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ORIGINAL ARTICLE

Microbiological characterization using combined culture dependent and independent approaches of Casizolu pasta filata cheese

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Enterococcus italicus, *Lactobacillus paracasei*, pasta filata cheese, raw milk, TTGE.

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Abstract

Aims: Casizolu is a traditional Sardinian (Italy) pasta filata cheese made with cow raw milk belonging to Sardo-Modicana and/or Bruno-Sarda breeds added with natural whey starter. This work aims to describe the traditional technology of this product and to evaluate the microbial groups/species involved in the first month of ripening.

Methods and Results: Raw milk, curd after stretching and Casizolu cheese samples from two different farmsteads were subjected to enumeration of microbial groups, isolation and genotypic characterization of isolates and PCR temporal temperature gel electrophoresis (TTGE) analysis. The counts of lactobacilli and lactococci groups in raw milk were about 5–6 log UFC ml^{-1} of milk. These counts tended to increase in curd and cheeses, reaching values higher than 8 log UFC g^{-1} of cheese. Culture dependent and independent approaches employed in this work highlighted the fundamental role of *Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus* and *Lactobacillus paracasei* in the manufacture and ripening of Casizolu cheese. Other species frequently isolated were *Enterococcus durans*, *Enterococcus faecium*, *Enterococcus italicus* while *Enterococcus lactis*, *Streptococcus parauberis*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus fermentum and Lactococcus raffinolactis* were isolated occasionally.

Conclusions: *Lactococcus lactis* subsp. *lactis*, *Strep. thermophilus* and *Lact. paracasei* were the principal bacterial species involved in the Casizolu cheese manufacturing and ripening. For the first time, *Ent. italicus* and *Ent. lactis* were isolated in the pasta filata cheese.

Significance and Impact of the Study: This study shows the first data on microbial groups and species involved in the manufacture of Casizolu cheese and highlights the role of *Lact. paracasei* and *Enterococcus* spp. from the earliest stages of ripening cheese; furthermore, provides evidence that raw milk cheese is a source of new strains and therefore a reservoir of microbial biodiversity.

Introduction

Pastoralism and small-scale agriculture has always been the backbone of the economy in Sardinia (Italy), where the dairy farming has been accompanied by the processing of its raw milk in cheese. Raw milk has also an important economic role in manufacturer's cheese production by increasing milk usefulness, compared to its current industrial price (Licitra 2010). In raw milk, cheese fermenting and ripening activities are carried out by native microbiota that gives typical organoleptic features to the cheese such as taste, aroma and texture, often much appreciated by the consumers. The Sardinia island (Italy) boasts a wide range of PDO (Manca *et al.*

2006; Mangia *et al.* 2013) and traditional cheeses (MiPAAF, 2000), typical products representing a specific geographic area whose protection is very important.

Casizolu, one of the most common and typical products of Sardinia is a pasta filata cheese manufactured from raw cow milk added with natural whey starter. The particular characteristics of this cheese type are strictly dependent to the production technology process as the use of cow raw milk belonging to Sardo-Modicana and/or Bruno-Sarda breeds and the high temperature (70–80°C) needed for the stretching process to which is subjected the curd after acidification. Casizolu cheese can be consumed after a brief period of ageing (average 30 days) or may undergo, rarely, extensive ageing time before being consumed (up to 9 months). Casizolu is characterized by a pear-like shape, an intense straw colour and a distinctive smell and flavour which make it much appreciated by consumers.

In Italy, there are several kinds of pasta filata cheeses, without aging, such as water bufalo mozzarella and Fior di Latte cheese and with brief or long ripening period such as Scamorza, Provolone and Caciocavallo cheese (Coppola *et al.* 2006; Aponte *et al.* 2008; De Pasquale *et al.* 2014; Guidone *et al.* 2015; Ricciardi *et al.* 2015). However, few study were made until now on the microbial community of these cheeses using both dependent and independent approach.

To present, there are not studies in the literature about the microbiota involved in fermentation and ripening of Casizolu cheese production. Therefore, the aim of this work is to describe the traditional technology of this product and to evaluate the microbial groups and lactic acid bacteria (LAB) involved in the ripening process. A polyphasic approach was carried out using both culture dependent (enumeration, isolation and genotypic characterization of bacteria strains on selective medium) and independent (PCR-TTGE analysis) methods.

Material and methods

Three batches of Casizolu were prepared in two farmsteads (small scale artisanal Casizolu cheese producing) indicated as FA and FB.

The manufacturing procedures, involved in the cheese-making trials, are shown in Table 1.

Sampling, enumeration of microbial groups, pH and water activity determination

Raw cow milk ($n = 3$) curd after stretching ($n = 3$) and 30 days ripened cheese samples ($n = 3$) were collected per farmstead and analysed. Ten grams of milk, curd and cheese samples were homogenized with 90 ml of ¼ strength Ringer solution and serially diluted. The following microbial groups were enumerated in different media: total mesophilic bacteria on Plate Count Agar (Oxoid, Milan, Italy) at 32°C for 48 h; presumptive lactic cocci on M17 agar (Microbiol, Cagliari, Italy), incubated at 37°C for 72 h in anaerobic conditions (Gas-Pack; Oxoid); lactobacilli in de Man, Rogosa and Shape agar (Oxoid) acidified to pH 5·4, incubated at 37°C for 72 h in anaerobic conditions; presumptive enterococci on Slanetz & Bartley medium (Oxoid), incubated at 37°C for 48 h; enterobacteria on Violet Red Bile Glucose agar (VRBGA; Oxoid), incubated at 30°C for 48 h; on Baird Parker agar (BPA; Oxoid) presumptive colony of coagulase positive staphylococci (CPS) were assayed for coagulase activity using the Staphylase test (Oxoid). *Escherichia coli* was determined using Most Probable Number methods in Brilliant Green Bile Broth (Oxoid) with Durham tube, at 44°C for 48 h and after the incubation time, a subculture (0·1 ml) of test positive tube was transferred and incubated at 44°C in peptone broth and tested for indole production using Kovac's reagent (Microbiol); *Pseudomonas* spp. on Pseudomonas Selective Agar (Microbiol); yeasts on Yeast extract Peptone Dextrose agar (1% w/v yeast extract, 2% w/v dextrose, 2% w/v peptone, 1·5% w/v agar, pH 4·5) at 25°C for 48 h. Spores of butyric clostridia were assessed in liquid media: Skim Milk added with A solution (sodium lactate 12 g; sodium acetate 5 g; yeast extract 5 g; cysteine 1 g; water q. s. to 100), incubated at 37°C for 48 h in anaerobic conditions after pasteurization at 80°C for 20 min (APHA 1985).

Milk, curd and cheese pH values, were measured with a pH meter (Crison Instruments SA, Barcelona, Spain), curd and cheese samples (10 g) were homogenized with equal quantities of distilled water (10 ml) and the pH of

Table 1 Traditional cheese making process of Casizolu

Milk	Raw cow milk
Heating of milk	37°C
Starter	Natural whey starter
Addition of rennet	30 ml 100 l^{-1} (Chr. Hansen, Horsholm, Denmark; strength 1 : 10·000)
Coagulation time	About 20 min
Heating	40°C
Break	15–20 min
Breaking curd	1–2 mm
Heating curd	40°C
Break	The curd is left in whey for about 1 h
Curd extraction	The curd is transferred into plastic mould
Curd acidification	18–20 h
Cutting	Of the acidified curd
Stretching	In hot water (70–80°C) for about 5 min
Shaping	The curd again hot is manually shaped
Cooling	First at 14–15°C then at 5–7°C
Salting	Immersion in saturated brine for 6–8 h per kg of cheese
Ripening	Usually 30 days

dispersion was measured. Water activity (a_w) of the curd after stretching was determined using the AW LAB SET H (Novasina, Lachen, Switzerland) according to the manufacturer's instructions.

Lactic acid bacteria strains isolation

Five colonies of different morphologies were randomly picked from each 25–250 colony of MRS and M17 agar plate. The isolates were purified by successive subculturing on MRS and M17 agar for lactobacilli and cocci, respectively, tested for Gram stain, catalase production and shape morphology (phase contrast microscopy; Zeiss, Gottingen, Germany) and stored at −80°C in broth media containing 30% of glycerol (v/v).

