Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

International Dairy Journal 18 (2008) 403–413

INTERNATIONAL DAIRY JOURNAL

www.elsevier.com/locate/idairyj

Lactic acid bacteria occurring during manufacture and ripening of Provolone del Monaco cheese: Detection by different analytical approaches

Maria Aponte*, Vincenzina Fusco, Rosamaria Andolfi, Salvatore Coppola

Dipartimento di Scienza Degli Alimenti, Università Degli Studi di Napoli Federico II, Via Università, 100-I 80055 Portici, Italy

Received 14 March 2007; accepted 30 October 2007

Abstract

Lactic acid bacteria occurring in Provolone del Monaco, an artisanal pasta filata cheese produced in Campania (Italy) from raw cows' milk and without starter addition, were investigated by a combination of conventional and molecular approaches. The microbial community was monitored during a cheese-making process giving rise to a premium quality product. *Streptococcus thermophilus* and *Streptococcus macedonicus* prevailed during cheese manufacture and survived along 9 months of ripening, together with enterococci and lactobacilli of the *casei* group, especially *Lactobacillus rhamnosus*. Phenotypic and genetic identification of 308 isolates largely reflected the results obtained by 16S rDNA sequencing analysis by polymerase chain reaction-denaturant gradient gel electrophoresis, with the significant exception of *Lb. fermentum* and four *Lb. delbrueckii* subspecies that were not detected by cultural methods. Each different analytical approach employed provided useful information. Their combination proved to be suitable to effectively describe the ecosystem of Provolone del Monaco cheese.
© 2007 Elsevier Ltd. All rights reserved.

1. Introduction

Provolone del Monaco is a pasta filata cheese ripened for a long time, traditionally produced in the Lattari mountains area of Campania (Italy) from cows' raw milk and without starter addition. Recently, the cheese has obtained the transitory PDO (Protected Designation of Origin), a European mark indicating that its unique sensory characteristics derive from local environmental conditions, including cattle breed and nutrition, as well as cheese-making practices. For its production, at least 20% of the milk should come from cows belonging to the "Agerolese" breed, typical of the cheese production area. Briefly, the manufacturing process involves: mixing of the morning and evening milk (the latter stored in tanks at 4–7 °C for 8–10 h, after cleaning by centrifugation); coagulation by adding lamb rennet paste; curd cutting to hazelnut size; cooking at 48 °C for 40 min; whey draining by squeezing curd blocks in flaxen cloths; curd acidification on a table at room temperature for 12–14 h; stretching in water at 80–90 °C; moulding in pieces of 3–5 kg; salting in brine (18 °Be for 10 h), and ripening at about 10 °C, 85% relative humidity, for 6–12 months.

No information is available on the evolution and nature of the microbial groups occurring during manufacture and ripening of this kind of cheese. The strongly traditional technique, based on the use of raw milk with no deliberate starter addition, enhances the role of autochthonous microbiota, significantly defining the sensory quality of the cheese. In the present study, results were obtained by conventional, molecular and culture-independent techniques to investigate the microbial population changes during the Provolone del Monaco cheese-making process, which gave rise to a better quality product. It was performed in a dairy factory located in Agerola (Province of Naples), known for its traditional technology and premium quality production. We considered the main technological phases potentially capable of affecting microbial diversity. The study also showed additional

*Corresponding author. Tel.: +39 081 2539398; fax: +39 081 2539407.
E-mail address: aponte@unina.it (M. Aponte).

0958-6946/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2007.10.011

evidence concerning the reliability and potential of different analytical procedures for detecting and identifying microorganisms occurring in dairy samples.

2. Materials and methods

2.1. Sampling

Eight samples were collected throughout the technological process: from the milk in the cheese vat (M), from three different locations of the curd after cooking at 48 °C for 40 min (C_0), from the curd at the end of ripening at room temperature (C_F), from the cheese after stretching in hot water (C_S), after 10 h of brining (C_B), and after 1, 5 and 9 months of ripening (C_1, C_5, and C_9). They were kept in ice during the transfer to laboratory and analysed within few hours.

2.2. Enumeration and isolation of microorganisms

Samples were homogenized in sterile quarter strength Ringer's solution (Oxoid, Basingstoke, UK) with a Stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min, serially diluted and plated in triplicate for both microbial enumeration and isolation using the spreading plate method. The following media and incubation conditions were used: M17 agar (Oxoid) with 1% of lactose (LM17) incubating at 30 and 44 °C for 48 h; Rogosa agar (Oxoid) acidified with acetic acid to pH 5.4 in anaerobiosis (Anaerogen kit, Oxoid) at 44 °C for 72 h; Slanetz & Bartley agar (Oxoid) at 37 °C for 72 h; MRS agar (Oxoid) supplemented with 30 $\mu g\,mL^{-1}$ of vancomycin (Sigma, Milan, Italy) at 30 °C for 72 h; and HF Isolini agar (prepared according to Isolini, Grand, & Gattli, 1990) in anaerobiosis at 37 °C for 72 h.

2.3. Preliminary characterization of isolates

A total of 308 colonies were randomly picked from agar plates seeded with the highest sample dilutions to analyse microbial populations. Each colony was purified by repeated streaking on the same medium, and incubating at 30 or 37 °C for 48 h. All isolates were characterized by Gram staining, catalase activity, spore formation and gas production from glucose by the hot loop test (Sperber & Swan, 1976). Rod-shaped isolates were characterized according to Schillinger and Lücke (1987), by detecting the growth at 15 °C, the optical nature of the isomer of lactate formed by an enzymatic method (Von Krush & Lompe, 1982) and the acid production from ribose and other carbohydrates fermented (Schillinger & Lücke, 1987). All isolates were stored at −25 °C in liquid MRS cultures after addition of 20% sterile glycerol.

2.4. Preparation of total DNA from pure cultures

Bacterial colonies were suspended in sterile water and harvested by centrifuging at 12,000 × *g* for 10 min. DNA was extracted by InstaGeneTM Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations.

2.5. Molecular identification of isolates

Cocci-shaped lactic acid bacteria (LAB) were identified by amplification of 16S–23S rDNA spacer region using synthetic oligonucleotide primers described by Jensen, Webster, and Straus (1993). Cocci characterized by a unique class of 360 bp spacer were identified at species level by carrying out species-specific polymerase chain reaction (PCR) for *Streptococcus thermophilus* (Lick, Keller, Bockelmann, & Heller, 1996), and *St. macedonicus* (Papadelli, Manolopoulou, Kalantzopoulos, & Tsakalidou, 2003). Rod-shaped LAB were referred to the genus *Lactobacillus* by means of 16S–23S rDNA spacer analysis (Jensen et al., 1993). Biochemical identification was validated by species-specific PCR by using primers described by Fortina, Ricci, Mora, Parini, and Manachini (2001), Torriani, Felis, and Dellaglio (2001) and Ward and Timmins (1999) for *Lb. helveticus*, *Lb. plantarum* and *Lb. casei* groups, respectively.

