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Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the Toma piemontese

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Abstract

We studied the natural bacterial population used in the production of artisanal protected denomination origin (PDO) Toma piemontese cheese in order to obtain preliminary information on the lactic acid bacteria involved in the traditional fermentation of this cheese. A total of 116 coccal isolates and 11 mesophilic lactobacilli were collected from different curd and cheese samples, and were identified by the combined use of PCR 16S–23S rDNA spacer analyses, species-specific probes and 16S rDNA sequencing. The results obtained were supported by DNA/DNA hybridization studies and by reference cultures used as markers. Lactococci constituted 67% of the coccal isolates. They were identified as *Lactococcus lactis* and *L. garvieae*, with an incidence of this latter species higher than expected. The majority of these lactococcal isolates displayed an atypical phenotype of growing in the presence of 6.5% NaCl and at 10°C. Enterococci were also isolated, principally from the cheese samples (16% of the coccal isolates), together with strains of *Streptococcus macedonicus* and *S. thermophilus*, the latter in a lower amount. Lactobacilli (principally *Lactobacillus paracasei* strains) were only detected in three samples and their incidence was very low (1, 3 and 7 cfu g^{-1}, respectively). The presence of undesirable micro-organisms in some samples of curd emphasizes the necessity of improving the general hygienic conditions of cheese production. The results suggest the possibility of preserving the wild bacterial population in order to protect the typical organoleptic characteristics of this traditional raw milk cheese and to select new strains for the dairy industry.

Keywords: Toma cheese; Lactic acid bacteria; Bacterial identification; Species-specific PCR

1. Introduction

In recent years, there has been a growing interest in genotypic and phenotypic studies on wild isolates from artisanal cheeses produced mainly without the addition of any starter cultures (Cogan et al., 1997; Desmasures et al., 1998; Baruzzi et al., 2000; Suzzi et al., 2000; Coppola et al., 2001). Increasing information on the natural microbial population present in dairy products can help to prevent the loss of microbial biodiversity in typical foods and consequently the loss of a wide range of cheeses produced by different methods whose typical features depend on local and regional traditions and on the indigenous microbial population present in raw milk and selected by the cheese-making environment.

Moreover, these studies could be the basis for the selection of new strains to be used either as specific cultures in a larger-scale production of traditional cheeses, or together with classical starter cultures for improving the existing dairy product manufacture. Indeed, the diversity of starters used in industrial dairy fermentation is low and there is an increasing demand from cheese makers for new strains, starter and non-starter lactic acid bacteria (LAB and NSLAB), that show advantageous effects on cheese characteristics. Although their composition and performance may be relatively variable, natural starter cultures have a number of interesting properties: they are relatively

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insensitive to attack by phages (Séchaud et al., 1992; Reinheimer et al., 1997), are capable of producing bacteriocin-like substances (McKay, 1990; Casla et al., 1996) and are a rich source of many different biotypes showing unusual properties which can contribute to the production of a typical cheese taste and flavour (Fortina et al., 1998; Gaya et al., 1999; Mannu et al., 2000; Xanthopoulos et al., 2000).

To analyse and rapidly identify microbial communities, classical physiological and biochemical tests are not adequately efficient, since the bacterial population involved often has similar nutritional requirements and grows under similar environmental conditions. In the past few years, the development of molecular techniques has opened up new perspectives for characterizing strains from raw milk and traditional cheeses. The 16S rDNA sequence, PCR 16S–23S rDNA spacer polymorphism and PCR-DGGE (denaturating gradient gel electrophoresis) analyses have been successfully performed for the genetic differentiation of several species of LAB, such as *Lactococcus lactis, Streptococcus thermophilus, Enterococcus faecium, Lactobacillus delbrueckii* (Nour et al., 1995; Moschetti et al., 1997, 1998; Coppola et al., 2001). Several species-specific probes have also been designed for LAB identification through PCR or multiplex PCR reactions that are more rapid and convenient than hybridization techniques (Garde et al., 1999; Ke et al., 1999; Fortina et al., 2001; Torriani et al., 2001). Finally, random amplified polymorphic DNA (RAPD) analysis has been used for rapid typing of lactococci, enterococci and lactobacilli strains (Corroler et al., 1998; Morea et al., 1999; Suzzi et al., 2000; De Angelis et al., 2001).