DNA extraction from LAB isolates

Total DNA of 105 LAB isolates was extracted using the ArchivePure DNA Yeast & Gram-+ Kit (5 PRIME GmbH, Hamburg, Germany), according to manufacturer's instructions. The concentration, integrity and purity of DNA was assessed by spectrophotometric measurements using LvisPLATE SpectroSTAR Nano (BMG Labtech, Ortenberg, Germany) and by agarose gel electrophoresis.

Randomly amplified polymorphic DNA-polymerase chain reaction fingerprint analysis

For amplification reaction in the randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and subsequent fingerprinting was used the primer M13 (5′GAGGGTGGCGGTTCT-3′) (Huey and Hall 1989). PCR reactions were performed in 50 μl final volume according to Rossetti and Giraffa (2005) with minor variation. Briefly, the reaction mixture containing 1× PCR buffer (Life Technology, Carlsbad, CA), 5 mmol l^{-1} of $MgCl_2$, 200 μmol l^{-1} of dNTPs, 2 μmol l^{-1} of primer, 1·25 U of TAQ polymerase® (Life Technology) and 50 ng of bacterial DNA. PCR amplification was performed with a thermal cycler MyCicler™ (Bio-Rad Laboratories, Hercules, CA) with the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 40 s, elongation at 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis for 3 h at a constant voltage of 2·6 V cm^{-1} on 1·5% (w/v) agarose gel (15 × 20 cm) containing 0·1 μl ml^{-1} of SYBR safe (Life Technology) in 1× TAE (40 mm l^{-1} Tris-acetate, 1 mm l^{-1} EDTA, pH 8). A 100–10 000 DNA molecular mass marker (Euroclone, Milano, Italy) was used as a standard. Digital pictures of the gels were captured using Chemidoc XRS (Bio-Rad Laboratories) and saved as TIFF file using the software QUANTITY ONE (Bio-Rad Laboratories) for following cluster analysis.

Repetitive element palindromic-polymerase chain reaction analysis

The repetitive element palindromic-polymerase chain reaction (rep-PCR) analysis was performed with $(GTG)_5$ oligonucleotide primer (5′-GTGGTGGTGGTGGTG-3′) in 50 μl final volume containing 1× PCR buffer (Life Technology), 3 mmol l^{-1} of $MgCl_2$, 200 μm l^{-1} of dNTPs, 1 μm l^{-1} of primer, 2 U of TAQ polymerase® (Life Technology) and 20 ng of bacterial DNA. PCR amplification was performed with a thermal cycler MyCicler™ (Bio-Rad Laboratories) with the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 1 min, elongation at 72°C for 4 min and a final extension at 72°C for 7 min. PCR products were separated by electrophoresis for 3·5 h at a constant voltage of 2·8 V cm^{-1} on a 1·5% (w/v) agarose gel (15 × 20 cm) containing 0·1 μl ml^{-1} of SYBR safe (Life Technology) in 1× TAE (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8). Digital analysis of gel were performed as above.

Evaluation of reproducibility of RAPD and rep-PCR methods

The reproducibility of fingerprint patterns of random amplification of polymorphic DNA (RAPD) and rep-PCR methods was assessed using DNA extracts from three different microbial colony randomly selected, according to Solieri *et al.* (2012).

Sequencing of 16S rDNA of LAB isolates

Isolated strains representative of each cluster and strains noncluster forming, were subjected at 16S rDNA sequencing. The DNA of single strain was extracted as reported above and the 16S ribosomal DNA fragment (1500 bp) was amplified using the universal primers W001 and W002 as previously described by Godon *et al.* (1997) Subsequently, amplicons were sequenced after being purified with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Sequencing with primer W001 was performed at BMR Genomics, University of Padova, Italy (http://www.bmr-genomics.it). An average of 800 bp per sequence were obtained and compared with those presents in the GenBank database using the BLAST program (http://www.ncbi.nih.gov/BLAST/) (Altschul *et al.* 1997), and with those in the Ribosomal Database project

(http://rdp.cme.msu.edu/edu/index.jsp). Sequences with 97% of identity or higher were considered to represent the same species (Stackebrandt and Goebel 1994; Palys *et al.* 1997).

Species-specific PCR for identification of the LAB strains

After 16S rDNA sequencing, on LAB strain clustered in *Lactococcus lactis*, *Streptococcus thermophilus*, *Enterococcus durans*, *Enterococcus faecium* and *Enterococcus italicus* taxon, species specific PCR was applied in order to confirm their phylogenetic affiliation. Since the sequencing of the 16S rDNA did not allow to distinguish among the species belonging to *Lactobacillus plantarum* groups a multiplex PCR to amplify *rec*A gene was performed according to Torriani *et al.* (2001). The primers Y1 and Y2 were used to discriminate *Lactobacillus casei*, *Lact. paracasei* and *Lactobacillus rhamnosum*, at the species level according to Ward and Timmins (1999). *Lactococcus lactis* was distinguish at subspecies level according to Pu *et al.* (2002).

All primer used were purchased from Invitrogen (*Life* Technology, Glasgow, UK) and are listed in Table 2 along with the size of the expected PCR fragment. Reaction mixture and PCR cycling were performed as described originally for each primer set. PCR reagent Taq polymerase, PCR buffer and $MgCl_2$ were purchased from *Life* Technology; dNTPs were purchased from EuroClone. The PCR products were analysed by electrophoresis in 2% agarose gel on 1× Tris-Acetate-EDTA (TAE) buffer. For each PCR, a reference strain was used as positive control (Table 3) and the PCR mixture (without DNA) as negative control.

Temporal temperature gradient gel electrophoresis analysis of Casizolu

Fingerprints of DNA extracted from milk, curd and cheeses were performed by temporal temperature gradient gel electrophoresis (TTGE) analysis of 16S rDNA. The variable region V3 of rDNA of each samples was amplified by PCR and separated along their differences in nucleotide sequence, as previously described (Parayre *et al.* 2007) with the following minor differences. After running, the gel was stained with SBYR safe (Life Technology), bands were excised from gels after staining with sterile scalpel, eluted overnight in 50 μl of sterile water, re-amplifed with the same protocol and primers used for

Table 2 Primers used in this work

Primer	Sequence	Specificity	Size of amplicon	Reference
M13	5′-GAGGGTGGCGGTTCT-3′	RAPD-PCR	Variable	Huey and Hall (1989)
$(GTG)_5$	5′-GTGGTGGTGGTGGTG-3′	REP-PCR	Variable	Versalovic *et al.* (1994)
W001	5′-AGAGTTTGATCMTGGCTC-3′	16S rDNA	1500 bp	Godon *et al.* (1997)
W002	5′-GNTACCTTGTTACGACTT-3′			
paraF	5′-GTCACAGGCATTACGAAAAC-3′	(*Multiplex PCR*)	318 bp (*Lact. plantarum*),	Torriani *et al.* (2001)
pentF	5′-CAGTGGCGCGGTTGATATC-3′	*Lactobacillus plantarum*,	218 bp (*Lact. pentosus*),	
planF	5′-CCGTTTATGCGGAACACCTA-3′	*Lact. pentosus and*	107 bp	
pREV	5′-TCGGGATTACCAAACATCAC-3′	*Lact. paraplantarum*	(*Lact. paraplantarum*)	
Casei	5′-TGCACTGAGATTCGACTTAA-3′	*Lactobacillus casei*	290 bp	Ward and Timmins (1999)
Y2	5′-CCCACTGCTGCCTCCCGTAGGAGT-3′			
Para	5′-CACCGAGATTCAACATGG-3′	*Lactobacillus paracasei*	290 bp	Ward and Timmins (1999)
Y2	5′-CCCACTGCTGCCTCCCGTAGGAGT-3′			
Rham	5′-TGCATCTTGATTTAATTTTG-3′	*Lactobacillus rhamnosus*	290 bp	Ward and Timmins (1999)
Y2	5′-CCCACTGCTGCCTCCCGTAGGAGT-3′			
IRL	5′-TTTGAGAGTTTGATCCTGG-3′	*Lactococcus lactis*	290 bp	Pu *et al.* (2002)
LacreR	5′-GGGATCATCTTTGAGTGAT-3′			
LacF	5′-GTACTTGTACCGACTGGAT-3′	*Lactococcus lactis* subsp *lactis*	290 bp	Pu *et al.* (2002)
LacreR	5′-GGGATCATCTTTGAGTGAT-3′			
creF	5′-GTGCTTGCACCGATTTGAA-3′	*Lactococcus lactis* subsp *cremoris*	290 bp	Pu *et al.* (2002)
LacreR	5′-GGGATCATCTTTGAGTGAT-3′			
itaF	5′-TACCGCATAATACTTTTTCTCT-3′	*Enterococcus italicus*	295 bp	Fortina *et al.* (2007)
itaR	5′-GTCAAGGGATGAACATTCTCT-3′			
F1	5′-GCAAGGCTTCTTAGAGA-3′	*Enterococcus faecium*	550 bp	Dutka-Malen *et al.*(1995)
F2	5′-CATCGTGTAAGCTAACTTC-3′			
DU1	5′-CCTACTGATATTAAGACAGCG-3′	*Enterococcus durans*	295 bp	Jackson *et al.* (2004)
DU2	5′-TAATCCTAAGATAGGTGTTTG-3′			
StrF	5′-CACTATGCTCAGAATACA-3′	*Streptococcus thermophilus*	968 bp	Lick *et al.* (1996)
StrR	5′- CGAACAGCATTGATGTTA-3′			