Strains characterized by atypical classes of spacer were identified by sequencing the 5′ end of the 16S rDNA. Amplification of the 16S rRNA gene was performed according to Weisburg, Barns, Pelletier, and Lane (1991). The 16S rRNA PCR fragments were purified from agarose gel 1.5% (w/v) by Qiaquick Gel Extraction Kit (Qiagen, Milan, Italy) according to the supplier's instructions. The DNA sequences were determined by the dideoxy chain termination method (Sanger, Nicklen, & Coulson, 1977) by using the forward primers (fD1) described by Weisburg et al. (1991). Research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank (Altschul et al., 1997). The strains listed in Table 1 were used as markers or reference cultures when required.

2.6. Population dynamics

2.6.1. DNA extraction from bulk cells

Bulks of cells were obtained by suspending all the colonies developed on the plate surfaces in a suitable volume of quarter strength Ringer's solution (Oxoid). DNA was extracted by the Wizard DNA purification kit (Promega, Madison, WI, USA) by a procedure modified according to Ercolini, Moschetti, Blaiotta, and Coppola (2001).

2.6.2. DNA extraction from dairy samples

DNA was directly extracted from dairy samples according to three different protocols developed by Bonaïti, Parayre, and Irlinger (2006), Baruzzi, Matarante, Caputo, and Morea (2005), and Ercolini et al. (2001), respectively. The last two methods are Wizard protocol modifications, while the first technique combines the action of a

Table 1
Reference strains used in this study

Species	Source[a]
Enterococcus faecalis	ATCC 19433^T
Enterococcus faecium	ATCC 19434^T
Enterococcus durans	ATCC 19432^T
Lactococcus lactis subsp. *lactis*	DSM 20481^T
Lactococcus garvieae	DSM 20684^T
Lactococcus raffinolactis	DSM 20443^T
Leuconostoc mesenteroides subsp. *mesenteroides*	DSM 20343^T
Streptococcus thermophilus	DSM 20617^T
Streptococcus macedonicus	LMG 18488^T
Streptococcus bovis	NCDO 2127
Streptococcus parauberis	SAP99 (Blaiotta et al., 2002)
Lactobacillus casei	ATCC 393
Lactobacillus paracasei	ATCC 334
Lactobacillus rhamnosus	CNRZ 212^T
Lactobacillus crispatus	DSM 20584^T
Lactobacillus acidophilus	DSM 20079^T
Lactobacillus helveticus	DSM 20075^T
Lactobacillus fermentum	ATCC 14931^T
Lactobacillus plantarum	ATCC 14917^T
Pediococcus acidilactici	DSM 20333^T

[a]DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; CNRZ: Centre National de Recherches Zootechniques, Jouy-en-Josas, France; ATCC: American Type Culture Collection, Rockville, MD, USA; LMG: collection of the Laboratorium voor Microbiologie, Gent, Belgium. A superscript T indicates a Type strain.

chaotropic agent (acid guanidinium thiocyanate), detergents (sodium dodecyl sulphate, *N*-laurylsarcosine), a chelating agent (ethylene-diamine-tetra-acetic acid) and a mechanical action (beat beating system).

2.6.3. PCR amplification of variable regions V3 and V6–V8 of 16S rDNA

The primers V3f and V3r (Muyzer, De Waal, & Uitterlinden, 1993) and primers U968 and L1401 (Zoetendal, Akkermans, & de Vos, 1998) were used to amplify the variable regions V3 and V6–V8 of the 16S rRNA gene, giving PCR products of about 200 and 450 bp, respectively. To the forward primers, a GC clamp was added according to Muyzer et al. (1993). Amplifications were performed in a programmable heating incubator (Techno, Progene, Italy). Each mixture (final volume, 25 μL) contained 1 μL of template DNA (about 25 ng), each primer at a concentration of 0.2 μM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 μL of PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl, Invitrogen, Milano, Italy) and 2.5 U of Taq polymerase (Invitrogen). Template DNA was denatured for 5 min at 94 °C. A "touchdown" PCR was performed as previously described (Ercolini et al., 2001) to increase the specificity of amplification and to reduce the formation of spurious by-products. The initial annealing temperature was 66 °C; it was reduced by 1 °C every cycle for 10 cycles, with 20 cycles finally being performed at 56 °C. The extension for each cycle was carried out at 72 °C for 3 min while the final extension was at 72 °C for 10 min. Aliquots (5 μL) of PCR products were routinely checked on 1.5% agarose gels.

2.7. Denaturing gradient gel electrophoresis (DGGE) analysis

PCR products were analysed by DGGE using a Bio-Rad D-code apparatus and the procedure first described by Muyzer et al. (1993). Samples were applied to 7% (V6–V8) or 8% (V3) (w/v) polyacrylamide gels in TAE buffer (40 mM Tris acetate, pH 8.3 and 1.0 mM EDTA). Parallel electrophoresis experiments were performed at 60 °C by using gels containing 25–55% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40%, w/v, formamide) increasing in the direction of the electrophoresis. The gels were run for 10 min at 50 V and 4 h at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water. Bands were visualized using a UV transilluminator (Gel Doc EQ, Bio-Rad).

2.8. Sequencing of DGGE bands

DGGE bands to be sequenced were purified in water according to Ampe, ben Omar, Moizan, Wacher, and Guyot (1999). One microlitre of the eluted DNA of each DGGE band was re-amplified by using the above-described primers and conditions. PCR products which gave a single band co-migrating with the original band were then purified by QIAquick PCR purification kit (Qiagen, Milan, Italy) according to the manufacturer's instructions, and sequenced as reported above for molecular identification of isolates.