The objective of this work was to study the natural bacterial population used in the production of artisanal protected denomination origin (PDO) Toma piemontese cheese in Piedmont, northern Italy, to obtain preliminary knowledge of the lactic acid bacteria involved in traditional fermentation of this cheese. Toma cheese has been attributed a protected designation origin (PDO) qualification, but the strain composition of the natural microbial population colonizing this traditional cheese is still unknown. Toma is a semi-cooked cheese which is produced in Piedmont from raw milk which has rested 12 h at 8–10°C. Without the addition of any natural or selected starter culture, the milk is warmed to 37–40°C for 40–60 min and rennet is added at a concentration of 0.15–0.20 ml l^{-1}. Clotting time is established visually by the cheese maker. The curd is cut into 5–10 mm particles and collected with a cloth, pressed and drained for 24 h. Cheese is ripened in suitable rooms or caverns at approximately 6–10°C and 85% relative humidity for 30–40 days. The production and the ripening process, which lacks temperature and pH control, depends entirely on the natural microbial population present in the milk.

2. Materials and methods

2.1. Reference strains

The reference strains used in this study are listed in Table 1. Lactococci, enterococci and streptococci were grown in M17 (Difco) supplemented with 5 g l^{-1} glucose and/or lactose, while for lactobacilli MRS (Difco) was used. The cultures were incubated at their optimal growth temperature under anaerobic conditions (Anaerocult A, Merck) for 24–48 h. For long-term maintenance, cell suspensions were stored at –80°C in broth cultures supplemented with 15% (w/v) glycerol.

2.2. Sampling and isolation of bacteria

Seven dairy farms were selected in different dairy regions of the PDO Toma cheese area. They were labelled A–G. Samples of curd (those having cu as their second labelling) were collected from four different origins on the day of the production, during the summer alpine pasture. Six cheese samples (those having ch as their second labelling) were at 30–40 days of ripening; three of these samples derived from the same curd producers (A and B). All samples were transported to the laboratory under refrigerated conditions (4°C) and analysed not more than 6 h from the collection. Curd and cheese samples were emulsified in sterile 2% (w/v) trisodium citrate, serially diluted in sterile saline solution and plated in duplicate on the following media: kanamycin aesculin azide agar (KAA) and M17 agar, incubated at 37°C for 24–48 h for enterococci, M17 agar, incubated at 30°C and 37°C for 48 h for mesophilic and thermophilic cocci and MRS agar at pH 5.8, incubated anaerobically at 30°C and 37°C for 48 h, for lactobacilli. Cycloheximide was added (100 mg l^{-1}) to

Table 1
Reference strains used in this study

Reference strains	Source
Streptococcus thermophilus	DSM 20617^T
Streptococcus macedonicus	BCCM-LMG 18487^T
Streptococcus suis	DSM 9682^T
Enterococcus faecium	ATCC 19434^T
Enterococcus faecalis	NCDO 588
Enterococcus durans	NCDO 596
Lactococcus garvieae	DSM 20684^T
Lactococcus lactis subsp. *lactis*	DSM 20481^T
Lactococcus lactis subsp. *cremoris*	DSM 20069^T
Lactobacillus paracasei	DSM 5622^T

T: Type strains; DSM: Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany; ATCC: American Type Culture Collection, Rockville, MD, USA; NCDO: National Collection of Dairy Organisms, Aberdeen, Scotland, UK: BCCM-LMG: Belgian Co-ordinated Collection of Microorganisms—Bacterial Collection, Gent, Belgium.

prevent yeast growth. After incubation, randomly selected colonies were purified by two subsequent subcultures and then submitted to microscopic examination, Gram staining, catalase test, production of gas from D-glucose, growth at 6.5% (w/v) NaCl, at 10°C and 45°C. Isolates were finally stored at –80°C.