16S rDNA PCR without the GC-clamp. The purified PCR amplicons were sequenced with primer V3P2 and nucleotide sequences compared with those listed in the GenBank database, as described above.

Data analysis

The cluster analysis of the band profiles obtained from Random Amplification of Polymorphic DNA (RADP) and rep-PCR, was performed by the software INFOQUEST (ver. 4.5; Bio-Rad) using the gel pictures saved in TIFF format as described above. Calculation of similarity between strains profiles was calculated using the Pearson's correlation similarity coefficient. RAPD and rep-PCR band profiles were combined and a unique dendrogram was obtained, for lactobacilli and cocci, respectively, using the unweighted-pair-group method with arithmetic averages (UPGMA). The discriminatory index was evaluated by calculating the Simpson's index of diversity at the specific cut off value of 85%, according to Coenye *et al.* (2002).

Results of microbiological counts were $\log_{10}$ transformed to normalized the data. The significance of difference between means counts for each microbial groups for milk, curd and cheese samples from farmstead A and B, respectively, was determined by Student's *t*-test ($P < 0{\cdot}05$). Calculated values of *t* were determined by using software STATGRAPHICS CENTURION XV (Statpoint Technologies, Inc., Warrenton, VA).

Results

Enumeration of microbial groups by culture dependent approach

Microbial counts at different sampling time of Casizolu cheeses from each of the two farmsteads are reported in Table 4. Raw milk from the farmstead FB generally exhibited greater microbial counts, only exception was observed for lactobacilli and *Pseudomonas* counts but significant differences in any group were not observed; while in curd and cheese FB samples Yeast, CNS, Enterobacteria and *Pseudomonas* counts were statistical significantly higher than FA samples.

In particular, microbial counts revealed an initial presumptive lactobacilli and lactococci population of 5–6 log CFU ml^{-1} in raw milk. These values tended to increase in curd and in the cheese where the presumptive lactobacilli reaching the highest value in farmstead FB. Presumptive enterococci were present in all milk samples analysed and their counts increased in cheese at 30 days of ripening.

Yeast counts in both raw milk and curd from different farmsteads were very similar. The yeast count in FA cheese at 30 days of ripening was one log unit lower than of milk yeast count, whereas in FB cheese and milk yeast count is almost the same.

The FA coagulase negative staphylococci (CNS) count decreases only after curd stretching, whereas in FB samples the count was lower in the curd compared to that of milk and increased at 30 days of ripening. Coagulase positive staphylococci (CPS) were below the enumeration limit in all samples. Enterobacteria were present in FB milk and curd samples, whereas the count in the cheese is very low (about 1 log CFU g^{-1}), they have also been detected in the FA milk, but in the other two sampling times their numbers decreased until being below the detection limit in the cheese.

Escherichia coli presumptive count were below the limit of detection in all samples analysed as the spores of butyric clostridia.

Presumptive *Pseudomonas* ssp. were initially detected in both raw milk samples, in FA completely disappeared during manufacturing process, whereas in FB *Pseudomonas* count increased in the curd and decreased in the cheese samples. The value of pH during FA Casizolu manufacturing decreased reaching the lowest value in the cheese, whereas in the FB batches show the lowest value in the curd and then increased in the cheese. Both curd samples presented similar water activity value but in FA cheese decreases until 0·930, significantly lower than FB during ripening time (Table 4).

Table 3 Reference strains used in this work

Lactic acid bacteria species	ID number
Lactococcus lactis subsp *lactis*	DSM 20481
Lactococcus lactis subsp *lactis*	DSM 20069
Streptococcus thermophilus	ATCC 19258
Enterococcus faecium	DSM 20477
Enterococcus durans	DSM 20633
Enterococcus italicus	DSM 15952
Lactobacillus plantarum	ATCC 8014
Lactobacillus pentosus	DSM 20314
Lactobacillus paraplantarum	DSM 10667
Lactobacillus casei	DSM 20011
Lactobacillus rhamnosus	ATCC 7469
Lactobacillus paracasei subsp. *paracasei*	DSM 5622

Clustering and identification of isolates

RAPD and rep-PCR fingerprinting methods were used to dereplicate the LAB collection of 105 isolates at strains level. The rep-PCR banding profiles showed a higher complexity than RAPD-PCR profiles, giving the former, on average, a greater number of PCR products. However, both fingerprinting methods showed a good reproducibility with

Table 4 Microbial count (log CFU g^{-1} ± standard deviation), pH and water activity in raw milk, curd after stretching and cheese at 30 days of ripening from two different farmsteads (FA and FB)

	Cow raw milk		Curd after stretching		Cheese	
Microbial groups	FA	FB	FA	FB	FA	FB
TMC*	5·6 ± 0·5	6·4 ± 0·2	6·0^{a}±0·6	7·9^{b}±0·7	6·6 ± 1·4	7·8 ± 0·6
Lactobacilli	5·5 ± 0·8	5·1 ± 0·2	7·4 ± 0·5	7·6 ± 1·4	7·6 ± 0·3	8·8 ± 0·5
Lactic cocci	5·9 ± 0·5	6·2 ± 0·3	8·1 ± 0·6	7·8 ± 0·7	8·2 ± 0·3	8·5 ± 0·6
Enterococci	3·0 ± 2·6	5·0 ± 0·5	5·6 ± 1·5	7·4 ± 1·2	7·7 ± 0·5	8·3 ± 0·5
Yeasts	3·3 ± 0·7	3·6 ± 0·8	3·5 ± 0·4	3·4 ± 0·4	2·2^{a}±0·6	3·6^{b}±0·2
Coagulase Negative Staphylococci	3·7 ± 0·3	4·3 ± 0·4	3·8 ± 0·8	3·6 ± 3·1	2·3^{a}±1·1	4·6^{b}±0·5
Coagulase Positive Staphylococci	<10	<10	<10	<10	<10	<10
Enterobacteria	2·7 ± 1·0	3·9 ± 0·6	1·8 ± 1·1	3·7 ± 1·6	<10^{a}	2·3^{b}±0·9
E. coli†	<3	<3	<3	<3	<3	<3
Butyric clotridia spore†	<3	<3	<3	<10	<3	<10
Pseudomonas spp.	3·2 ± 1·0	3·1 ± 0·7	<10^{a}	3·3^{b}±1·2	<10^{a}	2·2^{b}±0·4
pH	6·61 ± 0·1	6·53 ± 0·0	5·26 ± 0·0	5·24 ± 0·1	5·01^{a}±0·0	5·36^{b}±0·0
a_w	-	-	0·957 ± 0·0	0·960 ± 0·0	0·930^{a}±0·0	0·951^{b}±0·0

Different letters (a, b) on the same row, and only for the same ripening time, indicate statistically significant differences ($P < 0·05$).
*Total microbial count.
†MPN g^{-1} (MPN method).

a very similar banding pattern amongst three independent DNA preparations of three biological replicates of the same isolate. In fact, the reproducibility of the electrophoresis pattern was 91%.