3. Results

3.1. Enumeration and identification of microorganisms occurring during cheese-making

The microbial contents of the various samples analysed are reported in Table 2. The highest viable counts were detected at the end of curd ripening for each culture medium. The stretching procedure did not seem to affect bacterial numbers. Counts on LM17 agar at both 30 and 44 °C were higher than the numbers determined on Rogosa and Isolini agar throughout the whole cheese-making process. The highest population levels were detected from the end of curd fermentation up to the salting of the cheese, with counts ranging from about 10^7 cfu g^{-1} on MRS agar with vancomycin addition, to 10^9 cfu g^{-1} on LM17 agar incubated at 30 °C. During cheese ripening counts progressively decreased, reaching a range of 10^5–10^6 cfu g^{-1} in the cheese ready to be consumed. Isolates from plates containing between 30 and 300 colonies were picked randomly, purified and identified. The sample sources,

Table 2
Changes in microbial counts (log cfu mL^{-1} or g^{-1} and standard deviation) during manufacture and ripening of Provolone del Monaco cheese

Media and incubation conditions	Samples[a]							
	M	C_0	C_F	C_S	C_B	C_1	C_5	C_9
Rogosa agar (anaerobiosis; 44 °C; 72 h)	3.86±0.16	4.08±0.12	7.41±0.11	7.76±0.01	7.51±0.13	7.38±0.06	6.82±0.14	4.93±0.11
Isolini agar (anaerobiosis; 37 °C; 72 h)	5.23±0.16	5.04±0.13	8.73±0.10	8.77±0.03	7.92±0.13	7.97±0.09	7.08±0.01	5.98±0.12
LM17 agar (44 °C; 48 h)	5.28±0.17	7.45±0.18	8.04±0.14	8.76±0.14	8.43±0.15	7.65±0.22	7.09±0.19	6.03±0.24
LM17 agar (30 °C; 48 h)	6.81±0.18	7.36±0.24	9.08±0.21	9.00±0.76	8.26±0.41	7.95±0.41	7.22±0.21	6.93±0.13
MRS agar + vancomycin (30 °C; 72 h)	5.75±0.12	5.98±0.12	7.11±0.09	7.32±0.17	7.18±0.32	7.45±0.19	7.13±0.15	6.14±0.51
Slanetz & Bartley agar (37 °C; 72 h)	6.18±0.23	6.20±0.04	8.54±0.02	8.18±0.08	7.59±0.08	7.15±0.11	6.48±0.13	5.92±0.04

[a]M: raw milk in the cheese vat; C_0: curd after cooking; C_F: curd at the end of fermentation; C_S: cheese after stretching; C_B: cheese after brining; C_1: cheese after 1 month of ripening; C_5: cheese after 5 months of ripening; C_9: cheese after 9 months of ripening.

media, incubation conditions, and identification procedures of the isolates are presented in Table 3. A total of 287 isolates out of 308 were attributed to the LAB group. By means of PCR spacer analysis, LAB were identified at species and subspecies level in the following cases: *Enterococcus faecalis* (two bands located at about 300 and 400 bp); *Ent. faecium* (two bands located at about 410 and 510 bp), *Lactococcus lactis* subsp. *lactis* (one band located at about 380 bp) and *Lc. garvieae* (one band located at about 430 bp) (Blaiotta et al., 2002; Moschetti et al., 1998). LAB characterized by spacer regions of 360 bp were assigned to the genus *Streptococcus*. Species-specific PCR was carried out in order to determine the species according to Lick et al. (1996) for *St. thermophilus* and according to Papadelli et al. (2003) for *St. macedonicus*. Four isolates presenting a single class of spacer at about 650 bp were identified by 16S rDNA sequencing as *St. bovis* (99% similarity), while five isolates, all coming from milk samples, exhibiting a spacer region 500 bp long proved to belong to the species *St. parauberis* (99% similarity). Finally, eight isolates showing two bands at about 370 and 430 bp were assigned to the species *Ent. durans* (99% similarity) by means of 16S rDNA sequencing as well. Rod-shaped LAB assigned to the *Lactobacillus* genus by means of PCR spacer analysis (two bands located at 320 and 520 bp) were divided into four groups by combining the results of growth at 15 °C with the biochemical features. DL lactate producers, unable to grow at 15 °C and to ferment ribose and sucrose, were ascribed to the species *Lb. helveticus*, which was confirmed by species-specific PCR. DL lactate producers, able to grow at 15 °C and to ferment ribose and mannitol but not D-tagatose, were ascribed to the *pentosus-plantarum* group and identified by *recA* species-specific PCR. L(+) lactate producers, able to grow at 15 °C and to ferment mannitol, were tested by V1 16S rDNA region amplification in case of both positive and negative response to ribose fermentation. The latter were referred to the *Lb. casei* group. In all cases results obtained by molecular identification were consistent with the biochemical evidences (Table 3).

Rogosa agar appeared to be the most selective medium allowing very few species to be detected: two isolates showing the same spacer profile plus one isolate exhibiting a unique profile were ascribed to the species *Pediococcus acidilactici* by means of 16S rDNA sequencing (99% similarity). Only one isolate out of 48 lactobacilli could be assigned to the species *Lb. helveticus*, while the remaining were all referred to the species *Lb. rhamnosus* (Table 3). In contrast, several different species emerged by isolation on LM17 agar at both incubation temperatures. In addition to cocci, four isolates incubated at 30 °C were *Lb. paracasei* and one isolate at 44 °C was identified as *Lb. fermentum*. However, *St. thermophilus* and *St. macedonicus* represented the majority of the isolates, especially during cheese ripening. Poor selectivity was shown for the Isolini agar and for MRS plus vancomycin, used to detect LAB referable to the genus *Leuconostoc*. Actually, within 87 cultures randomly isolated from counting plates of modified MRS, only four could be presumptively reported to the genus *Leuconostoc* by means of biochemical features and were no further investigated (data not shown).

Twenty-nine isolates were identified as *Ent. faecalis*, another species occurring throughout the cheese-making process, and only eight as *Ent. faecium. Lc. lactis* was not detected during cheese ripening and the 11 isolates of this species were obtained from samples of the initial phases of the cheese manufacture.

3.2. Application of a PCR-DGGE method to monitor cultural microbial population dynamics during cheese manufacture and ripening

Diversity and dynamics of the dominating cultivable microorganisms during this manufacture of Provolone del Monaco cheese were also investigated by PCR-DGGE analysis of the microbial biomass grown on the countable agar plates. In order to estimate the margin of error inherent in this procedure, DNA was extracted from five progressive dilutions. No difference emerged from the comparison of the DGGE profiles of V3 amplicons of all the dilutions analysed (data not shown).

