species commonly associated with silage and defective cheese (24) but
9 rather to *C. lituseburensis*. This species was previously found to be predominant in cow manure
10 and dairy wastewaters, along with *Turicibacter sanguinis* (29). In addition, *Ralstonia* sp. and
11 *Dietzia* sp. have recently been found in oil field soil with high salinity (22). The culture-
12 dependent approach highlighted the presence of sub-dominant populations in cheese core, such as
13 coryneform bacteria, *Marinilactibacillus*, *Chryseobacterium*. Coryneform bacteria are commonly
14 isolated from the surface of smear-ripened cheeses (4, 31). Presumptive *Brevibacterium*,
15 *Arthrobacter* and *Micrococcus* strains were isolated from the rind of St. Nectaire-type cheese
16 after 20 days of ripening (27). *Marinilactibacillus psychrotolerans* originally isolated from the
17 marine environment (19) was recently found in French and German red-smear soft cheese surface
18 communities (17, 26). Our results support those of Duthoit *et al.* (14) who found coryneform
19 bacteria in the core of Salers cheese, another variety of uncooked, semi-hard cheese produced in
20 the Massif Central. However, although the rind was discarded, contamination of the core sample
21 by the cheese surface when sampling cannot be excluded.

22 Despite possible bias, SSCP fingerprinting of culturable communities on CRBM and
23 PCAI media was useful to overcome the problem of LAB dominance in cheese and to follow-up
24 the fate of subdominant microbial groups (*Actinobacteria*, *Staphylococcus*, *Alpha*- and

1 *Gammaproteobacteria, Flavobacteriaceae*) from milk to cheese. As in most cheeses (14, 39),
2 dramatic shifts in the composition of the microbial community were observed. The microbial
3 species added as starters (*S. thermophilus, L. lactis*) soon out-numbered the other species arising
4 from milk. However, considerable variation in the dynamics of subdominant populations between
5 cheeses was observed.

6 In conclusion, both direct and culture-dependent approaches are sources of bias but
7 produce complementary information on milk microbial ecology. The proposed method of
8 investigating the diversity of subdominant non-LAB bacteria in cheese by fingerprinting of
9 culturable communities is an interesting alternative to direct fingerprinting with general bacterial
10 primers by which these populations would be overlooked. They may be detected by specific PCR
11 amplification but this would require the use of multiple specific primers. However, group specific
12 primers may be useful to monitor populations that were only detected by the culture-independent
13 inventory of milk bacterial community, such as members of *Clostridiaceae, Aerococcaceae*
14 (*Facklamia*) and *Betaproteobacteria (Ralstonia)*. Microbial dynamics results from the combined
15 influence of microbial interactions and multiple technological and environmental factors (pH,
16 temperature, salinity...) affecting and being affected in return by microorganism activities.
17 Changes in biotic and abiotic conditions may be responsible for the limited increase in the counts
18 of the populations growing on PCAI medium, mainly gram-negative bacteria, during cheese
19 manufacture and ripening and for the concomitant drop in richness of the corresponding SSCP
20 patterns from day 1. Further work should be done to investigate the functions of persistent
21 subdominant populations, such as *Chryseobacterium*, in this system.

22

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Figure legends

FIG. 1. Principal Component Analysis of peak ratio from SSCP patterns obtained after direct amplification of milk and cheese DNA. A) Plot of peaks composing the patterns. Labels refer to SSCP peak numbers indicated in Table 1. B) Distribution of milk and cheese patterns along the first two principal components. Axis 1: first principal component, Axis 2: second principal component. \triangle milk F1, \circ milk F2, \square milk F3, \blacktriangle 1-day cheese F1, \bullet 1-day cheese F2, \blacksquare 1-day cheese F3, \blacktriangle 28-day cheese F1, \bullet 28-day cheese F2, \blacksquare 28-day cheese F3.

FIG. 2. Principal Component Analysis of peak ratio from SSCP patterns of milk and cheese subcommunities culturable on PCAI agar. A) Plot of peaks composing the patterns. Labels refer to SSCP peak numbers indicated in Table 3. B) Distribution of milk and cheese patterns along the first two principal components. Axis 1: first principal component, Axis 2: second principal component. \triangle milk F1, \circ milk F2, \square milk F3, \blacktriangle 1-day cheese F1, \bullet 1-day cheese F2, \blacksquare 1-day cheese F3, \blacktriangle 28-day cheese F1, \bullet 28-day cheese F2, \blacksquare 28-day cheese F3.

