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Application of Single Strand Conformation Polymorphism — PCR method for distinguishing cheese bacterial communities that inhibit *Listeria monocytogenes*

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Received 14 June 2006; received in revised form 17 November 2006; accepted 18 December 2006

Abstract

The aim of this study was to compare the microbial communities of different cheeses where *Listeria monocytogenes* either grew or did not grow. For this purpose, (i) isolates from the most inhibitory cheese ecosystem were identified and their ability to produce anti-*Listeria* substances was determined, (ii) bacterial communities of cheeses with and without *L. monocytogenes* growth were compared using the Single Strand Conformation Polymorphism method.

The study showed SSCP to be an effective tool for differentiating between the bacterial communities of different cheeses manufactured with the same technology. All the cheeses with the lowest *L. monocytogenes* counts on day 8 were distinguished by the dominance in their SSCP profiles, after amplification of the V2 region of the 16S rRNA gene, of 3 peaks whose nucleotide sequences comigrated with *Enterococcus faecium* and *Enterococcus saccharominimus*, *Chryseobacterium* sp and *Corynebacterium flavescens*, *Lactococcus garvieae* and *Lactococcus lactis* respectively. However, no anti-*Listeria* compounds were produced under our experimental conditions.

These six bacterial species were inoculated, separately or together, into pasteurised milk and their anti-listerial activity in cheese was evaluated. The area of inhibition between the control and trial curves confirmed that *L. monocytogenes* is inhibited by *E. saccharominimus*, *C. flavescens*, *L. lactis*, *L. garvieae* and the mixture of all six bacterial strains.

Further studies should be performed to determine the metabolites involved in *L. monocytogenes* inhibition.
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Keywords: Listeria monocytogenes; Inhibition; Raw-milk cheeses; SSCP; Anti-*Listeria* compounds

1. Introduction

The safety of dairy products has been greatly improved throughout the food production chain by the implementation of Good Manufacturing Practices (GMP) and Good Hygienic Practices (GHP) and by the development and increasing application of the semi-quantitative Hazard Analysis Critical Control Point (HACCP) system in "farm-to-fork" systems.

Safety can also be improved by eliminating milk pathogens by pasteurising or microfiltering raw milk. However, processing milk in these ways may have adverse effects on the typical organoleptic and sensory characteristics of traditional cheeses and does not prevent contamination by certain pathogens such as *Listeria monocytogenes*. Bio-preservation strategies based on the addition of inhibitory bacterial strains have been widely studied. Selecting bacterial strains that produce nisin (Maisnier-Patin et al., 1992; Olasupo et al., 1999) or other bacteriocins (Nunez et al., 1997; Ennahar and Deschamps, 2000; Buyong et al., 1998; Loessner et al., 2003) and/or organic acids (Ramsaran et al., 1998; Morgan et al., 2001; Millet et al., 2006) has been investigated as a method for inhibiting *L. monocytogenes* growth in cheeses. However, effective applications are scarce and the long-term effectiveness

of inhibitory strains has been questioned, mainly because of the occurrence of pediocin- and nisin-resistant pathogenic strains (Gravesen et al., 2002). A promising strategy could result from recent studies based on the hypothesis that natural flora can compete with food pathogens in cheese. Indeed, it has been shown that some ripening consortia from smear-ripened cheese (Maoz et al., 2003), and microbial communities from raw milk (Millet et al., 2006; Gay and Amgar, 2005) can inhibit *L. monocytogenes*.

The mechanism of inhibition in smear-ripened cheeses has yet to be understood (Maoz et al., 2003). The inhibition observed by Millet et al. (2006) in some raw-milk cheeses may be partly due to lower pH and acid production, but the microbial populations involved have not been identified. In complex cheese ecosystems, one method for identifying the populations involved in inhibition is to isolate the different strains that make up the bacterial community with anti-*Listeria* properties (Maoz et al., 2003). Such an approach is time-consuming and the strains may behave quite differently in laboratory media and in food products. A new approach for identifying the microbial populations associated with inhibition can be proposed, based on comparing the microbial communities of raw-milk cheeses where *L. monocytogenes* grows while others do not. This can be achieved by applying molecular tools that help describe the dynamics of microbial communities as a whole without having to culture any micro-organisms. Among these techniques, Temporal Temperature and Denaturing Gradient Gel Electrophoresis (TTGE and DGGE) (Cocolin et al., 2004; Ercolini et al., 2004; Ogier et al., 2004) and Single Strand Conformation Polymorphism (SSCP) (Duthoit et al., 2003, 2005) have been applied to food microbial ecology with increasing success.

The aim of this study was to identify microbial populations which can be involved in the inhibition of *L. monocytogenes* in cheeses previously studied by Millet et al. (2006). Two approaches were combined for this purpose. In the first, the isolates from most of the inhibitory cheese ecosystem were identified and their ability to produce anti-*Listeria* substances was determined. In the second, the SSCP method was used to compare the bacterial communities of cheeses with and without *L. monocytogenes* growth.