Overall, strains of the same species were grouped within the same major clusters in both lactobacilli and cocci LAB dendrogram (Figs 1 and 2). Some strains clustered separately from their respective reference species groups, i.e. 7AM3, 11 AM3 (*Lc. lactis*), 36AC3 (*Strep. thermophilus*), 1ACu2 and 18ACu3 (*Ent. faecium*).

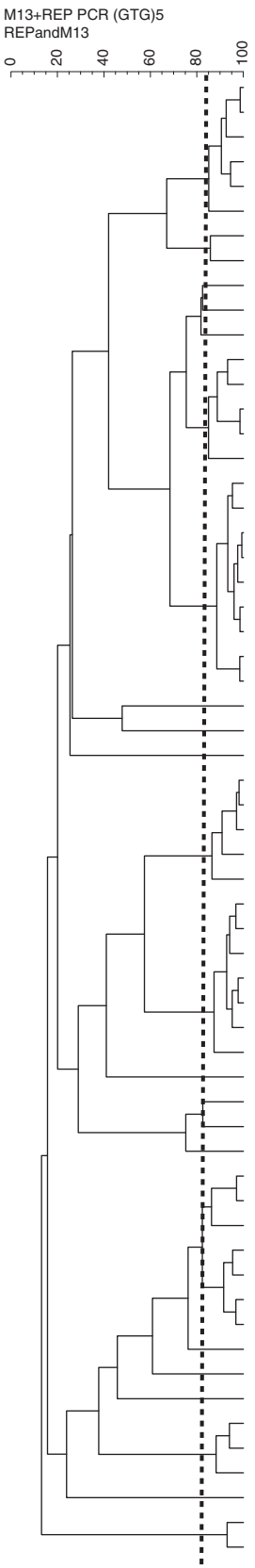
To increase the efficiency of the typing method, according to other authors (Bove *et al.* 2011; Pogacic *et al.* 2013), we combined, into a single cluster, the two series of RAPD and rep-PCR banding profiles. Cluster analyses were performed separately for lactobacilli and cocci LAB and different cluster and single cluster strain (singletons) were named biotype, according to Gatti *et al.* (2008). Regarding the lactobacilli (Fig. 1), a cut off value of 85% was fixed and 33 out of 45 isolates were grouped into seven biotypes. The remaining 12 strains are single strain biotype. At the same cut off value, 46 isolates out of 60 cocci LAB isolates were grouped into 10 biotypes while 14 isolates were single strain biotype (Fig. 2). The discriminatory index calculated on the basis of the combined fingerprinting methods was higher for lactic cocci (0·94) than lactobacilli (0·90). Both of these values are greater or equal at 0·90, according to Coenye *et al.* (2002), and corroborate the proper discriminating power of this typing method. The partial sequence of 16S rRNA gene from representative isolates of each cluster and from unclustered strains was amplified by PCR and sequenced. Then the phylogenetic affiliation of all isolates was confirmed by specific PCR using different species-specific primers as indicate in Table 2.

The rRNA sequencing (Tables 5 and 6) and species specific PCR revealed that *Lact. paracasei* (40 strains) clustered in 15 biotypes (8 were single strain biotypes), whereas *Lact. plantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis* and *Lactobacillus fermentum* were separated in single cluster.

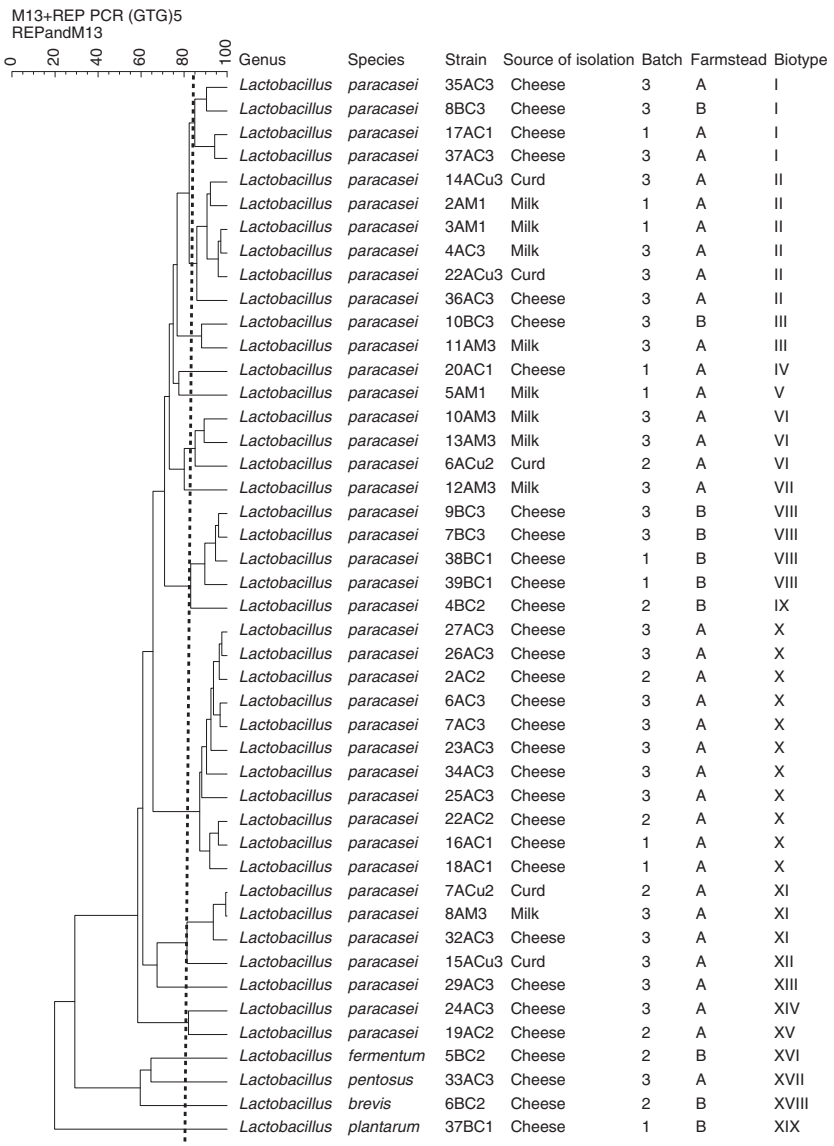
Some lactobacilli biotypes (Fig. 1) were found in all milk, curd and cheese FA samples after 30 days of ripening (biotype II and XI). Biotypes VIII and X were recovered only in cheese samples and were specific of farmstead B and A respectively. However, some biotypes (i.e. I and III) were recovered in both farmstead.

The cocci LAB (Fig. 2) were clustered in 24 biotypes (14 were single strain biotype), 3 biotypes of *Strep. thermophilus*, 5 biotypes of *Ent. durans*, 3 biotypes of *Ent. faecium*, 3 biotypes of *Lc. lactis* subsp. *lactis*, 2 biotypes of *Ent. italicus*, 2 biotypes of *Streptococcus parauberis*, one biotype of *Enterococcus lactis*, one of *Lactococcus garvieae*, one of *Lactococcus raffinolactis* and of *Leuconostoc lactis* respectively.

Figure 1 Genotyping of rods lactic acid bacteria isolated from Casizolu cheese from two different farmstead in three different batches by RAPD and REP-PCR. The unweighted-pair-group method with arithmetic averages dendrogram is based on Pearson correlation coefficient of composite data set of M13 and $(GTG)_5$ profiles. The dashed line indicates the cluster cut-off at 85% of similarity.