2.3. Dna preparation

Genomic DNA for all PCR reactions was extracted from a 100 µl of an overnight culture diluted with 300 µl of TE 1 × buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0) as described by Mora et al. (2000). For DNA/DNA hybridization experiments, total DNA was extracted as reported by Fortina et al. (1998). The extent of DNA reassociation was determined spectrophotometrically (Seidler and Mandel, 1971; Kurtzman et al., 1979) using a Gilford Response spectrophotometer (Ciba Corning Diagnostics Corp., USA). The reaction was carried out under optimal conditions (25°C below the melting temperature) in 5 × SSC buffer (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 20% (w/v) dimethylsulphoxide.

2.4. PCR reaction protocols

The DNA sequences (5′ to 3′) for the primers used in this study and their corresponding specificities were as follows: G1 (GAAGTCGTAACAAGG) and L1 (CAA GGCATCCACCGT) bacterial 16S/23S rRNA gene spacer region (Jensen et al., 1993); Ent1 (TACTGA CAAACCATTCATGATG) and Ent2 (AACTTCGT CACCAACGCGAAC) tuf gene in enterococci (Ke et al., 1999); EM1A (TTGAGGCAGACCAGATT GACG) and EM1B (TATGACAGCGACTCCGA TTCC) nucleotide sequence conserved in *E. faecium* (Cheng et al., 1997); E1 (ATCAAGTACAGTTA GTCTT) and E2 (ACGATTCAAAGCTAACTG) ddl gene in *E. faecalis* (Dutka-Malen et al., 1995); St1 (CACTATGCTCAGAATACA) and St2 (CGAACAG CATTGATGTTA) lacZ gene in *S. thermophilus* (Lick et al., 1996); His1 (CTTCGTTATGATTTTACA) and His2 (CAATATCAACAATTCCAT) histidine biosynthesis operon in *L. lactis* (Corroler et al., 1999); pLG-1 (CATAACAATGAGAATCGC) and pLG-2 (GCACCCTCGCGGGTTG) 16S rRNA gene in *L. garvieae* (Zlotkin et al., 1998); casei (TGCACTGAG ATTCGACTTAA), para (CACCGAGATTCAACAT GG), rham (TGCATCTTGATTTAATTTTG) and Y2 (CCCACTGCTGCCTCCCGTAGGAGT) 16S rRNA gene in Lactobacillus casei, *L. paracasei* and *L. rhamnosus* (Ward and Timmins, 1999); 16 (GCTGGAT CACCTCCTTTC) and Lc (TTGGTACTATTTAA TTCTTAG), Ls (ATGAAACTATTAAATTGGTAC) 16S/23S rRNA gene spacer region in *Lactobacillus curvatus* and *L. sakei* (Berthier and Ehrlich, 1998). Primer sets casei, para, rham and Y2 were used together in a multiplex PCR reaction, as were primer sets 16, Lc and Ls. The other primer sets were used singularly in separate PCR reactions. Primer, $MgCl_2$, dNTPs and Taq polymerase (Amersham, Pharmacia Biotech.) concentrations used in all PCR reactions were 0.5 µM, 2.5 mM, 200 µm and 0.5 U, respectively. The final volume was 25 µl. PCR reactions were performed in a Gene Amp PCR System 2400 (Perkin-Elmer) and the cycle parameters were the same reported by the different authors cited previously. Following amplification, 10 µl of product were directly electrophoresed at 5 V cm^{-1} in 1.5–3% agarose gel (with 0.2 mg ml^{-1} of ethidium bromide) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and photographed in UV light. A Gene-Ruler™ DNA ladder mix (MBI Fermentas, Germany) was used as a size marker.