2. Materials and methods

2.1. Cheese samples

The cheese samples were those studied by Millet et al. (2006). As described by those authors, they were manufactured according to a Saint-Nectaire type technology using raw milk from six different farms (F1, F2, F3, F4, F5 and F6). With each milk, cheese was made three times over a one-month period. This study specifically investigated cheeses ripened for 8 days, because the differences in *L. monocytogenes* counts have been shown to be greatest after this ripening period. Moreover, most of the microbial flora counted in different media have been shown to be at the highest level at day 8 (Millet et al., 2006). Two groups of cheeses were

selected according to their respective *L. monocytogenes* counts. The first group, the F1, F2 and F3 cheeses, consisted of those with the highest *L. monocytogenes* counts, ranging between 1.26 and 3.58 log (CFU/ml). The second group, F3, F4 and F5, had the lowest *L. monocytogenes* counts (0.1 to 2.31 log CFU/ml).

2.2. Total genomic DNA extraction

Total genomic DNA was extracted from 1 g cheese samples on day 8 by a phenol–chloroform method including treatment with zirconium beads as described by Duthoit et al. (2003).

2.3. PCR amplification

Total DNA extracted from cheeses sampled on day 8 was amplified with two sets of primers (Table 1): w34–w49 and V2F–V2R.

Forward primers w49 and V2F were labelled with 5′-fluorescein phosphoramidite HEX and reverse primers w34 and V2R with 3′-fluorescein phosphoramidite NED. All the primers were synthesised by Applied Biosystems (Foster City, USA).

PfuUltra™ High-Fidelity DNA polymerase (Stratagene Europe, Amsterdam Zuidoost, Netherlands) was used for all PCR amplifications. PCR was performed in a total volume of 25 μl containing 10 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1 μl of cheese-extracted DNA, 1× buffer and 1.25 U of PfuUltra™ High-Fidelity polymerase.

All the reaction mixtures were subjected to 30 amplification cycles in a thermocycler model 9700 (Perkin Elmer, Applied Biosystems, Foster City, USA). PCR amplification conditions are reported in Table 1. PCR products were electrophoresed on a 0.8% agarose gel, in 0.5× Tris borate EDTA buffer (89 mM

Table 1
PCR primers and amplification conditions used in this study

Primers	Sequence	Amplification conditions	References
w34	TTACCGCGGCGTGCTGGCAC	3 min at 96 °C	Zumstein et al. (2000)
w49	ACGGTCCAGACTCCTACGGG	30 cycles of: 30 s at 96 °C; 30 s at 61 °C; 30 s at 72 °C; 10 min at 72 °C	Delbes et al. (1998)
V2F	CGCGAACGGGTGAGTAA	3 min at 96 °C	Duthoit et al. (2003)
V2R	ACTGCTGCCTCCCGTAG	30 cycles of: 30 s at 96 °C; 30 s at 55 °C; 45 s at 72 °C; 10 min at 72 °C	Duthoit et al. (2003)
w02	NTACCTTGTTACGACTT	5 min at 96 °C	Godon et al. (1997)
w18	GAGTTTGATCMTGGCTCAG	25 cycles of: 60 s at 96 °C; 30 s at 50 °C; 60 s at 72 °C	Godon et al. (1997)

Table 2
Agar media, incubation time and incubation temperature used in this study

	Incubation time/ temperature (°C)		References
Medium 17 (M17)	72 h	30	Terzaghi and Sandine (1975)
Medium 17	48 h	42	Terzaghi and Sandine (1975)
Rabbit Plasma Fibrinogen (RPF)	24 h	37	De Buyser et al. (2003)
Slanetz Bartley (SB)	48 h	42	Slanetz and Bartley (1957)
Oxytetracycline Glucose Agar (OGA)	from 3 to 4 days	25	FIL-IDF 94B (1991)
Cephalidine Fucidine Cetramidine (CFC)	from 48 to 72 h	25	NF V 04-504 (1988)
Facultatively Heterofermentative (FH)	72 h	30	Isolini et al. (1990)
Turner (T)	72 h	30	Turner et al. (1963)
Turner + nalidixic acid (T + n.a.)	72 h	30	Turner et al. (1963)
Plate Count Agar + Milk (PCA + M)	3 days	30	FIL-IDF 100B
PCA + M + Gram-positive inhibitory	3 days	30	FIL-IDF 100B
Nickels and Leesment (NL)	72 h	25	Vogensen et al. (1987)
Mayeux Sandine Elliker (MSE)	72 h	30	Mayeux et al. (1962)
Cheese Ripening Bacteria Medium (CRBM)	5 and 10 days	25	Denis et al. (2001)
Violet Red Bile Glucose (VRBG) agar	24 h	42	NF V 08-021 (1985)

All the media were incubated aerobically except the FH medium which was incubated anaerobically.

Tris base, 89 mM borate, 2 mM EDTA) containing 5% ethidium bromide. DNA bands were visualised under UV light.

2.4. SSCP capillary electrophoresis

PCR products were purified with a Strata Prep™PCR purification Kit (Stratagene Europe, Amsterdam Zuidoost, Netherlands) following the manufacturer's recommendations.

A mixture containing 18.75 µl deionised formamide (Applied Biosystems, Foster City, USA), 0.25 µl internal DNA molecular weight standard Genescan 400 HD Rox (Applied Biosystems, Foster City, USA) and 1 µl purified PCR product was denatured for 5 min at 95 °C and immediately cooled on ice. The mixture was then electrophoresed by SSCP capillary electrophoresis on an ABI Prism 310 genetic analyser (Applied Biosystems, Foster City, USA) as described by Duthoit et al. (2003).