Genus	Species	Strain	Source of isolation	Farmstead	Biotype
Streptococcus	*thermophilus*	14ACu3	Curd	A	I
Streptococcus	*thermophilus*	20ACu3	Curd	A	I
Streptococcus	*thermophilus*	19ACu3	Curd	A	I
Streptococcus	*thermophilus*	29AC2	Cheese	A	I
Streptococcus	*thermophilus*	21ACu3	Curd	A	I
Streptococcus	*thermophilus*	34AC3	Cheese	A	I
Streptococcus	*thermophilus*	3BC2	Cheese	B	II
Streptococcus	*thermophilus*	25BCu2	Curd	B	II
Enterococcus	*durans*	28BCu3	Curd	B	III
Enterococcus	*durans*	30BC1	Cheese	B	IV
Enterococcus	*durans*	8BM2	Milk	B	V
Enterococcus	*durans*	13BCu2	Curd	B	VI
Enterococcus	*durans*	21BM3	Milk	B	VI
Enterococcus	*durans*	7BM2	Milk	B	VI
Enterococcus	*durans*	22BM3	Milk	B	VI
Enterococcus	*durans*	14BCu2	Curd	B	VI
Enterococcus	*durans*	1BM1	Milk	B	VII
Enterococcus	*durans*	18BCu2	Curd	B	VII
Enterococcus	*durans*	15BCu2	Curd	B	VII
Enterococcus	*durans*	37BCu2	Curd	B	VII
Enterococcus	*durans*	12BCu2	Curd	B	VII
Enterococcus	*durans*	5BCu1	Curd	B	VII
Enterococcus	*durans*	16BCu2	Curd	B	VII
Enterococcus	*durans*	38BC2	Cheese	B	VII
Enterococcus	*durans*	46BC2	Cheese	B	VII
Enterococcus	*faecium*	1ACu2	Curd	A	VIII
Streptococcus	*thermophilus*	36AC3	Cheese	A	IX
Lactococcus	*lactis subsp lactis*	11AM3	Milk	A	X
Enterococcus	*faecium*	11BM2	Milk	B	XI
Enterococcus	*faecium*	6BM2	Milk	B	XI
Enterococcus	*faecium*	34BM2	Milk	B	XI
Enterococcus	*faecium*	34BC2	Cheese	B	XI
Enterococcus	*faecium*	16BM2	Milk	B	XI
Enterococcus	*faecium*	17BCu2	Curd	B	XII
Enterococcus	*faecium*	26BCu2	Curd	B	XII
Enterococcus	*faecium*	9BM2	Milk	B	XII
Enterococcus	*faecium*	4BCu1	Curd	B	XII
Enterococcus	*faecium*	7BM1	Milk	B	XII
Enterococcus	*faecium*	29BC1	Cheese	B	XII
Enterococcus	*faecium*	44BC3	Cheese	B	XII
Enterococcus	*lactis*	9ACu3	Curd	A	XIII
Streptococcus	*parauberis*	23AM3	Milk	A	XIV
Streptococcus	*parauberis*	47AM1	Milk	A	XV
Enterecoccus	*faecium*	18ACu3	Curd	A	XVI
Enterococcus	*italicus*	41BC3	Cheese	B	XVII
Enterococcus	*italicus*	40BC3	Cheese	B	XVII
Enterococcus	*italicus*	45BC2	Cheese	B	XVII
Enterococcus	*italicus*	31BC1	Cheese	B	XVIII
Enterococcus	*italicus*	35BC2	Cheese	B	XVIII
Enterococcus	*italicus*	36BC2	Cheese	B	XVIII
Enterococcus	*italicus*	37BC2	Cheese	B	XVIII
Enterococcus	*italicus*	33BC2	Cheese	B	XIX
Lactococcus	*lactis subsp lactis*	7AM3	Milk	A	XX
Leuconostoc	*lactis*	3AM2	MIlk	A	XXI
Lactococcus	*lactis subsp lactis*	12AM3	Milk	A	XXII
Lactococcus	*lactis subsp lactis*	53AM3	Milk	A	XXII
Lactococcus	*lactis subsp lactis*	15ACu3	Curd	A	XXII
Lactococcus	*raffinolactis*	24AM3	Milk	A	XXIII
Lactococcus	*garvieae*	13AM2	Milk	A	XXIV
Lactococcus	*garvieae*	1AM1	Milk	A	XXIV



Genus	Species	Strain	Source of isolation	Batch	Farmstead	Biotype
Lactobacillus	*paracasei*	35AC3	Cheese	3	A	I
Lactobacillus	*paracasei*	8BC3	Cheese	3	B	I
Lactobacillus	*paracasei*	17AC1	Cheese	1	A	I
Lactobacillus	*paracasei*	37AC3	Cheese	3	A	I
Lactobacillus	*paracasei*	14ACu3	Curd	3	A	II
Lactobacillus	*paracasei*	2AM1	Milk	1	A	II
Lactobacillus	*paracasei*	3AM1	Milk	1	A	II
Lactobacillus	*paracasei*	4AC3	Milk	3	A	II
Lactobacillus	*paracasei*	22ACu3	Curd	3	A	II
Lactobacillus	*paracasei*	36AC3	Cheese	3	A	II
Lactobacillus	*paracasei*	10BC3	Cheese	3	B	III
Lactobacillus	*paracasei*	11AM3	Milk	3	A	III
Lactobacillus	*paracasei*	20AC1	Cheese	1	A	IV
Lactobacillus	*paracasei*	5AM1	Milk	1	A	V
Lactobacillus	*paracasei*	10AM3	Milk	3	A	VI
Lactobacillus	*paracasei*	13AM3	Milk	3	A	VI
Lactobacillus	*paracasei*	6ACu2	Curd	2	A	VI
Lactobacillus	*paracasei*	12AM3	Milk	3	A	VII
Lactobacillus	*paracasei*	9BC3	Cheese	3	B	VIII
Lactobacillus	*paracasei*	7BC3	Cheese	3	B	VIII
Lactobacillus	*paracasei*	38BC1	Cheese	1	B	VIII
Lactobacillus	*paracasei*	39BC1	Cheese	1	B	VIII
Lactobacillus	*paracasei*	4BC2	Cheese	2	B	IX
Lactobacillus	*paracasei*	27AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	26AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	2AC2	Cheese	2	A	X
Lactobacillus	*paracasei*	6AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	7AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	23AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	34AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	25AC3	Cheese	3	A	X
Lactobacillus	*paracasei*	22AC2	Cheese	2	A	X
Lactobacillus	*paracasei*	16AC1	Cheese	1	A	X
Lactobacillus	*paracasei*	18AC1	Cheese	1	A	X
Lactobacillus	*paracasei*	7ACu2	Curd	2	A	XI
Lactobacillus	*paracasei*	8AM3	Milk	3	A	XI
Lactobacillus	*paracasei*	32AC3	Cheese	3	A	XI
Lactobacillus	*paracasei*	15ACu3	Curd	3	A	XII
Lactobacillus	*paracasei*	29AC3	Cheese	3	A	XIII
Lactobacillus	*paracasei*	24AC3	Cheese	3	A	XIV
Lactobacillus	*paracasei*	19AC2	Cheese	2	A	XV
Lactobacillus	*fermentum*	5BC2	Cheese	2	B	XVI
Lactobacillus	*pentosus*	33AC3	Cheese	3	A	XVII
Lactobacillus	*brevis*	6BC2	Cheese	2	B	XVIII
Lactobacillus	*plantarum*	37BC1	Cheese	1	B	XIX

Figure 2 Genotyping of cocci lactic acid bacteria isolated from Casizolu cheese from two different farmstead in three different batches by RAPD and REP-PCR. The unweighted-pair-group method with arithmetic averages dendrogram is based on Pearson correlation coefficient of composite data set of M13 and $(GTG)_5$ profiles. The dashed line indicates the cluster cut-off at 85% of similarity.

Streptococcus thermophilus and *Ent. faecium* strains were grouped according to dairy farm origin. *Ent. durans* strains isolated from milk, curd and cheese samples were grouped in the same biotype.

TTGE analysis of Casizolu cheese

The band pattern of TTGE fingerprints showed a total ten bands with different migration pattern in the gel. In the tested samples, the number of bands ranged from two to seven (Fig. 3). Nine of ten encountered bands were identified by comparing their sequence with those present in GenBank. Seven of nine sequences were identified at species level and two at genus level. TTGE patterns of curd and cheese samples, taken from FB, showed a greater number of bands than FA.