Eluting, re-amplifying, and sequencing of the major bands from DGGE V3 amplicons, about 200 bp long, gave poor results for bulks from Rogosa, MRS with vancomycin,

Table 3
Bacterial species isolated during manufacture and ripening of Provolone del Monaco cheese

Culture identification	Identification procedure[a]	Sample[b]								Culture conditions
		M	C_0	C_F	C_S	C_B	C_1	C_5	C_9	
Staphylococcus spp.	Bi; Sp; Se		2(−6)[c]	1(−8)	1(−7); 1(−8)				1(−5); 3(−6)	LM17 agar at 30 °C
Ent. durans	Sp; Se			1(−8)					2(−5)	
Ent. faecalis	Sp	4(−5)		2(−8)					2(−5); 1(−7)	
Ent. faecium	Sp							1(−7)	2(−5)	
Lc. lactis	Sp	3(−5)		1(−7); 1(−8)	1(−7)					
St. bovis	Sp; Se		1(−6)	1(−8)						
St. macedonicus	Sp; Sm		1(−6)	3(−7); 2(−8)	6(−7); 1(−8)	5(−7); 1(−8)	1(−6); 5(−7)	2(−5); 7(−6); 1(−7)	1(−5)	
St. parauberis	Sp; Se	5(−5)								
St. thermophilus	Sp; St	1(−5)	7(−6); 1(−7)	1(−7); 2(−8)	2(−7); 2(−8)	2(−7); 2(−8)		1(−5)		
Lb. paracasei	Sp; Bi; Lc						1(−6); 1(−7); 1(−8)		1(−5)	
Staphylococcus spp.	Bi; Sp; Se		1(−6)	1(−8)	1(−7); 1(−8)		1(−8)	1(−5)	1(−5)	LM17 agar at 44 °C
Ent. durans	Sp; Se				1(−8)				2(−5)	
Ent. faecalis	Sp		2(−6)	1(−7); 1(−8)						
Ent. faecium	Sp			1(−8)					1(−5)	
Lc. garvieae	Sp	3(−5)								
St. bovis	Sp; Se		2(−7)							
St. macedonicus	Sp; Sm		2(−6); 1(−7)	4(−8)	2(−7); 4(−8)	5(−7)	1(−6); 1(−7)	6(−5); 2(−6)	5(−5); 1(−6)	
St. thermophilus	Sp; St	4(−5); 2(−6)	10(−6); 1(−7)	4(−7); 3(−8)	4(−7)	2(−7)	1(−6)			
Lb. fermentum	Sp; Bi; Se						1(−8); 3(−6)			
Ent. durans	Sp; Se								1(−4), 1(−5)	Slanetz & Bartley agar
Ent. faecalis	Sp	2(−5)	2(−6)	2(−7)	2(−7)	2(−7)	2(−6)	2(−5)	1(−5); 1(−6)	
Ent. faecium	Sp							1(−5)	1(−4)	
St. macedonicus	Sp; Sm							1(−5)		
Staphylococcus spp.	Bi; Sp; Se			2(−6)	1(−7)			1(−5)		Rogosa agar
Lb. helveticus	Sp; Bi; Lh	1(−2)								
Lb. rhamnosus	Sp; Bi; Lc	1(−2)	5(−6)	2(−6)	2(−7); 4(−6)	4(−6); 3(−7)	5(−6); 3(−7)	7(−5); 1(−6)	4(−3); 3(−4); 3(−5)	
Pc. acidilactici	Sp; Bi; Se	1(2); 1(−3)						1(−5)		
Staphylococcus spp.	Bi; Sp; Se	1(−4)								Isolini agar
Lb. paracasei	Sp; Bi; Lc	3(−4)					3(−7)	1(−6); 5(−5)	9(−5); 3(−6)	
Lb. helveticus	Sp; Bi; Lh						1(−6)			
Lb. plantarum	Sp; Bi; Lp		1(−5)		1(−7)					
Lb. rhamnosus	Sp; Bi; Lc		1(−5)				1(−7)	2(−6); 1(−5)		
Ent. faecium	Sp					1(−7)				
Lc. lactis	Sp	2(−4)		1(−8); 3(−7)	2(−7)	1(−7)				
St. macedonicus	Sp; Sm					1(−6)				

[a]Sp: PCR Spacer analysis (Jensen et al., 1993); Se: 16S rDNA sequencing; St: specific amplification for *St. thermophilus* (Lick et al., 1996); Sm: specific amplification for *St. macedonicus* (Papadelli et al., 2003); Bi: biochemical tests; Lc: specific amplification for *Lb. casei* group (Ward & Timmins, 1999); Lh: specific amplification for *Lb. helveticus* (Fortina et al., 2001); Lp: specific amplification for *Lb. plantarum* group (Torriani et al., 2001).
[b]Samples are named as in Table 2.
[c]Number of isolates from each sample with isolation dilution in parentheses.

and Isolini agar. Results from the other media are reported in Table 4. Further PCR-DGGE analyses were performed targeting V6–V8 regions of 16S rDNA giving PCR amplicons of about 450 bp. This approach allowed all major bands to be sequenced for the six media considered (Table 5). For both regions the analysis of samples taken during cheese-making, from raw milk to ripened cheese, showed quite dramatic changes in the profiles, with a noteworthy decrease in diversity during ripening for M17 agar at both incubation temperatures considered (Tables 4 and 5). In contrast, several species of lactobacilli appeared to be favoured by the ripening conditions (Table 5). Most of the amplicons (*Lc. lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *lactis*, *Lb. helveticus* and *Ent. faecium*) that dominated in the milk, milk with added rennet, and curd, disappeared during ripening (Tables 4 and 5); in contrast, some species such as *Lb. fermentum* and *Pd. acidilactici*, only occurred during the ripening phase (Table 5).

Amplicons showing 100% identity with *Lb. rhamnosus* and *Lb. paracasei*, were detected on Rogosa and Isolini agar, and even on MRS agar supplemented with vancomycin, confirming the resistance of both species to this antibiotic (Hamilton-Miller & Shah, 1998).

No significant difference emerged on comparing sequences of regions V3 or V6–V8 obtained from M17 agar at 30 and 44 °C or from Slanetz & Bartley agar. Sequencing of the V3 region seemed to be inadequate to define amplicons at the species level in the case of a major band characterizing the whole cheese-making process reported in Table 4 as *Streptococcus bovis/macedonicus*.

Results of band identification by DNA sequencing of both regions were at least 98% (data not shown), with the unique exception of a V6–V8 amplicon coming from DGGE patterns of bulks of cells derived by Slanetz & Bartley medium and reported in Table 5 as *Enterococcus* spp. (similarity of 95%).