2.5. Assignation of dominant peaks

Dominant peaks in the SSCP patterns were assigned by comparing their migration with the migration of cloned 16S rRNA gene sequences, or reference strains, or bacterial strains isolated from Saint-Nectaire cheese, or bacterial strains isolated from retentate raw-milk F5.

A clone library was prepared using genomic DNA extracted from the cheese sample showing the highest number of peaks on its SSCP profile. The 16S rRNA gene was amplified with universal primers w02 and w18. In a total volume of 50 µl, the PCR mixture contained 10 mM Tris HCl (pH 8.5), 25 mM KCl, 5 mM $(NH_4)2SO_4$, 2 mM $MgSO_4$, 0.1 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1 U of Pwo polymerase (Roche, Applied Science Division, Meylan Cedex, France) and 2 µl of extracted DNA. Amplification was performed as indicated in Table 1.

The amplified product was cloned into pCR-Blunt-II-TOPO vector and transformed in *Escherichia coli* cells following the manufacturer's instructions (Invitrogen SARL, Cergy Pontoise Cedex, France). Plasmid inserts of 94 clones were amplified by PCR with M13 forward and M13 reverse

Table 3
List of microbial species assigned on the basis of over 98% similarity between their partial 16S rRNA sequences and those in databases (NCBI databases using the BLAST program)

Bacteria	ni		
Lactococcus lactis	10		
Lactococcus garvieae	7		
Enterococcus faecalis	20		
Enterococcus saccharominimus	8		
Enterococcus malodoratus	1		
Enterococcus hirae	1		
Lactobacillus casei	1	Lactic bacteria	
Lactobacillus plantarum	2		
Lactobacillus farciminis	2		
Lactobacillus parabuchneri	2		
Lactobacillus paracase	3		
Lactobacillus curvatus	4		
Leuconostoc citreum	4		
Leuconostoc pseudomesenteroides	1		
Staphylococcus aureus	9		Gram-positive bacteria
Staphylococcus xylosus	4		
Staphylococcus saprophyticus	1		
Staphylococcus equorum	3		
Staphylococcys xylosus or Staphylococcus equorum	1		
Arthrobacter nicotianae	1		
Microbacterium sp.	1		
Corynebacterium flavescens	1	Non-lactic bacteria	
Corynebacterium casei	1		
Brevibacterium linens	1		
Rothia sp.	1		
Macrococcus caseolyticus	1		
Brachybacterium rhamnosus	1		
Brachybacterium alimentarium	1		
Kocuria sp.	3		
Kocuria rhizophilia	1		
Exiguobacterium sp.	2		
Pseudomonas sp	1		
Pseudomonas umsongensis	1		
Pseudomonas putida	16		
Stenotrophomonas malptophilia	2		Gram-negative bacteria
Enterobacter amnigenus	14		
Enterobacter sp.	3		
Chryseobacterium sp.	7		
Comamonas testoteroni	1		
Acinetobacterium sp.	2		

ni: number of isolates.

primers. The 16S rRNA gene clones were sequenced with primer w34 on an ABI prism 310 genetic analyser (Applied Biosystems, Foster City, USA) using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, USA). Each sequence (approximately 300 bp) was compared with sequences in the GenBank database, using the NCBI BLAST program.

2.6. Statistical analysis

The proportion of peaks present in the SSCP profiles obtained with each primer was calculated using the following formula: $P_i = a_i * 100 / \sum a_i$, where P_i was the ratio of peaks and a_i the area of one peak.

The relationship between the P_i values in the (V2F–V2R)–SSCP profiles of cheeses on day 8 was shown as a dendrogram constructed by the Unweighted Pair Group Method using Average (UPGMA) with statistical software (StatSoft 2003, Statistica version 6.1, Tulsa, USA).

Principal Component Analyses (PCA) were carried out with the P_i values in the (Gram1F–Gram2R)–SSCP profiles of cheeses on day 8 using statistical software (StatSoft 2003, Statistica version 6.1, Tulsa, USA).

2.7. Anti-Listeria activity assessment by the agar-well diffusion method

The anti-*Listeria* activity of the raw-milk microbial community expressing the strongest *Listeria*-inhibiting potential in cheese according to Millet et al. (2006) was assessed. The raw milk was then microfiltered on an MFS-1 microfiltration pilot plant with a capacity of 600 l/h (Alpha-Laval, Les Clayes-sous-Bois, France) and the composition of the microbial retentate (raw-milk microbial fraction trapped in a 1.2-µm pore membrane), which is representative of milk microbial community, was analysed. The various microbial populations composing the retentate were cultured in duplicate on 15 types of media (Table 2). All colonies grown on each nutrient agar medium were collected in a 5% solution of sterile glycerol and stored at −20 °C to be tested for their anti-*Listeria* activity as described below.

Ten to twelve colonies were also randomly picked from each duplicate culture agar medium, for identification. A total of 143 isolates were identified by sequencing approximately 300 bp of the 5′ end of the 16S rRNA gene (as described above) and comparing each sequence with sequences in GenBank using the NCBI BLAST program.