The band called as 'i' in the TTGE profile (Fig. 3), corresponded to *Lc. lactis*, was present in all curd and cheese samples taken from both FA and FB. The band 'i' was the most noticeable in Fig. 3 and this might reflects a high relative proportion of *Lc. lactis* in the total bacteria population of Casizolu (Muyzer *et al.* 1993). The band 'd', identified as corresponding to *Lc. raffinolactis*, appeared in almost all samples analysed.

A band with a sequence matching to *Strep. thermophilus* (band 'j') was observed in all three batches of curd and cheese samples of FA and in the cheese sample picked from batch number 3 of FB. Among the lactobacilli, *Lactobacillus helveticus* species (band 'g') was observed only in the cheese at 30 days ripening in one batch from FA and in two batches from FB. Only two bands corresponded to as *Lact. fermentum* (band

Table 5 Species assessment of rod lactic acid bacteria based on species specific PCR and 16S rRNA sequencing

Strains	(M13-(GTG)$_5$–PCR) Biotype*	Species specific PCR					16S rRNA gene sequence comparison (species, % identity and closest relative accession numbers)†
		Lpara	Lca	Lrha	Lpent	Lplant	
AC3	I	+	–	–			
8BC3	I	+	–	–			*Lact. casei/paracasei*, 100% KJ702471.1/HG931728.1
17AC1	I	+	–	–			
37AC3	I	+	–	–			
14ACu3	II	+	–	–			*Lact. casei/paracasei*, 99% HM188410.1/AP012541.1
2AM1	II	+	–	–			
3AM1	II	+	–	–			*Lact. casei/paracasei*, 100% JN188389.1/AP012541.1
4AC3	II	+	–	–			
22AC3	II	+	–	–			*Lact. casei/paracasei*, 100%, JN188389.1/AP012541.1
36AC3	II	+	–	–			
10BC3	III	+	–	–			
11AM3	III	+	–	–			*Lact. casei/paracasei*, 100% JN188389.1/AP012541.1
20AC1		+	–	–			*Lact. paracasei*, 100%, KC456365.1
5AM1		+	–	–			
10AM3	IV	+	–	–			*Lact. casei/paracasei*, 99%, KF245561.1/NR_121787.1
13AM3	IV	+	–	–			
6ACu2	IV	+	–	–			
12AM3		+	–	–			*Lact. casei/paracasei*, 99%, KF245561.1/NR_121787.1
9BC3	V	+	–	–			
7BC3	V	+	–	–			
38BC1	V	+	–	–			*Lact. casei*, 99%, KJ558389.1/CP002618.1
39BC1	V	+	–	–			*Lact. casei*, KF245561.1
4BC2		+	–	–			*Lact. casei/paracasei*, 99% KJ764646.1/KF245561.1
27AC3	VI	+	–	–			
26AC3	VI	+	–	–			
2AC2	VI	+	–	–			
6AC3	VI	+	–	–			*Lact. casei/paracasei*, 100%, KF245561.1/KJ764646.1
7AC3	VI	+	–	–			
23AC3	VI	+	–	–			
34AC3	VI	+	–	–			
25AC3	VI	+	–	–			
22AC2	VI	+	–	–			*Lact. casei/paracasei*, 100%, KF245561.1/KJ764646.1
16AC1	VI	+	–	–			
18AC1	VI	+	–	–			*Lact. casei/paracasei*, 99%, KF245561.1/KJ764646.1
7ACu2	VII	+	–	–			*Lact. paracasei*, 99%, NR_121787.1
8AM3	VII	+	–	–			
32AC4	VII	+	–	–			
15ACu3		+	–	–			*Lact. paracasei*, 99% AB362762.1
29AC3		+	–	–			*Lact. casei/paracasei*, 99%, KF245551.1/KJ702488.1
24AC3		+	–	–			
19AC2		+	–	–			
5BC2							*Lact. fermentum*, 99%, HM057964.1
33AC3					+		*Lact. plantarum/pentosus*, 99%, KF297814.1/KJ690918.1
6BC2							*Lact. brevis*, 99%, KJ702494.1
37BC1					–	+	*Lact. plantarum*, 99%, KJ690918.1

Lpara, *Lactobacillus paracasei;* Lca, *Lactobacillus casei;* Lrha, *Lactobacillus rhamnosum;* Lpent, *Lactobacillus pentosus;* Lplant, *Lactobacillus plantarum.*
Strains submitted to partial 16S rRNA sequencing were reported in bold.
*Cluster obtained combined (GTG)$_5$ and M13 PCR band pattern profile whit a similar cut-off value of 85%.
†When two sequence belonging to two different species presented the same % of identity with a isolates, both sequence accessions number were reported in the table.

'b') and were observed in two samples of raw milk taken from FA. Bands 'e', corresponding to *Ent. durans* were present in all raw milk samples with the exception in milk from batch 2 of FB. The band 'e' disappeared in curd and cheese samples of FA, whereas in samples from FB it was still observed in curd and

cheese after the manufacturing and ripening processes. *Streptococcus uberis* (band 'h'), was identified in 13 samples out of 18. In general, the intensity of this band was higher in the raw milk samples than in curd or cheese samples. Staphylococci ('c') were detected only in five sample (FB-Cu1, FB-C1, FB-C2, FB-Cu3 and FB-C3).

Discussion

The biodiversity of bacterial strains in the cheese is strictly dependent on many factors as the type of milk used (raw or pasteurized milk), the technological process, the supplementation of different starter cultures (natural or commercial starter) and the ripening conditions (temperature, relative humidity, etc.). This study reports the diversity of the natural microbiota involved in the manufacturing process of the Casizolu cheese by culture-dependent and culture-independent methods.

Microbial component of raw milk is influenced by many factors e.g.: season, location, pasture, animal's health and more generally by the hygiene practices implemented in the farmhouse. Given the low number of the farmstead object of this study, on the basis of our results we can make a few remarks: the two farmsteads are located in the same geographical context; the experimental trials were set up in the same period and more generally both farm have applied good hygiene practices, as highlighted even from the low presence of spoilage micro-organisms in both milk samples. The CNS population was present in the all samples, probably owing to their tolerance to heat treatment; however, is not possible to exclude a secondary contamination during cheese production, above all in the FB. Indeed, CNS were often found in different cheese (Irlinger 2008; Coton *et al.* 2010; Soares *et al.* 2011) and the several source of contamination of raw milk have been identified such teat surface, air of milking parlour, settled dust and hay (Vacheyrou *et al.* 2011).

FA cheese showed the pH and AW values significantly lower than FB cheese, this can have inhibited spoilage micro-organisms growth as Enterobacteria and *Pseudomonas* species. In both samples the absence of butyric clostridia may be related to the lack of silage in cow ration. However, the absence of certain bacteria potentially pathogenic micro-organisms as CPS and *E. coli* during the entire process has ensured the cheese safety.

LAB as lactobacilli and lactic cocci are predominant in raw cow milk and Casizolu cheese. Their concentration in cheese, after 30 days of ripening, is higher than that detected in other types of pasta filata cheese at the same ripening time (Mucchetti *et al.* 2008).

The stretching phase of the curd did not significantly affect the growth of LAB in the later stages. Although Aponte *et al.* (2008) did not detect a relevant reduction in the microbial content after the stretching phase. Probably, this step during the technological process positively selected thermoduric LAB strains (Monfredini *et al.* 2012).

RAPD and rep-PCR analysis displayed a great genetic diversity amongst the isolates. It is worth highlighting that some biotypes of *Lact. paracasei*, *Ent. durans* and *Ent. faecium* species persisted from milk to cheese and therefore might be considered typical of a particular farmstead (biotypes II and VIII, Fig. 1 and biotypes VII and XII, Fig. 2). While other biotypes (biotype I and III, Fig. 1) were found in both farmstead, suggesting that some strains were specific to the area of production of Casizolu cheese.