FIG. 3. Principal Component Analysis of peak ratio from SSCP patterns of milk and cheese subcommunities culturable on CRBM agar. A) Plot of peaks composing the patterns. Labels refer to SSCP peak numbers indicated in Table 3. B) Distribution of milk and cheese patterns along the first two principal components. Axis 1: first principal component, Axis 2: second principal component. \triangle milk F1, \circ milk F2, \square milk F3, \blacktriangle 1-day cheese F1, \bullet 1-day cheese F2, \blacksquare 1-day cheese F3, \blacktriangle 28-day cheese F1, \bullet 28-day cheese F2, \blacksquare 28-day cheese F3.

1 FIG. 4. Distribution of Operational and Taxonomic Units (OTUs) recovered from a clone library
2 obtained from milk DNA and individual isolates retrieved from milk on culture media, according
3 to phylogenetic groups. OTUs defined by 97 % sequence similarity or greater. ■ OTUs found
4 among culture isolates only, ■ OTUs found among clones only, □ OTUs shared by isolates and
5 clones presumably belonging to the same species according to their 97 % sequence similarity or
6 greater.

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TABLE 1. Phylogenetic affiliations of 125 cloned sequences obtained after cloning 16S rRNA genes comprised of DNA isolated from raw milk from farm F2

OTU name ^a	Number of clones ^b	SSCP peak no. ^c	Closest 16S rRNA gene sequence in Genbank	Accession no.	% Similarity	Affiliation
A7	7	5	<i>Arthrobacter arilaitensis</i>	AJ609628	99-100	<i>Actinobacteria</i>
F31	1	5	<i>Kocuria rhizophila</i>	AY030341	98	<i>Actinobacteria</i>
F13	1	5	<i>Corynebacterium sp. "Mali 33"</i>	AY211127	99	<i>Actinobacteria</i>
G27	1	6	<i>Microbacterium foliorum</i>	AJ249780	100	<i>Actinobacteria</i>
G51	1	6	<i>Dietzia maris</i>	X79291	98	<i>Actinobacteria</i>
F11	1	6	<i>Dietzia sp.</i>	X79291	96	<i>Actinobacteria</i>
G41	1	7	<i>Nocardioides dubius</i>	AY928902	98	<i>Actinobacteria</i>
F15	5	7	<i>Corynebacterium confusum</i>	Y15887	< 95	<i>Actinobacteria</i>
E20	1	10	<i>Arthrobacter psychrolactophilus</i>	AB097842	99	<i>Actinobacteria</i>
F29	1	ND	<i>Leucobacter komagatae</i>	AY838305	97	<i>Actinobacteria</i>
F44	1	ND	<i>Corynebacterium xerosis</i>	AF145257	100	<i>Actinobacteria</i>
E18	2	1-4	<i>Clostridium glycolicum</i>	AY007244	98	<i>Firmicutes, Clostridiales</i>
G14	2	1-4	<i>Clostridium lituseburense</i>	M59107	97	<i>Firmicutes, Clostridiales</i>
E28	6	1-4	<i>Clostridium lituseburense</i>	M59107	96	<i>Firmicutes, Clostridiales</i>
E10	4	1-4	<i>Clostridium lituseburense</i>	M59107	97	<i>Firmicutes, Clostridiales</i>
F17	1	1-4	<i>Clostridium lituseburense</i>	M59107	96	<i>Firmicutes, Clostridiales</i>
G1	2	1-4	uncultured <i>Clostridiaceae</i>	AB126280	98	<i>Firmicutes, Clostridiales</i>