The whole colonies collected on different agar media and the isolates were each grown individually for 24 h in an appropriate broth medium and at a suitable incubation temperature. Culture broths were centrifuged at 5000 *g* for 15 min at 4 °C. The pH of supernatants obtained was adjusted to pH 6. Supernatants were filtered on a 0.45 µm membrane filter (Minisart Sartorius, Hannover, Germany). The anti-listerial properties of the supernatants were tested using the Tagg and McGiven (1971) agar-well diffusion method modified by the use of a double agar layer. Petri dishes were filled with 20 ml

of neutral pH nutrient agar. The top layer consisted of 10 ml of Trypticase Soja–Yeast Extract (TS–YE) agar medium previously inoculated with 0.1 ml of an 18 h-old TS–YE broth culture of *L. monocytogenes*. Holes (∅ 6 mm) were pierced through the top agar, the wells were filled with 0.1 ml of culture filtrate and the pH was neutralised. The inoculated plates were incubated for 24 h at 37 °C. The diameter of the inhibition zone was measured.

2.8. Anti-Listeria activity assessment in pasteurised milk cheeses

The anti-listerial activities of some species were also evaluated by making pasteurised milk cheeses. The bacterial species tested were selected on the basis of the SSCP results.

All the milks pasteurised at 71 °C for 30 s were inoculated with the starter *Streptococcus thermophilus* at a concentration of 10^7 CFU/ml and *L. monocytogenes* strain S2 at 5 to 10 CFU/ 25 ml. Other species were then inoculated individually into the pasteurised milks as follows.

Chryseobacterium spp., *Enterococcus faecium* and *Enterococcus saccharominimus* were inoculated at 10^2 CFU/ml, *Corynebacterium* spp. at 10^3 CFU/ml, *Lactococcus lactis* and *Lactococcus garvieae* at 10^5 CFU/ml. The level of inoculation was chosen according to their bacterial counts in milk F5 (Millet et al., 2006 and unpublished data). One milk was only inoculated with *S. thermophilus* to act as a control.

Prior to inoculation, all the bacteria were prepared as described above (see above, Anti-Listeria activity assessment by the agar-well diffusion method).

For each set of experiment, seven batches of cheeses were made with milks inoculated in this way, according to Saint-Nectaire type technology.

After one day of ripening, the cheeses were coated with an orange wax containing 1000 ppm of natamycin (Coquard, Villefranche sur Saône, France).

All cheeses were ripened for 28 days in INRA's ripening cellars under identical conditions, at 10 °C with 95% relative humidity.

The experiment was performed twice over a two-week period.

2.9. Sampling and pH determination of pasteurised milk cheeses

The cheeses were sampled for microbial and chemical analysis on days 1, 8, 18 and 28. For each cheese sample, pH was measured with a 926 VTV pH-meter with Ingold electrode 406 MX (Mettler-Toledo S.A., Viroflay, France). Cheese samples were prepared as described by Millet et al. (2006).

2.10. Enumeration of bacteria in pasteurised milk cheeses

L. monocytogenes concentrations were determined on PALCAM media. The genus and species of five presumed *Listeria* colonies were then confirmed according to norm ISO 11290-2.

Populations of *S. thermophilus* were enumerated on M17, *Chryseobacterium* spp. on PCA + M + I medium (Table 2),

E. saccharominimus and *E. faecium* on SB medium, *Coryne-bacterium flavescens* on CRBM medium, *L. garvieae* and *L. lactis* on T + a.n. medium in the incubation conditions indicated in Table 2.

2.11. Calculation of area of inhibition

In order to easily quantify the differences in *L. monocytogenes* population between treatment and control throughout ripening, the area of inhibition (AI) between two days (t1 and t2) of ripening was calculated using the formula of Wenzel and Marth (1990):

$$AI = (t2-t1)/2 \times [(C_{t2} + C_{t1}) - (T_{t2} + T_{t1})]$$

where

C	count of *L. monocytogenes* in control cheese inoculated with *S. thermophilus*
T	count of *L. monocytogenes* in trial cheese inoculated with *S. thermophilus* and other bacterial species.

3. Results

3.1. Identification of isolates and the production of anti-Listeria compounds by the raw-milk microbial community expressing the strongest Listeria-inhibiting potential in cheese

One hundred and forty-three isolates were assigned to a species, based on a 98% similarity between their partial 16S rRNA gene sequences and those in the databases (NCBI databases using the BLAST program) (Table 3). Ninety-five isolates were identified as Gram-positive bacteria (63 lactic and 32 non-lactic bacteria), 48 as Gram-negative bacteria. The 143 isolates were classified into 41 microbial species.

With the agar-well diffusion method we used, none of the tests, either with individual isolates or with the retentate or with microbial populations collected on 15 agar media, produced any *L. monocytogenes*-inhibiting compound.

3.2. SSCP patterns obtained with w34–w49 primers amplifying the V3 region of the 16S rRNA gene

The various cheeses, prepared with three sets of experiments with each type of 6 different milks (F1 to F6), were analysed with primer pair w34–w49 on day 8. The V3 region of the 16S rRNA gene of 16 cheeses was amplified. Comparison of the SSCP patterns obtained with these primers revealed a dominant peak, named A ($P_A > 30\%$), in all SSCP profiles regardless of the cheese-making batch studied. As peak A was dominant and present in all SSCP patterns, it hampered the distinction of other, subdominant peaks that might have been useful for distinguishing between the different cheeses' bacterial communities (data not shown). The dominant peak corresponded to the *S. thermophilus* isolate sequence.