On the other hand, 26 of the 48 cluster were singletons and some species like *Lact. plantarum*, *Lact. fermentum*, *Lact. pentosus*, *Lact. brevis*, *Ent. lactis*, *Ent. Italicus*, were peculiar only for one of the two farmstead. These great presence of singletons suggests that Casizolu cheese is a reservoir of high LAB biodiversity and each farmstead may also be characterized by a unique microbial population (Carafa *et al.* 2015, 2016; Franciosi *et al.* 2015).

Although the diversity in certain species, such as *Lact. paracasei*, *Ent. durans* and *Ent. faecium* might be the result of the higher number of strains used for comparison and, therefore, of the increased probability of encountering more distantly related taxonomic units (Abriouel *et al.* 2008).

Both culture-dependent and independent methods highlight the predominant role of the starter bacteria *Lc. lactis* subsp *lactis* and *Strep. thermophilus* in Casizolu cheese process. Isolates of *Lc. lactis* subsp *lactis* species were found only in the milk and curd samples. However, the TTGE analysis showed that *Lc. lactis* subsp *lactis* species was present during ripening time of cheese. Viable counts did not support TTGE analysis, likely because *Lc. lactis* subsp *lactis* species detected during ripening was uncultivable or autolytic strains, releasing into the cheese enzyme (very important for the development of flavour compounds) and nucleic acid then detected with the TTGE analysis. Rantsiou *et al.* (2008) obtained similar results on Feta cheese using both DNA and RNA DGGE analysis. Moreover, a recent study of Ruggirello *et al.* (2014) point out that lactococci are able to grow on M17 medium when they are abundant and not stressed, as during milk and curd fermentation. These and our findings suggested that *Lc. lactis* population is for the most present in viable but nonculturable state during cheese ripening and culture dependent methods are not able to detect their presence and have to be complemented with direct analysis in cheese.

The TTGE analysis showed the presence of *Lc. raffinolactis* in milk, curd and cheese samples, whereas only one isolate was isolated from raw milk of FA. Despite the role

Table 6 Species assessment of cocci lactic acid bacteria based on species specific PCR and 16S rRNA sequencing

Strains	(M13-$(GTG)_5$-PCR) Biotype*	Species specific PCR							16S rRNA gene sequence comparison (species, % identity and closest relative accession numbers)†
		St	Ed	Ef	Ei	Lc	Lll	Lcc	
14ACu3	I	+							
20ACu3	I	+							*Strep. thermophilus*, 99% CP006819.1
19ACu3	I	+							*Strep. thermophilus*, 99% KJ833590.1
29AC2	I	+							
21ACu3	I	+							*Strep. thermophilus*, 99% KJ026696.1
34AC3	I	+							*Strep. thermophilus*, 99% CP006819.1
3BC2	II	+							
25BCu2	II	+							*Strep. thermophilus*, 99% KJ833590.1
28BCu3			+	–	–				
30BC1			+	–	–				
8BM2			+	–	–				*Ent. durans*, 99%, HE646381.1
13BCu2	III		+	–	–				
21BM3	III		+	–	–				*Ent. durans*, 99%, KJ725230.1
7BM2	III		+	–	–				*Ent. durans*, 99%, KJ725230.1
22BM3	III		+	–	–				
14BCu2	**III**		+	–	–				*Ent. durans*, 99%, KJ725230.1
1BM1	IV		+	–	–				
18BCu2	**IV**		+	–	–				*Ent. durans/faecium*, 100%, KJ702577.1/KJ728981.1
15BCu2	IV		+	–	–				*Ent. durans/faecium*, 100%, KJ702577.1/KJ728981.2
37BCu2	IV		+	–	–				
12BCu2	IV		+	–	–				
5BCu1	IV		+	–	–				
16BCu2	IV		+	–	–				
38BC2	IV		+	–	–				
46BC2	IV		+	–	–				*Ent. durans*, 99%, KJ725230.1
1ACu2			–	+	–				
36AC3		+							*Strep. thermophilus*, 99%, KJ833590.1
11AM3						+	+	–	*Lc. lactis* subsp *cremoris/lactis*, 100% KJ702499.1/FJ749847.1
11BM2	V		–	+	–				*Ent. faecium*, 100%, KF245564.1
6BM2	V		–	+	–				
34BM2	V		–	+	–				
34BC2	V		–	+	–				*Ent. faecium*, 100%, KJ728981.1
16BM2	V		–	+	–				*Ent. faecium*, 99%, KJ728981.1
17BCu2	VI		–	+	–				
26BCu2	VI		–	+	–				
9BM2	VI		–	+	–				*Ent. faecium*, 99%, KJ698643.1
4BCu1	VI		–	+	–				
7BM1	VI		–	+	–				*Ent. faecium*, 100%, KJ726575.1
29BC1	VI		–	+	–				
44BC3	VI		–	+	–				
9ACu3			–	–	–				*Ent. lactis*, 98%, KF826014.1
23AM3			–	–	–				*Strep. parauberis*, 99%, NR_102798.1
47AM1			–	–	–				*Strep. parauberis*, 99%, KC836715.1
18ACu3			–	+	–				
41BC3	VII		–	–	+				
40BC3	VII		–	–	+				
45BC2	VII		–	–	+				*Ent. italicus*, 99%, KF148999.1
31BC1	VIII		–	–	+				
35BC2	VIII		–	–	+				*Ent. italicus*, 99%, FR865170.1
36BC2	VIII		–	–	+				*Ent. italicus*, 100%, HQ721276.1
37BC2	VIII		–	–	+				
33BC2			–	–	+				*Ent. italicus*, 99%, JF757229.1
7AM3						+	+	–	*Lc. lactis*, 99%, HE962097.1
3AM2									*Lc. lactis*, 98%, KJ720573.1

Table 6 (*Continued*)

Strains	(M13-(GTG)$_5$-PCR) Biotype*	Species specific PCR							16S rRNA gene sequence comparison (species, % identity and closest relative accession numbers)†
		St	Ed	Ef	Ei	Lc	Lll	Lcc	
12AM3	IX					+	+	–	
53AM3	IX					+	+	–	
15ACu3	IX					+	+	–	*Lc. lactis*, 100%, KJ690920.1
24AM3									*Lc. raffinolactis*, 99%, AB593336.1
13AM2	X								
1AM1	X								*Lc. garvieae*, 99%, JF831155.1

St, *Streptococcus thermophilus;* Ed, *Enterococcus durans;* Ef, *Enterococcus faecium;* Ei, *Enterococcus italicus;* Lc, *Lactococcus lactis;* Lll, *Lactococcus lactis* subsp. *lactis;* Llc, *Lactococcus lactis subsp. cremoris.*

Strains submitted to partial 16S rRNA were reported in bold.

*Cluster obtained combined (GTG)$_5$ and M13 PCR band pattern profile whit a similar cut-off value of 85%.

†When two sequence accession numbers belonging to two different species present the same % of identity both were reported in the table.

of *Lc. raffinolactis* in dairy production is still not clear (Lopez-Diaz *et al.* 2000; Jokovic *et al.* 2008); recently was demonstrate by Kimoto-Nira *et al.* (2012) an interaction among this bacteria and a strains of *Lc. lactis*.

Streptococcus thermophilus strains was found both in curd and cheese but not in milk samples, intriguingly isolated from different farmsteads, clustered differentially (cluster I and II) demonstrating that some strains are typical of a particular dairy ecosystems. Given the low genetic polymorphism of *Strep. thermophilus* species, the selection of new strains could be useful in dairy processing (Rasmussen *et al.* 2008; Delorme *et al.* 2010).

A high percentage of the cultivable microbiota of Casizolu cheese was represented by *Enterococcus*. Enterococci overcome the texturizing phase, since they thrive under harsh conditions as high temperature, high salt concentration and low pH, compared to other LAB species. This is confirmed by the fact that certain enterococci strain (biotype VII and XII) persist during the ripening of Casizolu cheese.