3.3. Culture-independent identification of the dominant species

As a third approach, DNA was directly extracted from the same samples considered above. Patterns obtained by using the DNA extraction method proposed by Bonaïti et al. (2006) appeared significantly different from the others, while no significant difference emerged between the two Wizard's variants considered (Baruzzi et al., 2005;

Table 4
Identification by sequencing of DGGE V3 fragments from bulk cells harvested from viable counts media[a]

Media	Samples[b]								Closest relative accession no.
	M	C_0	C_F	C_S	C_B	C_1	C_5	C_9	
LM17 at 30 °C		*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	AY324612.1 AF459431.1
	Lc. lactis lactis[c]	*Lc. lactis lactis*	*Lc. lactis lactis*						AY533297.1
		St. thermophilus	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*				CP000024.1
	(−5)	(−5)	(−7)	(−7)	(−6)	(−6)	(−5)	(−4)	
LM17 at 44 °C			*Ent. faecium*		*Ent. faecium*	*Ent. faecium*	*Ent. faecium*	*Ent. faecium*	AJ968570.1
	Sp. Haem./croceolyticus	*Sp. haem./croceolyticus*	*Sp. haem./croceolyticus*		*Sp. haem./croceolyticus*	*Sp. haem./croceolyticus*			AY953148.1 AY688062.1
	Sp. warneri/pasteuri	*Sp. warneri/pasteuri*	*Sp. warneri/pasteuri*						AY186059.1 AJ717376.1
			St. bovis/macedonicus	*St. bovis/macedonicus*	*St. bovis/macedonicus*	St. bovis/macedonicus	*St. bovis/macedonicus*	*St. bovis/macedonicus*	AY324612.1 AF459431.1
	St. thermophilus		*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*			CP000024.1
				Shigella boydii					AJ575433.1
	(−4)	(−5)	(−7)	(−7)	(−6)	(−6)	(−5)	(−4)	
Slanetz & Bartley		*Ent. faecium/durans*	*Ent. faecium/durans*	*Ent. faecium/durans*	*Ent. faecium/durans*	*Ent. faecium/durans*	*Ent. faecium/durans*	*Ent. faecium/durans*	DQ411813.1 DQ239693.1
	Ent. faecalis	*Ent. faecalis*	*Ent. faecalis*						DQ411814.1
		St. bovis/macedonicus	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	*St. bovis/macedonicus*	AY324612.1 AF459431.1
	(−4)	(−5)	(−6)	(−6)	(−6)	(−5)	(−4)	(−4)	

[a]Sequences are reported following the order of migration on DGGE patterns.
[b]Samples are named as in Table 2.
[c]*Lc.*: *Lactococcus*; *St.*: *Streptococcus*; *Ent.*: *Enterococcus*; *Sp.*: *Staphylococcus*; *haem.*: *haemolyticus*.

Table 5
Identification obtained by sequencing of DGGE V6–V8 fragments from bulk cells harvested from viable counts media[a]

Media	Samples[b]								Closest relative accession no.
	M	C_0	C_F	C_S	C_B	C_1	C_5	C_9	
LM17 at 30 °C	*Staph. pasteuri*[c]		*Staph. pasteuri*						AF041361.1
	Staph. aureus	*Staph. aureus*							CP000255.1
	Lc. lactis lactis								AE006288.1
	St. thermophilus	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*				AY675258.1
				St. macedonicus	*St. macedonicus*	*St. macedonicus*	*St. macedonicus*	*St. macedonicus*	SMZ94012
				Lb. delb. lactis	*Lb. delb. lactis*	*Lb. delb. lactis*	*Lb. delb. lactis*	*Lb. delb. lactis*	AY773950.1
	(−5)	(−5)	(−7)	(−7)	(−6)	(−6)	(−5)	(−4)	
LM17 at 44 °C	*Staph. pasteuri*	*Staph. pasteuri*							AF041361.1
	Staph. aureus	*Staph. aureus*		*Staph. aureus*					CP000255.1
	Macr. caseolyticus	*Macr. caseolyticus*	*Macr. caseolyticus*	*Macr. caseolyticus*	*Macr. caseolyticus*				MCY15711
	Lc. lactis lactis	*Lc. lactis lactis*	*Lc. lactis lactis*	*Lc. lactis lactis*					AE006288.1
	St. thermophilus	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*			AY675258.1
				St. macedonicus	*St. macedonicus*	*St. macedonicus*	*St. macedonicus*	*St. macedonicus*	SMZ94012
				Shigella boydii					AY696681.1
	(−4)	(−5)	(−7)	(−7)	(−6)	(−6)	(−5)	(−4)	
Slanetz & Bartley			*Ent. faecalis*	*Ent. faecalis*	*Ent. faecalis*	*Ent. faecalis*	*Ent. faecalis*	*Ent. faecalis*	DQ411814.1
	Enterococcus spp.	*Enterococcus* spp.	*Enterococcus* spp.	*Enterococcus* spp.					DQ411813.1
									AM292057.1
	(−4)	(−5)	(−7)	(−7)	(−6)	(−5)	(−5)	(−4)	
Rogosa						*Pd. acidilactici*	*Pd. acidilactici*	*Pd. acidilactici*	DQ523486.1
						Lb. rham./paracasei	*Lb. rham./paracasei*	*Lb. rham./paracasei*	AY773957.1
									AY773956.1
	Lb. helveticus	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*				AM113779.1
						Lb. delb. bulgaricus	*Lb. delb.bulgaricus*	*Lb. delb. bulgaricus*	AY773948.1
					Lb. delb. delbrueckii	*Lb. delb. delbrueckii*	*Lb. delb. delbrueckii*	*Lb. delb. delbrueckii*	AY773949.1
				Lb. fermentum	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	LCE575812
	(−2)	(−2)	(−5)	(−6)	(−6)	(−5)	(−4)	(−3)	
Isolini	*Staph. pasteuri*	*Staph. pasteuri*							AJ717376.1
			St. macedonicus	*St. macedonicus*	*St. macedonicus*				SMZ94012
						Lb. rham./paracasei	*Lb. rham./paracasei*	*Lb. rham./paracasei*	AY773957.1
									AY773956.1
				Lb. delb. bulgaricus	*Lb. delb. bulgaricus*	*Lb. delb. bulgaricus*			AY773948.1
					Lb. delb. indicus	*Lb. delb. indicus*			AY421720.1
				Lb. delb. lactis	*Lb. delb. lactis*	*Lb. delb. lactis*			AY773950.1
							Lb. fermentum	*Lb. fermentum*	LCE575812
	(−3)	(−4)	(−7)	(−7)	(−6)	(−6)	(−5)	(−4)	
MRS + vancomycin					*Lb. rham./paracasei*	*Lb. rham./paracasei*	*Lb. rham./paracasei*	*Lb. rham./paracasei*	AY773957.1
									AY773956.1
	Leuc. mesen. lactis	*Leuc. mesen. lactis*	*Leuc. mesen. lactis*	*Leuc. mesen. lactis*	*Leuc. mesen. lactis*				AB023968.1
						Lb. fermentum	*Lb. fermentum*	*Lb. fermentum*	LCE575812
	(−4)	(−4)	(−5)	(−5)	(−5)	(−5)	(−5)	(−4)	

[a]Sequences are reported following the order of migration on DGGE patterns.
[b]Samples are named as in Table 2.
[c]*Staph.*: *Staphylococcus*; *Lc.*: *Lactococcus*; *St.*: *Streptococcus*; *Lb.*: *Lactobacillus*; *delb.*: *delbrueckii*; *Macr.*: *Macrococcus*; *Ent.*: *Enterococcus*; *Pd.*: *Pediococcus*; *rham.*: *rhamnosus*; *Leuc.*: *Leuconostoc*; *mesen.*: *mesenteroides*.