G2	3	1-4	uncultured <i>Clostridiaceae</i>	AY511259	98	<i>Firmicutes, Clostridiales</i>
F26	1	1-4	uncultured <i>Clostridiaceae</i>	AY457751	< 95	<i>Firmicutes, Clostridiales</i>
F34	1	1-4	uncultured <i>Clostridiaceae</i>	DQ238609	< 95	<i>Firmicutes, Clostridiales</i>
F48	1	1-4	uncultured <i>Clostridiaceae</i>	AY978077	< 95	<i>Firmicutes, Clostridiales</i>
B12	2	1-4	uncultured <i>Clostridiales</i>	AJ409008	95	<i>Firmicutes, Clostridiales</i>
B43	2	1-4	uncultured <i>Clostridiales</i>	AB100469	< 95	<i>Firmicutes, Clostridiales</i>
G29	1	1-4	uncultured rumen <i>Clostridiales</i>	DQ937239	99	<i>Firmicutes, Clostridiales</i>
G30	1	1-4	uncultured <i>Clostridiales</i>	AY578501	< 95	<i>Firmicutes, Clostridiales</i>
G47	1	1-4	uncultured <i>Clostridiales</i>	AF018544	97	<i>Firmicutes, Clostridiales</i>
C1	1	1-4	uncultured rumen Gram +	AB009232	< 95	<i>Firmicutes, unaffiliated</i>
C4	1	5	uncultured Gram +	AY511299	< 95	<i>Firmicutes, unaffiliated</i>
A9	1	NC	adult human fecal bacterium	AF132283	98	<i>Firmicutes, unaffiliated</i>
E27	1	8	uncultured Gram +	AB107470	97	<i>Firmicutes, unaffiliated</i>
E8	1	10	uncultured rumen bacterium	AB034006	98	<i>Firmicutes, unaffiliated</i>
E11	1	13	<i>Enterococcus faecalis</i>	AB098122	97	<i>Firmicutes, Lactobacillales</i>
G50	1	14	<i>Staphylococcus warneri</i>	AY186059	98	<i>Firmicutes, Bacillales</i>
E4	6	14	<i>Turcibacter sanguinis</i>	AF349724	97-98	<i>Firmicutes, Bacillales</i>
E9	1	15	<i>Staphylococcus equorum</i>	AY688054	99	<i>Firmicutes, Bacillales</i>
C33	1	15	swine manure pit bacterium PPC98	AF445247	95	<i>Firmicutes, unaffiliated</i>
A5	3	15	swine manure pit bacterium PPC16	AF445295	99-100	<i>Firmicutes, unaffiliated</i>
G39	1	16	<i>Jeotgaliococcus psychrophilus</i>	AY028926	98	<i>Firmicutes, Bacillales</i>
G5	4	16	<i>Lactobacillus casei</i>	AY244628	97-99	<i>Firmicutes, Lactobacillales</i>
A34	5	17	<i>Streptococcus dysgalactiae</i>	AY584478	98-100	<i>Firmicutes, Lactobacillales</i>
E6	1	18	uncultured Gram +	AY916353	< 95	<i>Firmicutes, unaffiliated</i>
E15	3	18	<i>Facklamia tabacinasalis</i>	Y17820	96	<i>Firmicutes, Lactobacillales</i>
E26	1	19	<i>Lactobacillus kefiranofaciens</i>	AM113782	99	<i>Firmicutes, Lactobacillales</i>
B52	5	19	<i>Lactococcus lactis</i>	AF515226	99-100	<i>Firmicutes, Lactobacillales</i>
B9	1	19	<i>Streptococcus thermophilus</i>	AY188354	100	<i>Firmicutes, Lactobacillales</i>
F23	1	ND	uncultured rumen Gram +	AB185814	98	<i>Firmicutes, unaffiliated</i>
F30	1	ND	uncultured rumen Gram +	AB185646	< 95	<i>Firmicutes, unaffiliated</i>
G40	1	ND	uncultured rumen Gram +	AF018557	95	<i>Firmicutes, unaffiliated</i>
G35	1	5	<i>Mesorhizobium amorphae</i>	DQ022832	97	<i>Alphaproteobacteria</i>