3.3. SSCP patterns obtained with V2F–V2R primers amplifying the V2 region of the 16S rRNA gene

The different cheeses prepared with three sets of experiment with each type of 6 different milks (F1 to F6) were also obtained on days 1 and 8 with the V2F–V2R primer pair (Fig. 1). As there were no marked differences in SSCP profiles between days 1 and 8 and for the reasons explained in the Materials and methods section above, only SSCP profiles on day 8 were analysed. All the 8-day cheeses were classified in a dendrogram according to the SSCP pattern peak ratios obtained after amplifying the V2 region of the 16S rRNA gene (Fig. 2). The cheeses with the highest *L. monocytogenes* counts were clustered in 5 groups defined at an aggregative distance of 42: two cheeses prepared in two experiments from F2 and F3 were in group V and two cheeses from F1 were in group III. However, some F1, F2, F3 cheeses manufactured in the different experiments were in groups II or IV, indicating some variation in bacterial community composition from one cheese-making day to another. In contrast, cheeses with low *L. monocytogenes* counts (<2.31 CFU/g) were clustered in group I (F4, F5 and F6 cheeses from all three experiments). This clustering shows that the balance between the different populations in the bacterial communities of these cheeses, as amplified by the V2F–V2R primer pair, was stable from one cheese-making day to another.

Peak F was the dominant peak in the SSCP profiles of cheeses clustering in groups II and IV, but was proportionately smaller in the SSCP profiles of group I, III and V cheeses. There was no peak clearly characteristic of the cheeses with the highest *L. monocytogenes* counts. The cheeses in group I were distinctive in that peaks C, G and D were dominant in their SSCP profiles, whereas these peaks were absent or subdominant in the SSCP profiles of cheeses in groups II to V.

Twenty-two clones were analysed to identify the peaks with the highest ratios in their SSCP profiles. Considering only 16S rDNA sequences with at least 98% similarity with database sequences, 52% of clones were identified as *S. thermophilus*, 27% as *L. lactis*, 3% as *Streptococcus parauberis* and 14% as *Streptococcus* sp. As diversity was greater with isolate identification, the peaks were also assigned by comparing their migrations with those of the nucleotide sequences of the bacterial isolates and reference strains.

Peak F, whose ratios were among the highest in all SSCP patterns, comigrated with nucleotide sequences of *S. thermophilus* clones and isolates. Peak C comigrated with isolate sequences identified as *E. faecium* and *E. saccharominimus*, and peak G with *Chryseobacterium* sp. and *C. flavescens* isolate sequences. Similarly, peak D coeluted with nucleotide sequences of *L. garvieae* isolate and also with nucleotide sequences of clones and isolates of *L. lactis*.