Table 6
Identifications obtained by blast comparison in GenBank of V6–V8 16S rDNA region sequences after PCR-DGGE analysis[a]

DNA extraction technique	Samples[b]								Closest relative Accession No.
	M	C_0	C_F	C_S	C_B	C_1	C_5	C_9	
Ercolini et al. (2001)	*Mor. Osloensis*[c]	*Mor. osloensis*	*Mor. osloensis*	*Mor. osloensis*					AY730714.1
		Macr. caseolyticus		*Macr. caseolyticus*	*Macr. caseolyticus*	*Macr. caseolyticus*			MCY15711
	Lactococcus spp.	*Lactococcus* spp.							DQ212979.1
		Weissella spp.	*Weissella* spp.	*Weissella* spp.	*Weissella* spp.				M23036.1
	St. thermophilus	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	AY188354.1
				Lb. helveticus	*Lb. helveticus*				AY369116.1
						Lb. delbrueckii lactis	*Lb. delbrueckii lactis*	*Lb. delbrueckii lactis*	AY773950.1
Baruzzi et al. (2005)					*Staph. haem/aureus*				AP006716.1 BX571857.1
		Rahanella spp.							DQ405246.1
		Mor. osloensis	*Mor. osloensis*	*Mor. osloensis*					AY730714.1
		Macr. caseolyticus		*Macr. caseolyticus*	*Macr. caseolyticus*	*Macr. caseolyticus*			MCY15711
	Lactococcus spp.	*Lactococcus* spp.							DQ212979.1
	Weissella spp.	*Weissella* spp.	*Weissella* spp.	*Weissella* spp.	*Weissella* spp.				M23036.1
	St. thermophilus	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	*St. thermophilus*	AY188354.1
	Lb. helveticus			*Lb. helveticus*	*Lb. helveticus*				AY369116.1
			Lb. delbrueckii lactis		*Lb. delbrueckii lactis*	*Lb. delbrueckii lactis*	*Lb. delbrueckii lactis*		AY773950.1
Bonaïti et al. (2006)		*Aeromonas simiae*	*Aeromonas simiae*	*Aeromonas simiae*	*Aeromonas simiae*	*Aeromonas simiae*	*Aeromonas simiae*	*Aeromonas simiae*	AJ536821.1

[a]DNA directly extracted from samples collected during cheese-making of Provolone del Monaco. Sequences are reported following the order of migration on DGGE patterns.
[b]Samples are named as in Table 2.
[c]*Mor.*: *Moraxella*; *Macr.*: *Macrococcus*; *St.*: *Streptococcus*; *Lb.*: *Lactobacillus*; *Staph.*: *Staphylococcus*; *haem*: *haemolyticus*.

Ercolini et al., 2001), and no benefit was achieved by additional steps performed to hydrolyse caseins.

In Table 6 we report the species detected after elution, purification, and sequencing of V6–V8 amplicons. One species, referred to *Aeromonas simiae* (98%), emerged by profiles obtained with DNA extracted according to Bonaïti et al. (2006). Surprisingly, this species was not detected when DNA was extracted with Wizard's variants.

In DGGE patterns obtained from DNAs extracted with Wizard's variants one band appeared throughout the cheese-making process, being the only dominating species up to the end of ripening. Sequencing revealed a 100% identity with *St. thermophilus* strain ATCC 19258, confirming the fundamental role of this species in the production of Provolone del Monaco cheese (Table 6). With the only exception of *Lb. delbrueckii* subsp. *lactis*, the other LAB species, specifically *Lb. helveticus, Lactococcus* spp. and *Weissella* spp., were no longer detectable during cheese ripening. *Enterobacteriaceae* occurring in the raw milk produced in this area (Coppola et al., 2006) were detected only in the early stages of cheese production. Band intensity shows that these microorganisms are affected by curd stretching temperatures and cannot survive in the ripening conditions.

Amplicons reported in Table 6 as *Lactococcus* spp. and *Weissella* spp. presented a percentage of similarity with

known sequences of GenBank (Altschul et al., 1997) of 93% and 95%, respectively. In the other cases similarity was at least 98%.

4. Discussion

Provolone del Monaco cheese is a ripened pasta filata cheese made from raw cows' milk according to a traditional process. Producers firmly sustain that their product is distinct from other similar cheeses that are produced with raw milk but with the addition of a milk or whey culture of lactic acid bacteria as a starter, as well as, of course, from cheeses of the same category produced with pasteurized milk and commercial starters. In the case of this Provolone cheese, only the native microflora arising from the milk and dairy environment is involved.

In our study, evolution of the LAB community during a cheese-making process, successfully performed in a dairy factory known for its recognized premium quality production, was revealed by viable counting on different media and using a combination of conventional and molecular methods. Microbial enumeration closely matches findings obtained during a survey about Fior di latte di Agerola (an unripened pasta filata cheese) performed on samples coming from the same traditional farmer (Coppola et al., 2006), although, in Provolone del Monaco viable counts of both mesophilic and thermophilic LAB in milk were at least one log lower. The observed lower values could be related to the choice of producers to use commonly the best quality lots of milk for the cheese-making of Provolone del Monaco. Plate count methods allowed the effects of the selective pressure exerted by technology during cheese manufacture and ripening to be shown only to a limited extent. This may be due to the poor selectivity of the media and incubation temperatures as well as to the intrinsic ability of lactic microflora to grow in a rather wide range of conditions or to tolerate technological stress. Our results clearly show that the initial phases of cheese manufacture, up to the end of the phase of curd cooking, promote the growth of microorganisms able to give countable colonies on the media employed, and, in regard with LM17 agar, at both 30 and 44 °C. With the long phase of cheese ripening, as expected, all counts significantly decrease. However, within this study, the reduction in the microbial content, usually detected due to curd stretching, was quantified as not particularly relevant, since, in this case, curd stretching is typically performed in the presence of minimal quantities of hot water. Subsequently, the cheese microbial content decreases with brining followed by ripening.