2.5. DNA sequence analysis

A 500 bp portion of the 16S rRNA gene was sequenced for some isolates. Amplification was performed as previously reported, using the primer (5′-AGAGTTTGATCCTGGCTCAG-3′) position 8-27 of *Escherichia coli* (Lane, 1991). The PCR mixtures were subjected to the following thermal cycling: 2 min at 94°C, then 5 cycles of 45 s at 94°C, 45 s at 50°C, 1 min at 72°C, and then 30 cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C, with a 7-min final extension at 72°C. Nucleo Spin Extract (Macherey-Nagel GmbH & Co., Germany) was used to purify PCR products that were sequenced using the dideoxy chain-termination principle (Sanger et al., 1977), employing ABI Prism Big Dye Terminator Kit (Applied Biosystems). The reaction products were analysed with the ABI Prism™310 DNA Sequencer. Taxonomic identification and S_ab calculations were performed using The Ribosomal Database Project (RPD-II) (Maidak et al., 2001). The 16S rRNA sequences of the isolates were compared with more than 1000 aligned sequences present in Similarity Matrix as well as with more than 34 000 unaligned sequences present in the Sequence Match of the RPD-II.

3. Results

3.1. Isolates

Altogether, 116 coccal isolates were collected, 53 from five different curd samples and 63 from six different cheese samples, with 4–20 colonies randomly selected from each sample (Table 2). The viable counts on M17 plates varied from 10^5 to 10^6 cfu g^{-1} for curd samples, with pH values ranging between 6.0 and 6.6. Higher levels (10^8 cfu g^{-1}) were reached in cheese samples with pH values dropping to 4.9. Seventy-eight per cent of

Table 2
Isolation and identification of cocci from Toma samples

Source of isolates	Total isolates	Identification	Number of strains
A_{cu}	16	*L. lactis* subsp. *lactis*	6
		L. lactis subsp. *cremoris*	1
		S. suis	6
		S. agalactiae	1
		S. dysgalactiae	2
$A1_{ch}$	12	*L. lactis* subsp. *lactis*	4
		Enterococcus spp.	3
		E. faecalis	1
		S. macedonicus	4
$A2_{ch}$	5	*L. lactis* subsp. *lactis*	2
		L. lactis subsp. *cremoris*	1
		E. durans	1
		S. thermophilus	1
$B1_{cu}$	17	*L. garvieae*	14
		E. faecium	1
		E. durans	1
		S. thermophilus	1
$B2_{cu}$	4	*L. garvieae*	4
B_{ch}	20	*L. lactis* subsp. *lactis*	6
		L. garvieae	3
		E. faecium	8
		E. faecalis	1
		S. macedonicus	2
C_{cu}	10	*L. garvieae*	8
		S. agalactiae	1
		S. uberis	1
D_{ch}	9	*L. lactis* subsp. *lactis*	3
		L. garvieae	6
E_{ch}	10	*L. garvieae*	7
		E. durans	2
		Enterococcus spp.	1
F_{cu}	6	*L. lactis* subsp. *lactis*	2
		L. lactis subsp. *cremoris*	2
		L. garvieae	2
G_{ch}	7	*L. lactis* subsp. *lactis*	1
		L. lactis subsp. *cremoris*	6

cocci isolates were able to grow at 10°C and 73% in the presence of 6.5% NaCl, but only 16% could be differentiated for the ability to produce the blackening reaction in media containing aesculin. Lactobacilli were only detected in three samples, F_{cu}, $A1_{ch}$, G_{ch} and their incidence was very low (1, 7 and 3 cfu g^{-1} respectively). All *Lactobacillus* isolated were able to utilize pentoses, while only one was able to produce gas from glucose.