E29	2	5	<i>Bradyrhizobium japonicum</i>	DQ133342	100	<i>Alphaproteobacteria</i>
A1	3	6	<i>uncultured bacterium from aphids</i>	AB074647	96-97	<i>Proteobacteria, unaffiliated</i>
A30	1	NC	<i>unidentified rumen bacterium</i>	AF001775	96	<i>Proteobacteria, unaffiliated</i>
C41	1	NC	<i>uncultured bacterium Pla 09</i>	AY578361	< 95	<i>Proteobacteria, unaffiliated</i>
C26	1	10	<i>Enterobacter agglomerans</i>	AY315451	100	<i>Gammaproteobacteria</i>
A12	15	15	<i>Ralstonia pickettii</i>	AY268177	97-100	<i>Betaproteobacteria</i>
C17	1	16	<i>Alcaligenes sp.</i>	X86512	95	<i>Betaproteobacteria</i>
A14	2	16	<i>Acinetobacter lwoffii</i>	AF189695	97	<i>Gammaproteobacteria</i>
B34	2	11	<i>Chryseobacterium sp.</i>	AJ279493	95	<i>Bacteroidetes, Flavobacteriales</i>
A2	1	NC	<i>clone B1hii40</i>	AJ318144	< 95	<i>Bacteroidetes, unaffiliated</i>
A24	1	NC	<i>uncult rumen Bacteroidetes</i>	AB185710	< 95	<i>Bacteroidetes, unaffiliated</i>
A31	1	17	<i>Sphingobacterium sp.</i>	AF492000	< 95	<i>Bacteroidetes, unaffiliated</i>
Total	125					

^aOperational Taxonomic Units (OTUs) defined by 97 % sequence similarity or greater.

^bNumber of clones of this OTU in the library

^cRefers to number (no.) of peak on the SSCP pattern of milk DNA reported in Fig. 1 with which SSCP peak of clone was found to co-migrate

ND: undetermined, NC: not co-migrating with any of the dominant peaks of milk SSCP pattern

TABLE 2. Number of peaks^a of SSCP patterns obtained after direct amplification of milk or cheese DNA of farms F1, F2 and F3, microbial counts^b in milks and cheeses and number of peaks^a of SSCP patterns obtained after cultivation on four culture media.

Time (days)	Farm	Direct amplification		Subcommunities culturable on media:															
		Number of SSCP peaks		M17		PCAM		CRBM		PCAI									
				Number of SSCP peaks	Microbial count	Number of SSCP peaks	Microbial count	Number of SSCP peaks	Microbial count	Number of SSCP peaks	Microbial count	Number of SSCP peaks	Microbial count						
0	F1	12,7	a	9,7	NS	3,05	b	11,7	a	3,62	ab	10,0	NS	2,91	NS	7,7	ab	2,18	a
0	F2	19,3	b	10,3	NS	2,85	ab	18,0	b	3,82	b	10,7	NS	2,87	NS	11,0	b	3,31	b
0	F3	13,7	a	8,3	NS	2,37	a	11,3	a	3,00	a	9,3	NS	2,56	NS	5,0	a	1,96	a
1	F1	1,0	NS	1,0	NS	8,30	ab	1,0	NS	7,73	NS	11,0	NS	3,34	NS	4,3	NS	4,32	NS
1	F2	1,0	NS	1,0	NS	7,40	a	1,0	NS	8,43	NS	7,0	NS	4,27	NS	5,0	NS	3,67	NS
1	F3	1,0	NS	1,0	NS	8,70	b	1,0	NS	7,51	NS	9,7	NS	4,00	NS	2,3	NS	3,57	NS
28	F1	1,0	NS	1,0	NS	8,15	b	1,0	NS	8,14	NS	9,3	NS	4,81	NS	5,7	NS	4,15	NS
28	F2	1,0	NS	1,0	NS	7,38	a	1,0	NS	8,25	NS	9,3	NS	5,18	NS	5,3	NS	4,20	NS
28	F3	1,0	NS	1,0	NS	8,60	b	1,0	NS	7,95	NS	9,7	NS	4,75	NS	2,3	NS	4,21	NS

^a Only peaks with relative area above 1% of the total area of the profile were considered.

^b Microbial counts are in log (CFU/ml) for milk or log (CFU/g) for cheese.

The values are the means obtained from the three sampling periods. For each sampling day, letters a, b and c in the same column indicate whether number of peaks or microbial count were significantly different between the three farms according to Fisher's LSD statistical test, with a<b<c. P<0.01, NS : non significant.