As far as suitability of the culture media employed, we noted that in addition to streptococci and lactococci, enterococci, lactobacilli as well as catalase-positive cocci were isolated on LM17 agar at both incubation temperatures. Moreover, Isolini agar (Isolini et al., 1990) proposed for the culture of heterofermentative *Lactobacillaceae*, allowed us to isolate strains referable to the *Lb. casei* group.

From the cultures, a variety of 16 different taxa were detected after identification of the isolates by classical phenotypic procedures, PCR-based methods, and 16S rDNA sequence analysis. The dominant LAB species throughout the cheese-making process were the thermophilic *St. thermophilus* and *St. macedonicus* among the cocci-shaped LAB, and *Lb. rhamnosus* among the rods: 72 out of 308 isolates (about 23%) could be referred to the first species and 52 (about 17%) to each of the other two. These three species occurred in all the samples analysed and were isolated from the highest sample dilutions.

The presence of *St. macedonicus* in cheese manufacture is quite interesting as this species, first isolated from Greek Kasseri cheese (Tsakalidou et al., 1998) has been recently found in several European (Callon, Millet, & Montel, 2004; Lombardi et al., 2004; Poznanski, Cavazza, Cappa, & Cocconcelli, 2004) and specifically Italian cheeses (Pacini, Cariolato, Andrighetto, & Lombardi, 2006). Although the technological properties of the isolates collected in this study were not investigated, it is widely held that this microorganism may play a role in the sensory property of cheeses since strains of this species may possess lipolytic and proteolytic activity (Lombardi et al., 2004), and may be able to produce exopolysaccharides (Vincent, Faber, Neeser, Stingele, & Kamerling, 2001).

Among rod-shaped LAB, lactobacilli belonging to the *Lb. casei* group seemed to dominate when classical isolation procedures were performed. *Lb. rhamnosus* significantly occurs throughout the production process, confirming that its prevalence as a natural component of the microflora of dairy environments has been underestimated previously (Desai, Shah, & Powell, 2006). Remarkably, *Lb. delbrueckii*, by PCR-DGGE analysis indicated as quite dominant in the ripening cheese, was never isolated on the selective media. This phenomenon has been noted before in dairy environments (Ercolini, Hill, & Dodd, 2003; Obodai & Dodd, 2006; Randazzo, Torriani, Akkermans, de Vos, & Vaughan, 2002). The relatively small number of identified strains may be insufficient to detect all the LAB species.

The PCR-DGGE of V3 region amplicons provided poor results even when applied to the bulk of colonies harvested from M17 agar plates: one major band could not be identified at species level, since it fitted both *Streptococcus bovis* and *St. macedonicus* equally. Presumably, according to evidence emerging from V6–V8 amplicons sequencing, this band could be assigned to the species *St. macedonicus*.

Higher microbial diversity and more interesting information were obtained by amplifying the V6–V8 regions: in addition to the most significant species found by analysing the isolates, *Macrococcus caseolyticus*, *Leuconostoc mesenteroides* subsp. *lactis*, *Lb. fermentum*, and four subspecies of *Lb. delbrueckii* (namely *delbrueckii*, *bulgaricus*, *lactis*, and *indicus*) were also detected. During manufacture and especially during ripening, thermophilic lactobacilli, including *Lb. delbrueckii* and *Lb. fermentum*, emerged, presumably selected by the modified cheese environment.

None of the species was isolated when a conventional approach was used. In contrast, DGGE analysis failed to show the presence in the initial steps of cheese-making of species such as *St. bovis*, *St. parauberis*, and *Lc. garvieae* obtained by isolate identification.

Culture-independent PCR-DGGE analysis was carried out in order to circumvent the limitations of conventional culture methods already pointed out in many previous studies (Ampe et al., 1999; Ercolini et al., 2001). Within this approach, DNA was directly extracted from the cheese samples. When investigating whole communities, a reliable extraction method of DNA from sample is critical, since all further analyses assume the complete and representative presence of accessible nucleic acids. Wizard's variants (Baruzzi et al., 2005; Ercolini et al., 2001), allowed more reproducible profiles and more bands to be obtained, compared to those achieved with DNA extracted according to Bonaïti et al. (2006). *St. thermophilus* represented the only detectable species up to the end of ripening and can thus be considered the most dominant and metabolically important bacteria in Provolone del Monaco, probably involved in flavour and aroma development (Chammas, Saliba, Corrieu, & Béal, 2006). Surprisingly, band sequencing highlighted the genus *Weissella* which had never appeared before, although MRS supplemented with vancomycin is a suitable medium for isolating this bacterium.

5. Conclusions

The microbiota involved in the Provolone del Monaco cheese-making investigated in the present study can be considered largely dominated, up to the end of cheese ripening, by thermophilic streptococci such as *St. thermophilus* and *St. macedonicus*, enterococci and lactobacilli belonging to the *casei* group, with *Lb. rhamnosus* significantly occurring throughout the production process. In contrast, homofermentative thermophilic *Lb. helveticus*, generally suggested as the main component of starter culture for this type of cheese, was detected only occasionally.

The use of various approaches allowed many other species to be detected. The molecular procedures used to support the identification of isolates provided various contributions to describe microbial diversity for the different samples. Among the results obtained by PCR-DGGE analysis, the most important were those achieved targeting the V6–V8 region of 16S rDNA when DNA extracted from bulks of colonies grown on different culture media was used as PCR template. Nevertheless, each analytical procedure provided useful information. Therefore, these results confirm the need of a polyphasic approach to investigate microbial successions and their connection to cheese quality, and highlight the different capability of the employed methods to detect LAB species. Other further studies performed by similar approaches on different production processes will be necessary in order to definitively assess the microbiological events that can be considered characteristics for achieving the typical quality of this cheese variety, in absence of defects or spoilage.

Acknowledgements

This study was partially supported by a grant from the Italian MIPAF (Ministero per le Politiche Agricole e Forestali) within the Program "Valorizzazione e salvaguardia della microflora autoctona caratteristica delle produzioni casearie italiane". The authors thank Drs. Giuseppe Blaiotta and Danilo Ercolini for their useful suggestions, and Renata Solipano for her technical collaboration.

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402.

Ampe, F., ben Omar, N., Moizan, C., Wacher, C., & Guyot, J.-P. (1999). Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Applied and Environmental Microbiology*, *65*, 5464–5473.

Baruzzi, F., Matarante, A., Caputo, L., & Morea, M. (2005). Development of a culture-independent polymerase chain reaction-based assay for the detection of lactobacilli in stretched cheese. *Journal of Rapid Methods and Automation in Microbiology*, *13*, 177–192.