3.2. Genotypic identification of isolates

A first clustering step was reached by a PCR amplification of the 16S–23S rRNA spacer region (RSA) of the 116 coccal isolates, with primers G1 and L1, as reported previously. This analysis allowed us to obtain five different RSA clusters. Fig. 1 gives an example of the typical profile of each cluster.

Cluster I was composed of 38 isolates and its profile was characterized by a unique band migrating at approximately 380 bp. Cluster II, including 45 isolates, exhibited a typical 400 bp band. For eight isolates one amplification band at about 350–370 bp was obtained (cluster III). In cluster IV were grouped six isolates showing a major band at 500 bp; in some cases one additional band at about 700 bp was also visible. Finally, in cluster V we grouped 19 isolates ascribing to genus *Enterococcus* for the presence of two main amplification bands. Within this cluster four subgroups were distinguishable: subgroup Va, comprising nine isolates and showing the typical profile of *E. faecium* species, subgroup Vb, comprising four isolates and characterized by two bands migrating at 400 and 380 bp, subgroup Vc, in which two isolates showed the typical RSA profile for *E. faecalis* species. The remaining subgroup, comprising four isolates, was characterized by an RSA profile similar but not identical to *E. faecalis*, and for this reason the isolates were grouped separately.

Further PCR experiments using species-specific primers were carried out for each RSA cluster obtained.

Within cluster I, characterized by the 380 bp band concerning the spacer region of *L. lactis*, 34 isolates were further discriminated between the two major subspecies of *L. lactis*. Particularly, 24 isolates had the pattern characteristic of *L. lactis* subsp. *lactis*, exhibiting an approximately 930 bp band with primer pair His1 and His2. The other 10 isolates were identified as *L. lactis* subsp. *cremoris* because of an approximately 1100 bp band (Fig. 2). The remaining four isolates grouped in cluster I could not be identified as *L. lactis*. In this case, taxonomic identification was reached by 16S rRNA sequencing. With a Similar matrix (Sm) and a S_ab values of 1.00 and 0.99, respectively, two isolates were identified as *Streptococcus dysgalactiae* and the other two (Sm of 0.99 and S_ab of 0.97) as *S. agalactiae*.

The 16S rRNA sequence analysis of a representative strain included in cluster II showed the high correlation between the isolates of this cluster and *L. garvieae* (Sm of 0.99 and S_ab of 0.93), an emerging zoonotic agent phenotypically very similar to *L. lactis,* originally isolated from a mastitic udder (Collins et al., 1984) and then recovered from different fish species (Eldar et al., 1999). A PCR assay developed for its identification (Zlotkin et al., 1998) was positive for 44 isolates of the cluster II (Fig. 2). The remaining isolate, although showing the same RSA profile of *L. garvieae*, was not

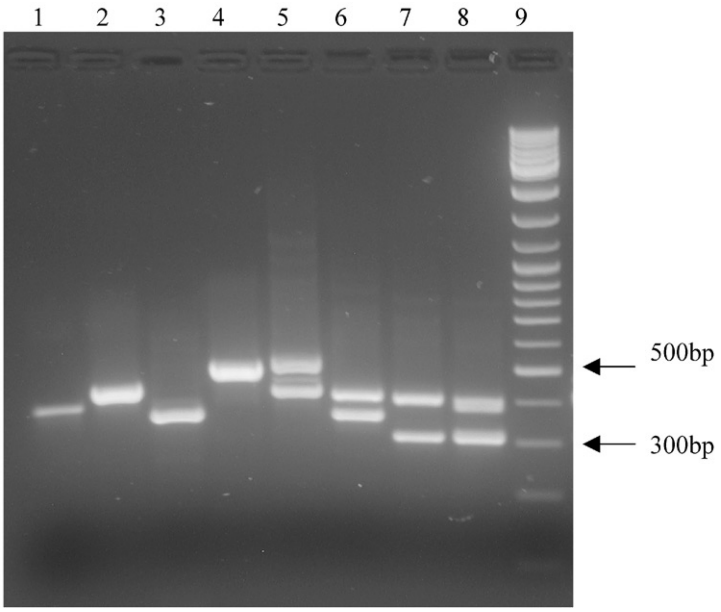

Fig. 1. RSA profiles of representative coccal strains of each cluster obtained. Lanes 1–4: Cluster I, II, III, IV; lanes 5–8: cluster V, subgroups a, b, c, d; lane 9: DNA Ladder Mix (MBI).