TABLE 3. Phylogenetic affiliations of isolates recovered from milk and cheese from farm F2, on four culture media

Medium	Strain name	Number in milk	Number in 28-day cheese	SSCP peak no. ^a	Closest 16S rRNA sequence in Genbank	Accession no.	% Similarity	Affiliation
M17	M25	1			<i>Streptomyces</i> sp.	X80827	98	Actinobacteria
M17	M27	1			<i>Brachybacterium</i> sp. <i>S21F1</i>	AF041790	97	Actinobacteria
M17	M3	2			<i>Enterococcus faecalis</i>	DQ239694	99	Firmicutes
M17	M2	2			<i>Bacillus pumilus</i>	AB020208	99	Firmicutes
M17	M4	1			<i>Staphylococcus pasteurii</i> MS031	AY126212	98	Firmicutes
M17	M1	4			<i>Staphylococcus haemolyticus</i>	AY688061	97	Firmicutes
M17	M12	7	1		<i>Lactobacillus casei</i>	AY699577	99	Firmicutes
M17	M11	2			<i>Streptococcus dysgalactiae</i>	AY584478	98	Firmicutes
M17	M10	3	34		<i>Lactococcus lactis</i>	AE006456	99	Firmicutes
M17	MJ1		12		<i>Streptococcus thermophilus</i>	CP000024	100	Firmicutes
M17	M9	1			<i>Lactococcus garvieae</i>	AY438044	97	Firmicutes
Total		24	47					
		(-1)*	(-5)*					
PCAM	PA1	10			<i>Microbacterium oxydans</i>	AJ717357	99	Actinobacteria
PCAM	PA9	1			<i>Microbacterium lacticum</i>	X77441	96	Actinobacteria
PCAM	PA24	3			<i>Microbacterium laevaniformans</i>	AF535159	99	Actinobacteria
PCAM	PA2	3			<i>Sphingomonas</i> sp. <i>SKJH-30</i>	AY749436	98	Alphaproteobacteria
PCAM	PA27	6			<i>Chryseobacterium</i> sp. <i>JIP 17/96</i>	AY468461	97	Bacteroidetes
PCAM	PA6	2			<i>Flavobacterium</i> sp. <i>EP241</i>	AF493644	99	Bacteroidetes
PCAM	PA26	1			<i>Flavobacterium</i> sp.	DQ205296	96	Bacteroidetes
PCAM	PA3	1			<i>Luteibacter rhizovicina</i>	AY785744	99	Gammaproteobacteria
PCAM	PA19	1			<i>Psychrobacter faecalis</i>	AJ421528	99	Gammaproteobacteria
PCAM	PA35	1			<i>Psychrobacter</i> sp. <i>AI-2</i>	AY437611	99	Gammaproteobacteria
PCAM	PA36	2			<i>Moraxella osloensis</i>	AJ508366	99	Gammaproteobacteria
PCAM	PA13	1			<i>Stenotrophomonas maltophilia</i>	AJ293464	99	Gammaproteobacteria
PCAM	PJA 7		1		<i>Lactobacillus casei</i>	AY699577	98	Firmicutes
PCAM	PA39	6	36		<i>Lactococcus lactis</i>	AE006456	98	Firmicutes
Total		38	37					
		(-2)*	(-6)*					
CRBM	C2	3		c1-c4	<i>Rothia-like</i> sp. <i>CCUG 35957</i>	AJ131121	97	Actinobacteria
CRBM	C3	2		c1-c4	<i>Brevibacterium linens</i> AC825	AY017070	99	Actinobacteria
CRBM	C4	1		c1-c4	<i>Brachybacterium</i> sp. <i>S21F1</i>	AF041790	96	Actinobacteria
CRBM	C20	1		c1-c4	<i>Kocuria rhizophila</i>	AF542072	100	Actinobacteria
CRBM	C6	1		c1-c4	<i>Kocuria carniphila</i>	AJ622907	98	Actinobacteria
CRBM	C15	1	2	c1-c4	<i>Arthrobacter arilaitensis</i>	AJ609628	97-100	Actinobacteria
CRBM	CJ43		1	c5'	<i>Corynebacterium flavescens</i>	X84441	99	Actinobacteria
CRBM	CJ6		21	c8, 9, 14	<i>Staphylococcus fleuretti</i>	AY688058	100	Firmicutes
CRBM	CJ20		1	c8, 9, 14	<i>Staphylococcus saprophyticus</i>	AP008934	99	Firmicutes
CRBM	CJ30		1	c8, 9, 14	<i>Staphylococcus vitulinus</i>	AB009946	98	Firmicutes
CRBM	C1	3	2	c11	<i>Enterococcus faecalis</i>	DQ239694	99	Firmicutes
CRBM	C5	2		c12	<i>Staphylococcus haemolyticus</i>	AJ309920	98	Firmicutes
CRBM	C13	1		c12	<i>Aerococcus viridans</i>	M58797	99	Firmicutes
CRBM	C14	2		c12	<i>Bacillus pumilus</i>	AB020208	99	Firmicutes
CRBM	C19	1		c13	<i>Staphylococcus epidermidis</i>	AY458861	100	Firmicutes
CRBM	C8	2		c15	<i>Staphylococcus equorum</i>	AY688055	99	Firmicutes
CRBM	CJ9		10	c16	<i>Marinilactibacillus psychrotolerans</i>	AB159717	99	Firmicutes
Total		20	38					
		(-1)*	(-2)*					
PCAI	P33	1		-	<i>Brevundimonas nasdae</i>	AB071954	98	Alphaproteobacteria
PCAI	P7	7		p1	clone <i>NOS7.108WL</i>	AY043628	98	Alphaproteobacteria
PCAI	P28	1	1	p2	<i>Enterobacter agglomerans</i>	AF130907	99	Gammaproteobacteria
PCAI	P4	3		p3-4, 8	<i>Flavobacterium</i> sp. <i>EP241</i>	AF493644	97	Bacteroidetes
PCAI	P1	10	6	p7, p10	<i>Chryseobacterium</i> sp. <i>JIP 17/96</i>	AY468461	98	Bacteroidetes
PCAI	PI11		4	p8	<i>Klebsiella oxytoca</i>	AY873801	99	Gammaproteobacteria
PCAI	PI 19		1	p9	<i>Klebsiella terrigena</i>	Y17670	99	Gammaproteobacteria
PCAI	PI37		1	p11	<i>Klebsiella trevisani</i>	AF129444	99	Gammaproteobacteria