3.4. Evaluation of anti-listerial activity in pasteurised milk cheeses

The fact that peaks C, D and G have the highest ratios on SSCP profiles of cheeses with the lowest *L. monocytogenes* counts suggests that the bacterial species corresponding to
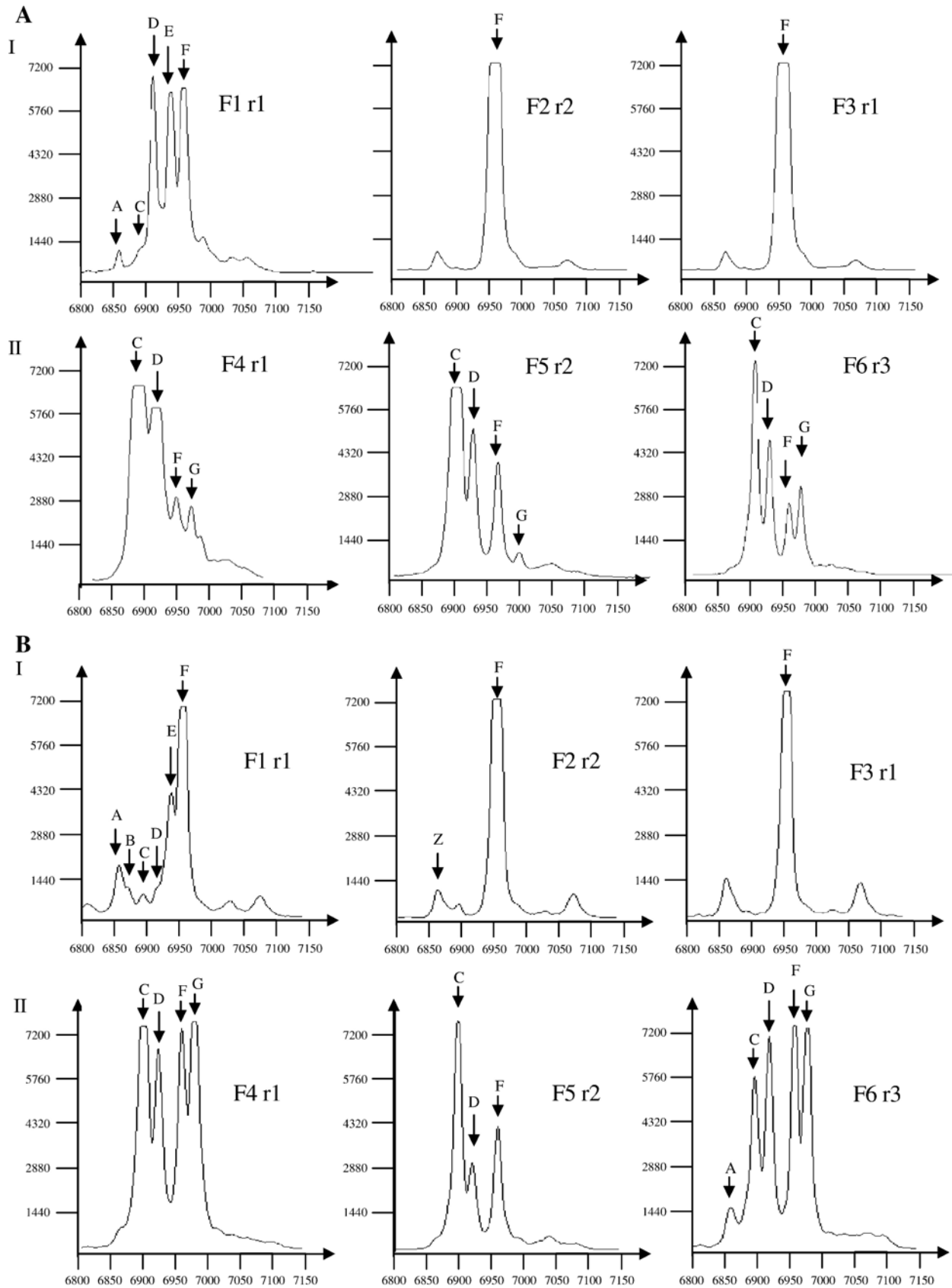
Fig. 1. Example of V2 SSCP patterns of PCR-amplified 16S rRNA gene fragments from bacterial communities of raw-milk cheeses on day 1 (A) and on day 8 (B) with highest counts in *L. monocytogenes* (I) and with lowest counts in *L. monocytogenes* (II). *y* axis, fluorescence; *x* axis, elution in scans (unit of Genescan software). F*i* (*i* from 1 to 6): origin farm of cheeses. r*j* (*j* from 1 to 3): experiment. Letters from A to G: name of peaks.
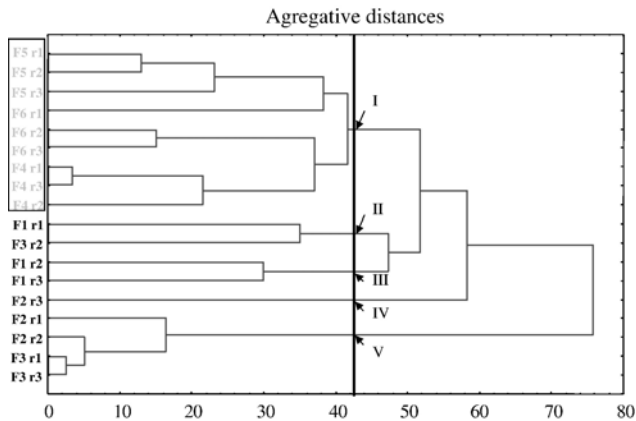
Fig. 2. Dendrogram of the distances (unweighted pair group average, Euclidean distances) between cheeses on day 8 of ripening. Classification of cheeses from the peak ratio data of their SSCP patterns of V2 region amplified 16S rRNA gene. Peak ratio data were calculated using the following formula: $P_i = a_i * 100 / \sum a_i$, where P_i was the ratio of peaks and a_i the area of one peak. Fi (i from 1 to 6): origin farm of cheeses; rj (j from 1 to 3): experiment. Fi rj framed and coloured in grey correspond to the name given to cheeses on day 8 with the lowest counts in *L. monocytogenes*.

these sequences may be involved in *L. monocytogenes* in-hibition. To verify this hypothesis, the ability of *E. faecium*, *E. saccharominimus*, *Chryseobacterium* sp., *C. flavescens*, *L. garvieae* and *L. lactis* to inhibit *L. monocytogenes* was evaluated in pasteurised milk cheeses. Cheeses made from milk inoculated with *S. thermophilus*, the species that was dominant in cheeses with high *L. monocytogenes* counts, acted as a control. *L. monocytogenes*, *S. thermophilus* and all other bacteria in the cheeses were enumerated (Table 4). All the bacterial species developed mainly during the first day. After day 1, their counts remained more or less stable. The highest counts were observed for *L. garvieae* and *L. lactis* and the lowest for *E. faecalis*. *E. saccharominimus* was not detected under our counting conditions. Between days 18 and 28, *C. flavescens* showed weaker growth when inoculated in the mixture of 6 bacterial strains than when inoculated alone.

The development of *L. monocytogenes* was evaluated by calculating Area of Inhibition (AI) values as described in the Materials and methods section. The AI data and pH values are shown in Table 5. The higher the AI values, the stronger the inhibition of *L. monocytogenes*.

At day 1, AI values were not significantly different. After day 1, *L. monocytogenes* developed differently depending on the bacterial inoculation.

Throughout the ripening, *L. monocytogenes* AI values were the lowest in trial cheeses manufactured with *Chryseobacterium* sp. and *E. faecium*. After day 18, *L. monocytogenes* was not inhibited in presence of *E. faecium* (AI<0).