Blaiotta, G., Pepe, O., Mauriello, G., Villani, F., Andolfi, R., & Moschetti, G. (2002). 16S–23S rDNA intergenic spacer region polymorphism of *Lactococcus garvieae*, *Lactococcus raffinolactis* and *Lactococcus lactis* as revealed by PCR and nucleotide sequence analysis. *Systematic and Applied Microbiology*, *25*, 520–527.

Bonaïti, C., Parayre, S., & Irlinger, F. (2006). Novel extraction strategy of ribosomal RNA and genomic DNA from cheese for PCR-based investigations. *International Journal of Food Microbiology*, *107*, 171–179.

Callon, C., Millet, L., & Montel, M. C. (2004). Diversity of lactic acid bacteria isolated from AOC Salers cheese. *Journal of Dairy Research*, *71*, 231–244.

Chammas, G. I., Saliba, R., Corrieu, G., & Béal, C. (2006). Characterisation of lactic acid bacteria isolated from fermented milk "laban". *International Journal of Food Microbiology*, *110*, 52–61.

Coppola, S., Fusco, V., Andolfi, R., Aponte, M., Blaiotta, G., Ercolini, D., et al. (2006). Evaluation of microbial diversity during the manufacture of Fior di Latte di Agerola, a traditional raw milk pasta-filata cheese of the Naples area. *Journal of Dairy Research*, *73*, 264–272.

Desai, A. R., Shah, N. P., & Powell, I. B. (2006). Discrimination of dairy industry isolates of the *Lactobacillus casei* group. *Journal of Dairy Science*, *89*, 3345–3351.

Ercolini, D., Hill, P. J., & Dodd, C. E. R. (2003). Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology*, *69*, 3540–3548.

Ercolini, D., Moschetti, G., Blaiotta, G., & Coppola, S. (2001). The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo Mozzarella cheese production: Bias of "culture dependent" and "culture independent" approaches. *Systematic and Applied Microbiology*, *24*, 610–617.

Fortina, M. G., Ricci, G., Mora, D., Parini, C., & Manachini, P. L. (2001). Specific identification of *Lactobacillus helveticus* by PCR with

pepC, pepN and htrA targeted primers. *FEMS Microbiology Letters*, *198*, 85–89.

Hamilton-Miller, J. M. T., & Shah, S. (1998). Vancomycin susceptibility as an aid to the identification of lactobacilli. *Letters in Applied Microbiology*, *26*, 153–154.

Isolini, D., Grand, M., & Gattli, H. (1990). Selektivmedien zum nachweis von obligat und fakultativ heterofermentative Laktobazillen. *Schweizerische Milchwirtschaftliche Forschung*, *19*, 57–59.

Jensen, M. A., Webster, J. A., & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology*, *59*, 945–952.

Lick, S., Keller, M., Bockelmann, W., & Heller, K. J. (1996). Rapid identification of *Streptococcus thermophilus* by primer-specific PCR amplification based on its lacZ gene. *Systematic and Applied Microbiology*, *19*, 74–77.

Lombardi, A., Gatti, M., Rizzotti, L., Torriani, S., Andrighetto, C., & Giraffa, G. (2004). Characterization of *Streptococcus macedonicus* strains isolated from artisanal Italian raw milk cheeses. *International Dairy Journal*, *14*, 967–976.

Moschetti, G., Blaiotta, G., Aponte, M., Catzeddu, P., Villani, F., Deiana, P., et al. (1998). Random amplified polymorphic DNA and amplified ribosomal DNA spacer polymorphism: Powerful methods to differentiate *Streptococcus thermophilus* strains. *Journal of Applied Microbiology*, *85*, 25–36.

Muyzer, G., De Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, *59*, 695–700.

Obodai, M., & Dodd, C. E. R. (2006). Characterization of dominant microbiota of a Ghanaian fermented milk product, *nyarmie*, by culture and nonculture-based methods. *Journal of Applied Microbiology*, *100*, 1355–1363.

Pacini, F., Cariolato, D., Andrighetto, C., & Lombardi, A. (2006). Occurrence of *Streptococcus macedonicus* in Italian cheeses. *FEMS Microbiology Letters*, *261*, 69–73.

Papadelli, M., Manolopoulou, E., Kalantzopoulos, G., & Tsakalidou, E. (2003). Rapid detection and identification of *Streptococcus macedonicus* by species-specific PCR and DNA hybridization. *International Journal of Food Microbiology*, *81*, 231–239.

Poznanski, E., Cavazza, A., Cappa, F., & Cocconcelli, P. S. (2004). Indigenous raw milk microbiota influences the bacterial development in traditional cheese from alpine natural park. *International Journal of Food Microbiology*, *92*, 141–151.

Randazzo, C. L., Torriani, S., Akkermans, A. D. L., de Vos, W. M., & Vaughan, E. E. (2002). Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Applied and Environmental Microbiology*, *68*, 1882–1892.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA*, *74*, 5463–5467.

Schillinger, U., & Lücke, F.-K. (1987). Identification of lactobacilli from meat and meat products. *Food Microbiology*, *4*, 199–208.

Sperber, W. H., & Swan, J. (1976). Hot loop test for the determination of carbon dioxide production from glucose by lactic acid bacteria. *Applied and Environmental Microbiology*, *31*, 990–991.

Torriani, S., Felis, G. E., & Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. *Applied and Environmental Microbiology*, *67*, 3450–3454.

Tsakalidou, E., Zoidou, E., Pot, B., Wassill, L., Ludwig, W., Devriese, L. A., et al. (1998). Identification of streptococci from Greek Kasseri cheese and description of *Streptococcus macedonicus* sp. nov. *International Journal of Systematic Bacteriology*, *48*, 519–527.

Vincent, S. J. F., Faber, E. J., Neeser, J. R., Stingele, F., & Kamerling, J. P. (2001). Structure and properties of the exopolysaccharide produced by *Streptococcus macedonicus* Sc136. *Glycobiology*, *11*, 131–139.

Von Krush, U., & Lompe, A. (1982). Schnellest zum qualitativen nachweis von L(+) und D(−) milchsaure fur due bestimmung von milchesaurebakterien. *Milchwissenschaft*, *37*, 65–68.

Ward, L. J. H., & Timmins, M. J. (1999). Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. *Letters in Applied Microbiology*, *29*, 90–92.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*, 697–703.

Zoetendal, E. G., Akkermans, A. D., & de Vos, W. M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Applied and Environmental Microbiology*, *64*, 3854–3859.