PCAI	P12	9	20	p11	<i>Enterococcus faecalis</i>	DQ239694	99	<i>Firmicutes</i>
PCAI	P46			p11	<i>Luteibacter rhizovicina</i>	AY785744	99	<i>Gammaproteobacteria</i>
PCAI	P19	2	4	-	<i>Psychrobacter faecalis</i>	AJ421528	99	<i>Gammaproteobacteria</i>
PCAI	PII0		2	-	<i>Enterobacter aerogenes</i>	AB099402	99	<i>Gammaproteobacteria</i>
PCAI	PII5		1	-	<i>Citrobacter freundii</i>	AB210978	98	<i>Gammaproteobacteria</i>
PCAI	P20	3		p12	<i>Moraxella osloensis</i>	AJ508366	100	<i>Gammaproteobacteria</i>
PCAI	PII4		1	p12	<i>Staphylococcus pasteurii</i>	AB009944	100	<i>Firmicutes</i>
PCAI	PII		3	p13	<i>Stenotrophomonas maltophilia</i>	AJ293464	99	<i>Gammaproteobacteria</i>
PCAI	P38	1		p14	<i>Pseudomonas reactans</i>	AY747594	99	<i>Gammaproteobacteria</i>
PCAI	P26	1		p15	<i>Streptococcus dysgalactiae</i>	AY584478	98	<i>Firmicutes</i>
PCAI	P2	4		p16	<i>Lactococcus lactis</i>	AE006456	98	<i>Firmicutes</i>
PCAI	P24	1	4	-	<i>Streptococcus parauberis</i>	AF284579	98	<i>Firmicutes</i>
Total		44	48					
		(-1)*	(-2)*					

^aRefers to peak labels indicated on Fig. 2 and Fig. 3

* dilution at which isolates were recovered.