Between days 1 and 8, AI values were significantly the highest in cheeses manufactured with *L. lactis*. Between days 8 and 18, *L. monocytogenes* was most inhibited (AI values highest) in presence of *L. garvieae* and the mixture of the 6 bacterial strains.

On days 1, 8 and 28, the pH values of the cheeses did not differ significantly. On day 18, the pH value was significantly lowest in the control cheese, whose *L. monocytogenes* count was among the highest.

4. Discussion

The SSCP technique was successfully used for monitoring microbial community dynamics in cheeses (Duthoit et al., 2003). This study revealed that SSCP after DNA amplification with the proper primer set can also be an effective tool for distinguishing between cheese bacterial communities. The dominance of one peak on the SSCP profiles resulting from amplification of the V3 region of the 16S rRNA gene shows one of the limitations of the direct molecular approach when analysing bacterial communities in which one species is dominant. In the Saint-Nectaire cheeses studied, *S. thermophilus* was dominant since it is routinely used as a starter culture for Saint-Nectaire cheese. Amplification of the V2 region of the 16S rRNA gene proved more relevant for distinguishing the bacterial communities of the different cheeses and for forming a hypothesis on the bacterial flora involved in the inhibition of *L. monocytogenes*. The only feature common to the cheeses with the highest *L. monocytogenes* counts was the peak assigned to *S. thermophilus*. Their SSCP profiles had no other common characteristics. By contrast, all the cheeses with the lowest *L. monocytogenes* counts on day 8 were characterised by the dominance of 3 peaks whose nucleotide sequences comigrated with *E. faecium* and *E. saccharominimus*, *Chryseobacterium* spp. and *C. flavescens* and *L. garvieae* and *L. lactis* sequences respectively. By inoculating these species in pasteurised milk with *L. monocytogenes*, it was shown that not all of them were able to inhibit it in cheeses prepared with these milks. *Chryseobacterium* spp. and *E. faecium* may not be involved in the inhibition under our experimental conditions. Inhibition occurred when *L. lactis* and *L. garvieae* were inoculated and, to a lesser extent, when *E. saccharominimus* and *C. flavescens* were inoculated. According to the literature, *L. lactis* can inhibit *L. monocytogenes* (1) by producing bacteriocins such as nisin and pediocin (Olasupo et al., 1999; Rodriguez et al., 2005), (2) by producing organic acids (Wenzel and Marth, 1990; Hicks and Lund, 1991) and (3) by lowering pH values as observed by Pitt et al. (2000) in pasteurised milk. However, our results reveal that *L. monocytogenes* inhibition was not due to a decrease of pH alone, nor to the ability of strains to produce anti-listerial compounds. Lowering pH values below 5.2 was not a sufficient condition to inhibit *L. monocytogenes*, although this value has been considered important for controlling *L. monocytogenes* in raw-milk Saint-Nectaire cheeses (Millet et al., 2006). A number of studies have found it difficult to identify the compounds involved in the inhibition of *L. monocytogenes* in cheeses. Maoz et al. (2003) failed to understand why *L. monocytogenes* was inhibited on cheese surfaces as the different microbial isolates of the inhibitory consortium exhibited no anti-listerial activity. Gay and Amgar (2005) suggest that the microbial community had some role in explaining why *L. monocytogenes* developed less in raw-milk cheeses than in pasteurised milk cheeses but they did not study the nature of the inhibition (pH, organic acids, bacteriocins, etc.).

Table 4
Bacterial counts (log CFU/ml) in control and trial cheeses on days 1, 8, 18 and 28