Enterococci were frequently detected in cheese made in the southern Europe (Coppola *et al.* 2001; Ercolini *et al.* 2003; Martin-Platero *et al.* 2008). In particular, *Ent. italicus* is widely diffused in artisanal cheeses (Fornasari *et al.* 2008; Dušinský *et al.* 2010; Yu *et al.* 2011; Akabanda *et al.* 2013; Terzic-Vidojevic *et al.* 2014) it accumulates high quantity of intracellular glutathione (Borgo *et al.* 2013) and shows low risks for human health (Fortina *et al.* 2008), although this species is able to disseminate antibiotic-resistance genes (Zago *et al.* 2010). Regarding *Ent. lactis*, a recent work of Morandi *et al.* (2013) showed the absence of antibiotic resistance in strains isolated from artisanal Bitto cheese, despite Rao Thumu and Halami (2012) demonstrated the presence of *ermB* gene in isolates of the same species. To our knowledge, *Ent. lactis* species has never been detected in the pasta filata cheese. Usually, the presence of enterococci suggests a potential faecal contamination, and some strains of the *Enterococcus faecalis* and *Ent. faecium* species often carry multiple antibiotic resistances and virulence factors such

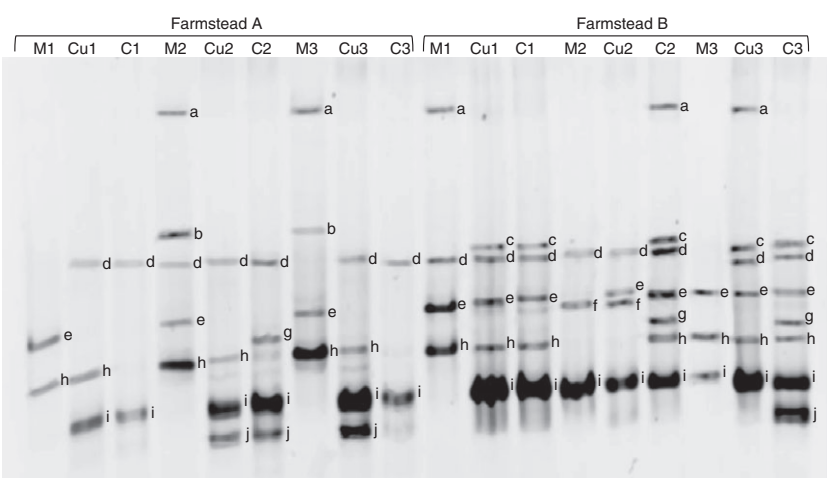


Figure 3 Temporal temperature gel electrophoresis analysis of V3 16s rDNA fragments from samples of three batches of Casizolu cheese at various stages of manufacturing and ripening. M, Cu and C indicate samples of milk, curd and cheese at 30 days of ripening and number indicate batches 1, 2, 3 respectively. Band indicated by letters were selected for sequencing. a sequencing failure, b *Lactobacillus fermentum*, c *Staphylococcus* spp., d *Lactococcus raffinolactis*, e *Enterococcus durans*, f *Enterococcus* spp., g *Lactobacillus helveticus*, h *Streptococcus uberis*, i *Lactococcus lactis*, j *Streptococcus thermophilus.*

as adhesins, invasins and hemolysins, which raise concern for the human diseases that could cause. On the other hand, different authors (Schirru *et al.* 2012, 2014; Favaro *et al.* 2014) demonstrated strong antimicrobial activity of *Ent. faecium* strains against several pathogens. *Streptococcus uberis* was a stable component of raw cow milk analysed in this work, as demonstrated by TTGE analysis. The presence of this species is often associated with subclinical and clinical mastitis (Bradley *et al.* 2007; Pitkala *et al.* 2008; Riekerink *et al.* 2008), although *Strep. uberis* with *Strep. parauberis* have been recurrently isolated from milk and cheese samples (Fuka *et al.* 2013; Pangallo *et al.* 2014). *Streptococcus uberis* secretes an extracellular protein named streptokinase which forms a complex with plasminogen making it active without prior proteolytic cleavage. Although heat treatment inactivates most of the chymosin, plasmin resists to cooking. Therefore, it is possible to assume that this process accelerated cheese proteolysis, as demonstrated in Cheddar cheese by Upadhyay *et al.* (2004). It would be interesting to study if *Strep. uberis* is present in raw milk pasta filata cheese and how its presence influences ripening.

Discrepancies in the *Lactobacillus* species detected by culture dependent and independent methods were also observed. In fact, the predominant species isolated by culture dependent approaches was *Lact. paracasei*, whereas culture independent method evidenced the presence of only *Lact. helveticus*. Guidone *et al.* (2015) found *Lact. helveticus* in Scamorza cheese by both approaches. These conflicting results may be due to temperature of 37°C used in our study for lactobacilli counts that does not well supports the growth of *Lact. helveticus*, similar results were found on Castelmagno cheese by Dolci *et al.* (2010a,b). Moreover, this species was frequently found in ripened pasta filata cheeses (Gobbetti *et al.* 2002; Piraino *et al.* 2005).

These differences could be attributed to some of the limitations of TTGE method, such as the presence of bacterial type in viable but not cultivable state, differential lysis of the microbial populations, amplified DNA from dead cells and differential amplification of some sequences (Ercolini *et al.* 2003; Ogier *et al.* 2004; El-Baradei *et al.* 2007). *Lact. plantarum* and *Lact. pentosus* are, occasionally, found in the Casizolu cheese, whereas in Oaxaca pasta filata cheese (Caro *et al.* 2013), *Lact. plantarum* is the predominant species. On the other hand, Gobbetti *et al.* (2002) observed in Caciocavallo pugliese cheese a prevalence of *Lact. paracasei* subsp *paracasei* in lactic microflora after 42 and 60 days of ripening. *Lactobacillus paracasei* was the main LAB involved in aging of Casizolu cheese. This species showed a high intraspecific biodiversity according to different authors (Zàrate *et al.* 1997; Sánchez *et al.* 2006). Some isolates of this species were able to grow at 45°C, likely due to a strong selection of high temperatures exerted on cheese microbiota during the stretching phase. A similar microbial pattern was already observed in milk (Franciosi *et al.* 2009), whey (Giraffa *et al.* 1997) and cheese (Monfredini *et al.* 2012). For such reasons, *Lact. casei* groups are the most important lactic acid bacteria species during ripening of most pasta filata and cooked cheese. This is confirmed in our study by the fact that *Lact. paracasei* isolates were found in milk, curd and Casizolu cheese samples (biotype II and XI).

The TTGE profile did not evidences bands corresponding to *Lact. paracasei* in this work. Similarly, Martín-Platero *et al.* (2009) have demonstrated a clear interference of *Lc. lactis* and *Lact. plantarum* on *Lact. paracasei* PCR amplification when all three species were amplified together finally influencing the results of TTGE analysis.

Heterofermentative lactobacilli were isolated a low frequency in Casizolu cheese. Although *Lact. fermentum* have been found in raw milk and cheese by culture dependent (Gobbetti *et al.* 2002) and by independent approaches such as Mozzarella (Ercolini *et al.* 2004) and Provolone del Monaco (Aponte *et al.* 2008).

The direct DNA extraction from dairy samples followed by PCR does not allows us to discriminate between alive, viable nonculturable cells and dead cells. Our work is limited from this point of view, although the use of both approaches (cultural dependent and independent) restricts the misinterpretations of the actual composition of the bacterial community. Moreover, in literature, can be found contrasting findings on the discrimination between alive and dead cells using both propidium monoazide/ethidium monoazide (PMA/EMA, that destroy DNA) and rRNA-DGGE methods in cheese, environmental and soil samples (Rantsiou *et al.* 2008; Villarreal *et al.* 2013; Ricciardi *et al.* 2014; Wagner *et al.* 2015).

In conclusion, this work highlight the role of *Strep. thermophilus*, *Lc. lactis*, *Lact. paracasei* and *Enterococcus* spp. during manufacture of Casizolu cheese. These results show that the technological process and dairy environment selected specific bacteria strains and confirmed the need of employ a polyphasic approach for a deep investigation of cheese microbiota. This study, as well as giving new information on traditional pasta filata cheeses microbiota and provides evidence that raw milk cheese is a source of new strains and therefore, a reservoir of microbial biodiversity.

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Conflict of Interest

No conflict of interest declared.

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