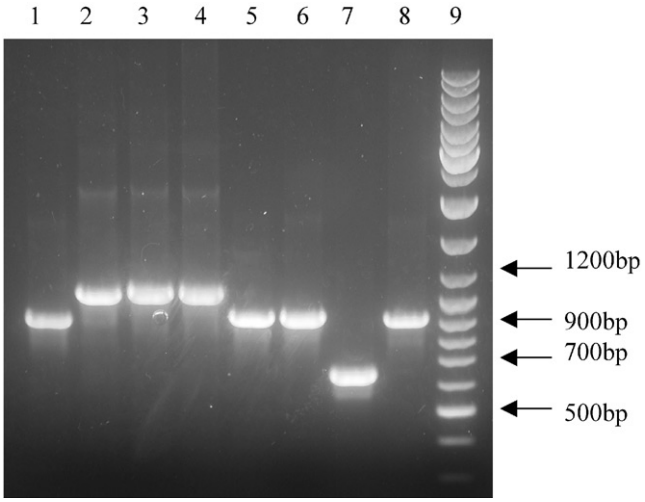

Fig. 2. PCR specific products of coccal isolates. Lane 1: *L. lactis* subsp. *lactis*; lane 2: *L. lactis* subsp. *cremoris*; lanes 3–4: *L. garvieae*; lanes 5–6: *S. thermophilus;* lane 7: *E. faecium*; lane 8: *E. faecalis*; lane 9: DNA Ladder Mix (MBI).

positive to the specific PCR experiment. The 16S rRNA sequence analysis showed that the isolate could belong to the species *S. uberis* (Sm of 1.00 and S_ab of 0.99).

16S rRNA sequence analysis was also necessary to identify isolates included in cluster IV, for which no indication was obtainable by RSA analysis. Through this determination, the isolates were identified as *Streptococcus suis* (Sm of 0.99, S_ab of 0.97). From these data, it is possible to deduce that, using G1 and L1 primers in RSA experiments, strains belonging to *S. suis* species can be detected by the presence of a principal band migrating at 500 bp (Fig. 1).

The isolates grouped in cluster III showed in RSA experiments typical spacer regions referable to *S. thermophilus* group. Further species-specific PCR carried out for the detection of *S. thermophilus* was positive for two of the eight isolates (Fig. 2). Through 16S rRNA analyses, the other six isolates were identified as *S. macedonicus* strains (Sm of 1.00, S_ab of 0.99).

Regarding the last cluster grouping *Enterococcus* spp. strains, the isolates within subgroups Va and Vc, in agreement with their RSA profiles, were identified as *E. faecium* and *E. faecalis*, respectively, by specific PCR experiments, as reported above (Fig. 2). A representative isolate of subgroup Vb was subjected to 16S rRNA sequence analysis that revealed that the isolates of this subgroup belonged to *E. durans* species. By sequence analysis of 16S rRNA, the four isolates of the last subgroup seem to be ascribing to a new species of *Enterococcus*. Indeed, the higher 16S rRNA similarity value found was 95% with the species *E. saccharolyticus.* It is known that at sequence homology values below 97–98%, it is unlikely that two organisms are related at the species level. Further identification studies are in progress.