Trial	D	L. monocytogenes	S	S. thermophilus	S	Mean [Chryseobacterium sp.]	S	Mean [E. saccharominimus / E. faecium]	S	Mean [C. flavescens]	S	Mean [L. garvieae / L. lactis]	S
Control	0	0.60	0.00	7.00	0.00								
Control	1	2.27	0.38	9.02	0.12								
Control	8	2.90	0.14	9.09	0.05								
Control	18	2.83	0.18	9.12	0.08								
Control	28	3.01	1.07	9.08	0.01								
Chryseobacterium sp.	0	0.60	0.00	7.00	0.00	2.00	0.00						
Chryseobacterium sp.	1	1.97	0.10	9.04	0.08	4.25	0.03						
Chryseobacterium sp.	8	2.58	0.16	9.20	0.11	4.04	0.52						
Chryseobacterium sp.	18	2.39	0.39	9.09	0.01	5.64	0.14						
Chryseobacterium sp.	28	3.30	0.88	9.04	0.02	5.55	0.27						
E. saccharominimus	0	0.60	0.00	7.00	0.00			ND	0.00				
E. saccharominimus	1	2.04	0.27	9.11	0.16			ND	0.12				
E. saccharominimus	8	2.27	0.38	9.19	0.06			ND	0.08				
E. saccharominimus	18	2.60	0.09	9.13	0.07			ND	0.37				
E. saccharominimus	28	1.39	0.55	9.06	0.03			ND	0.36				
E. faecium	0	0.60	0.00	7.00	0.00			2.00	0.00				
E. faecium	1	1.60	0.00	9.08	0.18			3.86	0.12				
E. faecium	8	2.77	0.03	9.22	0.06			3.95	0.08				
E. faecium	18	3.40	0.40	9.07	0.01			4.38	0.37				
E. faecium	28	3.00	0.74	9.06	0.03			4.39	0.36				
C. flavescens	0	0.60	0.00	7.00	0.00					3.00	0.00		
C. flavescens	1	2.01	0.23	9.09	0.22					4.81	0.06		
C. flavescens	8	2.63	0.12	9.15	0.18					5.96	0.47		
C. flavescens	18	2.09	0.68	9.16	0.07					6.29	0.97		
C. flavescens	28	2.19	0.06	9.01	0.05					6.22	0.90		
L. garvieae	0	0.60	0.00	7.00	0.00							5.00	0.00
L. garvieae	1	1.83	0.49	9.16	0.08							6.69	0.97
L. garvieae	8	1.39	0.55	9.24	0.13							7.01	0.38
L. garvieae	18	1.90	0.08	9.10	0.04							6.91	1.17
L. garvieae	28	1.94	0.65	9.17	0.14							7.21	0.35
L. lactis	0	0.60	0.00	7.00	0.00							5.00	0.00
L. lactis	1	1.00	0.00	9.26	0.04							8.55	0.02
L. lactis	8	1.68	0.96	9.27	0.00							8.51	0.02
L. lactis	18	1.65	0.07	9.17	0.12							8.62	0.08
L. lactis	28	1.42	0.60	9.16	0.16							8.58	0.02
Mixture	0	0.60	0.00	7.00	0.00	2.00	0.00	2.30	0.00	3.00	0.00	5.00	0.00
Mixture	1	1.30	0.43	9.26	0.03	4.29	0.18	3.72	0.14	4.86	0.79	8.60	0.01
Mixture	8	1.80	0.45	9.28	0.02	3.67	0.41	3.89	0.22	5.10	0.06	8.61	0.05
Mixture	18	1.15	0.21	9.29	0.12	5.51	1.28	4.09	0.07	5.50	0.35	8.66	0.11
Mixture	28	1.00	0.00	9.23	0.10	4.35	2.90	3.91	0.33	5.49	0.36	8.61	0.11

Notes on the added-species Mean columns: for the Mixture trial the fourth Mean column heading is *E. faecium, E. saccharominimus* and the last Mean column heading is *L. garvieae, L. lactis*.

All cheeses were manufactured with pasteurised milk inoculated with *S. thermophilus* and *L. monocytogenes*.
Values are the mean of 2 experiments. D: day, ±S: Standard deviation, ND: not detected.
Control: no inoculation of other bacteria, Trial: inoculation with single bacterium, Mixture: inoculation with 6 bacterial species.
L.: Listeria, S.: Streptococcus, L.: Lactococcus, E.: Enterococcus, C.: Corynebacterium.

Table 5
AI and pH values in pasteurised milk cheeses

Trial	AI (1–0)	AI (8–1)	AI (18–8)	AI (28–18)
Chryseobacterium sp.	0.15	2.94 ab	3.81 ab	0.78 ab
E. saccharominimus	0.12	2.09 a	5.61 ab	11.92 ab
E. faecium	0.33	2.78 a	2.23 a	−2.78 a
Corynebacterium flavescens	0.13	3.76 ab	3.76 ab	5.18 ab
L. garvieae	0.22	6.85 ab	12.19 b	10.01 ab
L. lactis	0.64	8.72 b	11.97 b	13.83 ab
Mixture	0.49	7.26 ab	13.9 b	18.45 b
p	NS	**	**	**

Trial	pH 1	pH 8	pH 18	pH 28
Control	5.51	5.36	5.17 a	5.34
Chryseobacterium sp.	5.50	5.37	5.24 ab	5.31
E. saccharominimus	5.46	5.33	5.24 ab	5.33
E. faecium	5.52	5.36	5.24 ab	5.30
Corynebacterium flavescens	5.52	5.35	5.26 b	5.37
L. garvieae	5.45	5.33	5.21 ab	5.28
L. lactis	5.34	5.24	5.20 ab	5.30
Mixture	5.32	5.25	5.21 ab	5.23
p	NS	NS	*	NS

All cheeses were manufactured with pasteurised milk inoculated with *S. thermophilus* and *L. monocytogenes*.
Control: no inoculation of other bacteria, Trial: inoculation with single bacterium, Mixture: inoculation with 6 bacterial species.
The values are the mean of 2 experiments. In the same column, letters (a, b and c) indicate homogeneous statistical processing groups. Numbers with different letters are not equal. a, b and c are classified in increasing order ($a < b < c$). Means in the same column with different letters were significantly different according to Newman–Keuls statistical test. p = the significance levels (NS = non-significant, * = $p < 0.05$, ** = $p < 0.01$).
AI: area of inhibition.

$$AI = (t2-t1)/2 \times [(C_{t2} + C_{t1}) - (T_{t2} + T_{t1})]$$

where C = count of *L. monocytogenes* in control cheese inoculated with *S. thermophilus* and T = count of *L. monocytogenes* in trial cheese inoculated with *S. thermophilus* and other bacterial species.
t1 and t2 = two days of ripening. AI(t2−t1): area of inhibition between two days of ripening.

Further studies, including more accurate determination of the anti-listerial metabolites produced, are needed to achieve a better understanding of *L. monocytogenes* in cheeses.

Acknowledgements

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