ACCEPTED

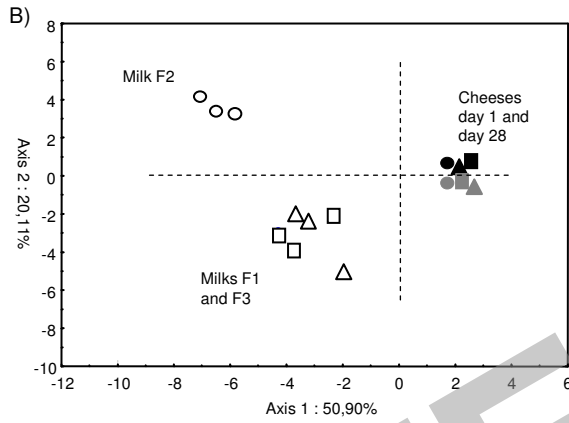
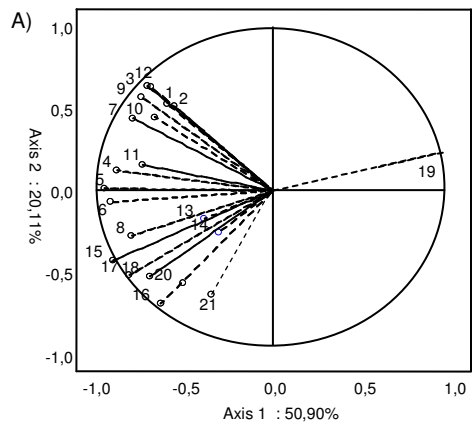


FIG. 1.

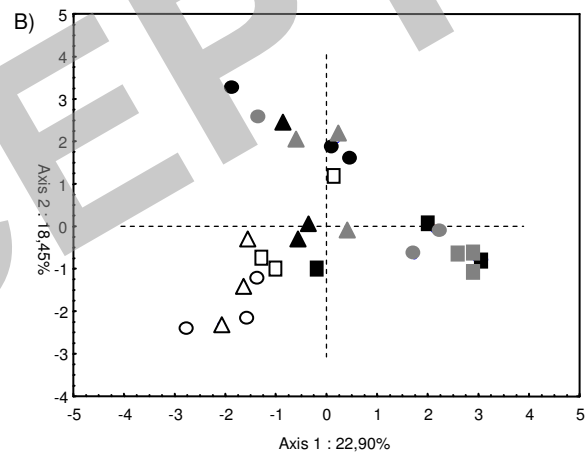
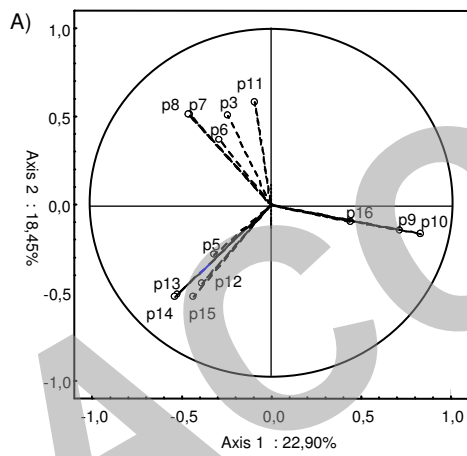


FIG. 2.

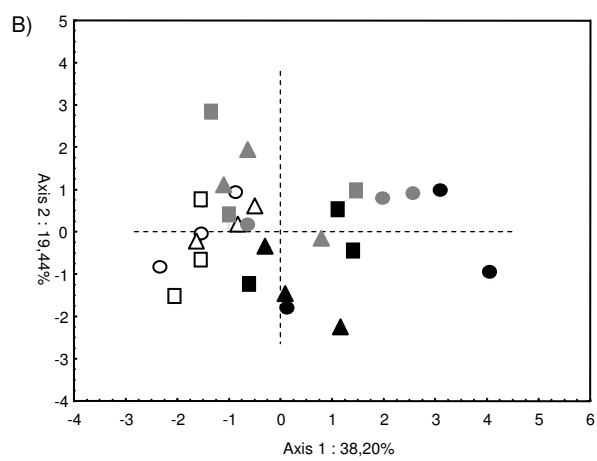
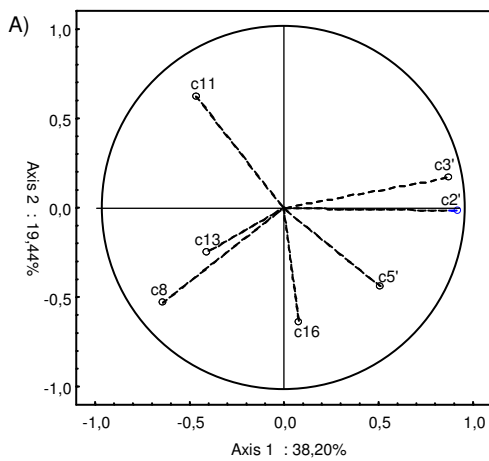


FIG. 3.

FIG. 4.