Regarding the 10 facultatively heterofermentative lactobacilli isolates, the RSA analysis has been poorly discriminating. Specific PCR and multiplex PCR amplification experiments permitted us to identify six isolates, originating principally from one cheese sample (G_{ch}), as *L. paracasei*, two as *L. curvatus* and one as *L. rhamnosus.* The remaining isolate was identified by 16S

rRNA analysis as *L. coryniformis* (Sm of 0.99, S_ab of 0.90). The only obligately heterofermentative strain found was ascribing to *L. fermentum* species (Sm of 0.98, S_ab of 0.98). In Figs. 3a and b are reported RSA profiles and PCR specific products of some of these lactobacilli isolates.

Representative isolates of the main clusters obtained were subjected to DNA/DNA reassociation experiments with the relative type strain or reference strain. The homology values obtained in all cases (>75%) confirmed that the specific PCR experiments and sequencing of 16S rRNA employed may replace, especially if the type strains are included in the analyses, time-consuming and laborious DNA/DNA hybridization technique for a rapid and reliable species identification. RSA analysis is an effective and useful technique for achieving a first clustering step of a great quantity of different isolates, but because its response is not always species-specific, it has to be followed by more specific experiments.

4. Discussion

Even today in Italy, many cheeses are produced with raw milk without the use of industrial starter cultures. The specific characteristics, such as taste, aroma and texture of these traditional fermented foods, may be attributed to the presence of an adventitious microbial population. To better understand the relationship between the organoleptic quality of cheese and the responsible microbial population, large numbers of isolates from a wide variety of fermented substrates need to be sampled and submitted to a rapid and reliable identification and typing method.

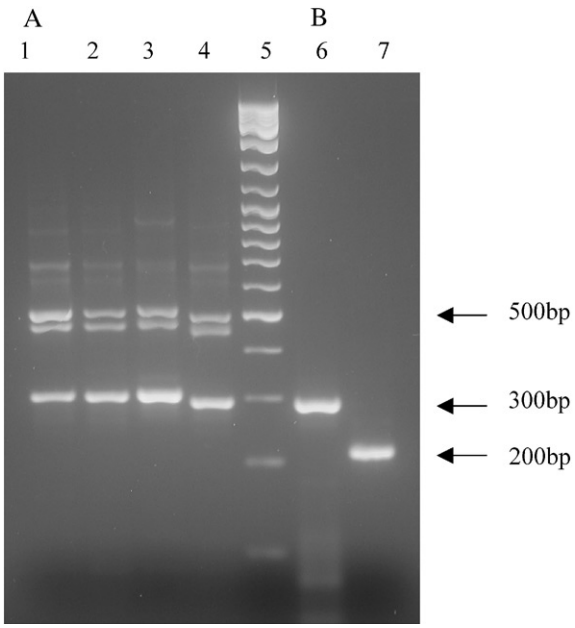

Fig. 3. (a) RSA profiles and (b) PCR specific products of some lactobacilli isolates: lanes 1A–2A–6B: *L. paracasei*; lanes 3A–7B: *L. curvatus*; lane 4A: *L. coryniformis*; lane 5: DNA Ladder Mix (MBI).

In the present work, a preliminary taxonomic study was performed on several isolates from naturally fermented Toma piemontese cheese, with the aim to identify some of the lactic acid bacteria involved in the production of this typical Italian cheese.

The presumptive coccal counts (M17 medium) were reasonably high in the curd and increased in the cheese during ripening. On the contrary, viable cells were not found on MRS plates for most samples analysed. Although MRS medium is not always considered very selective for this bacterial group, the presence of lactobacilli at very low levels and in only three samples, suggests that they cannot be considered as important in Toma fermentation.

Regarding the coccal population, we randomly selected 53 isolates from curd samples and 63 from cheese samples. Lactococci predominated (67% of the isolates from M17 agar) either in the curd samples (73%), or in the Toma cheese samples (62%) (Table 2). The major part of the isolated lactococci displayed the "atypical" phenotype of growing in the presence of 6.5% NaCl and/or at 10°C; this ability of surviving in hostile conditions can explain their presence in the cheese at 30–40 days of ripening. The lactococcal isolates were identified as *L. lactis* and *L. garvieae*, with an incidence of this latter species higher than expected. *L. garvieae* (senior synonym of *Enterococcus seriolicida*) is considered an important agent of septicaemia in fish (Eldar et al., 1999) and it has been related, together with *L. lactis*, in a few human infections, even if their primary role as pathogens remains to be established (Adams and Marteau, 1995). According to our observations, *L. garvieae* is not a rare species, because other researchers have found this species in various dairy sources, together with *L. lactis*, although in a lower proportion (Klijn et al., 1995; Morea et al., 1999). We think that *L. garvieae*, phenotypically very similar to *L. lactis* and showing many typical characteristics of *Enterococcus* species, could be commonly misidentified if no specific molecular typing is carried out. The distribution of these two lactococcal species is relatively variable in the samples analysed, with samples characterized by the presence of only *L. garvieae*, samples with only *L. lactis* and samples with a balanced mixture of both species. This variability could denote the artisanal production of Toma cheese, where changes in environmental conditions related to milk production and to the lack of controlled conditions employed in the production of curd (no temperature and pH control) can influence the access of different strains of lactococci in raw milk, and therefore the presence of different strains in the cheese.

Regarding the two subspecies of *L. lactis*, it is interesting to note that *L. lactis* subsp. *lactis* is the predominant subspecies found; this is in agreement with the observations that *L. lactis* subsp. *lactis* strains are commonly isolated from environmental sources other than from various dairy products, while strains with an *L. lactis* subsp. *cremoris* genotype are infrequent isolates because of their inability to survive in nature and are exclusively confined to dairy environments (Klijn et al., 1995; Corroler et al., 1998). Moreover, though the specificity and consistent presence of wild-type lactococcal strains of the two subspecies have to be confirmed further, it is possible to presume that the distribution of the two subspecies could be related to the area in which the samples were obtained. *L. lactis* subsp. *cremoris* was found more easily in cheese samples F and G that come from the same production area.

As a component of the complex population involved in the fermentation of Toma piemontese cheese, strains of *E. faecium, E. durans* and *E. faecalis,* in addition to some atypical *Enterococcus* strains, were found in a lower amount (16% of the coccal isolates). Enterococci have been found as a relevant component in cheeses produced in environmental conditions and with technological procedures fairly similar to Toma such as Fontina from Valle d'Aosta (Battistotti et al., 1977). Their presence is found more frequently in cheese after ripening, where enterococci can contribute to the typical aroma of the finished product, as the result of citrate metabolism and proteolytic and lipolytic activities (Cogan et al., 1997).

S. thermophilus and *S. macedonicus* can be considered minor components of PDO Toma piemontese cheese: they were found in a low amount (7% of the total coccal isolates) and mainly in cheese samples.

Finally, we found a high degree of contamination in curd sample A_{cu}, comprising several *S. suis* isolates. This contamination could be attributed to environmental conditions and management factors related to cow milk production in springtime, i.e. grazing on natural pastures. Although these undesirable micro-organisms were absent in corresponding cheeses from the same producer ($A1_{ch}$ and $A2_{ch}$) and in the other cheese samples analysed, their isolation emphasizes the necessity of improving milk quality, refrigerating milk at the farm and establishing better hygienic conditions in Toma production.

This preliminary examination revealed that in the samples of Toma piemontese analysed, lactobacilli were isolated in a very low number and only in one curd and two cheeses samples. Although mesophilic lactobacilli are commonly found in cheeses with long-term maturation, their presence is not relevant in other short-time ripening cheeses (Centeno et al., 1996; Menéndez et al., 2001).

The results of the present work represent the first approach to understanding the bacterial population involved in traditional PDO Toma piemontese cheese. Further determination of the technological performances of the strains isolated in cheese-making trials, together with further investigations on other specific groups of bacteria, could allow designation of specific starters to be used in the production of Toma piemontese cheese in order to improve and standardize product quality.

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