Microbial characterization of an artisanal production of Robiola di Roccaverano cheese

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ABSTRACT

Robiola di Roccaverano, from the Piedmont region of Italy, is a Protected Designation of Origin soft cheese made with raw goat milk. The peculiarity of this cheese is that during the manufacturing process, a natural starter culture (NC) is added to raw milk. This study examined the viable microorganisms of technological interest, including lactic acid bacteria and fungal populations, in samples of raw milk, NC, and fresh and ripened cheese collected from one dairy using culture-dependent techniques. First, the isolated colonies were analyzed using random amplification of polymorphic DNA (RAPD) PCR, and strains with similar fingerprints were clustered together. Further, representative isolates of each group were subjected to 16S or 26S ribosomal DNA sequencing. Finally, species-specific PCR was conducted to distinguish the *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*. Among the studied lactic acid bacteria, 13 RAPD profiles were obtained, corresponding to 9 different bacterial species or subspecies. Concerning mold and yeast isolates, 5 species were found that coincided with 5 RAPD types. Observing the strains isolated in the study, *Lc. lactis* was the most prevalent species in raw milk and NC samples, and *Leuconostoc mesenteroides* was the predominant species identified in 5- and 15-d cheese isolates. Furthermore, whereas only these 2 species were detected in NC, *Enterococcus* and *Lactobacillus* genera were found in raw milk and cheese, respectively. Concerning the mold and yeast isolates, in NC *Kluyveromyces* spp. was mainly found, and in cheese samples the representative species were *Geotrichum candidum* and *Yarrowia lipolytica*. Finally, raw milk and cheese safety were evaluated, and the samples complied with the standard required by European Commission regulation number 2073/2005.

INTRODUCTION

Italy has an ancient tradition in the production of dairy products, with a wide variety of cheeses that are strongly related to their place of origin. Some of these products have received Protected Designation of Origin (**PDO**) status along with the strict production requirements that this designation entails. Among these, Robiola di Roccaverano cheese holds high social and economic interest in the Piedmont region (northwest Italy), and it is the only Italian goat milk cheese that has been awarded with a PDO. Robiola di Roccaverano is a soft and creamy cheese made with at least 50% raw goat milk, with the possible addition of cow or ewe milk. Technical procedures establish that natural starter culture (**NC**) obtained from a back-slopping process must be used during artisanal Robiola di Roccaverano production. In detail, fermented milk from a previous fermentation process is added at room temperature to fresh raw milk from 2 consecutive milking sessions (afternoon and morning). Curd, obtained with goat or cow rennet, is inverted into a circular mold to allow the whey to release. The Robiola di Roccaverano can be consumed as fresh cheese after 4 d or allowed to ripen for up to 15 d (Gazzetta Ufficiale della Repubblica Italiana, 2006).

In traditional cheeses, such as Robiola di Roccaverano, the organoleptic features and quality of the final product are strongly influenced by the microbial ecosystem of raw materials that are not thermally treated (Quigley et al., 2011; Montel et al., 2014). Moreover, the traditional manufacturing practices and the addition of NC obtained by a back-slopping process rather than commercial starter allow the development of a unique independent microbial community that is adapted to the artisanal dairy environment (Smid et al., 2014; Bassi et al., 2015).

The microorganisms mainly involved in dairy production are lactic acid bacteria (**LAB**), naturally pres-

ent in raw milk, due to their ability to rapidly ferment lactose to lactate. Among this group, the most relevant to Robiola di Roccaverano are the *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* genera. In the first steps of cheese production, mesophilic starter LAB (**SLAB**), principally *Lactococcus lactis* and *Leuconostoc* spp., are found. During ripening, nonstarter LAB (**NSLAB**), mainly lactobacilli and pediococci, are present (Wouters et al., 2002). Together with LAB, molds and yeasts in dairy products also contribute to cheese ripening through lactose fermentation, proteolysis, lipolysis, lactate consumption, and aroma compound production (Quigley et al., 2013).

Traditionally, microbial species in fermented products were detected and identified using biochemical and phenotypic tests (Rossetti and Giraffa, 2005; Feligini et al., 2012). However, these methods alone are not optimal for studying microbial communities in complex matrices because they lack specificity and, in some cases, do not permit certain identification of species. In the last 2 decades, with the advent of molecular-based techniques, culture-dependent approaches to identifying microbial populations have been improved. The most useful techniques are based on DNA analysis, such as restriction fragment length polymorphism PCR, 16S ribosomal DNA (**rDNA**) sequencing, and random amplification of polymorphic DNA (**RAPD**)-PCR, applied singularly or in combination. Random amplified polymorphic DNA PCR has been widely used to study the dairy environment, allowing interspecies and to some extent intraspecies differentiation (Rossetti and Giraffa, 2005; Gala et al., 2008; Randazzo et al., 2009; Quigley et al., 2011; Soto Del Rio et al., 2016). In contrast, culture-independent methods are the ultimate tools for studying the microbial community in dairy products. Among these, PCR denaturing gradient gel electrophoresis (**DGGE**), PCR temporal temperature gradient gel electrophoresis, and, more recently, high-throughput DNA sequencing are widely applied (Flórez and Mayo, 2006; Martín-Platero et al., 2009; Dalmasso et al., 2016). These techniques do not allow discrimination between live and dead microorganisms. Consequently, the combination of culture-dependent and culture-independent methods has been demonstrated to be the most reliable approach to studying food-associated microbiota (Temmerman et al., 2004).

Several studies investigating the microbial populations involved in the manufacturing processes of high-value commercial cheese products, such as Grana Padano, Mozzarella di Bufala, and Feta cheese, have been conducted (Ercolini et al., 2004; Santarelli et al., 2013; Bozoudi et al., 2016). In recent years, research interest has been directed toward niche products whose production has a historical and traditional relevance

and is confined to a specific geographical region (Alessandria et al., 2010; Riquelme et al., 2015; Dalmasso et al., 2016).

The microbial population of Robiola di Roccaverano cheese has been investigated using classical microbiology and PCR-DGGE in studies focusing on cheese from different dairies (Bonetta et al., 2008a,b). The main objective of this study was to characterize the biodiversity of LAB, molds, and yeasts through the analysis of the viable microbiota involved in the production of Robiola di Roccaverano cheese. Culture-dependent techniques were applied to samples of raw milk, NC, and fresh and ripened cheese, and a safety assessment was conducted.

MATERIALS AND METHODS

Strains Isolation Using Classical Microbiology

The production of Robiola di Roccaverano from one artisanal cheese factory was analyzed. Samples of NC (n = 12), raw goat milk (n = 12), 5-d ripened cheese (n = 12), and 15-d ripened cheese (n = 12) from 12 independent batches were collected in different periods of the year. Within the same batch, samples were matched. Raw milk (before starter addition) and NC were collected on the first day of production, and the corresponding fresh and matured cheeses were sampled after 5 and 15 d, respectively. Samples were transported in sterile conditions and analyzed within 2 to 3 h of sampling.

Ten grams or 10 mL of each sample was first homogenized with a sterile physiological saline solution and peptone (85:15 vol/vol; 90 mL; Oxoid Ltd., Basingstoke, UK) using a Stomacher 400 Circulator (Seward Ltd., Worthing, UK) at 230 rpm for 1 min. Subsequently, serial dilutions were prepared with the same saline solution.

Lactobacilli were isolated using De Man, Rogosa and Sharpe (MRS) agar (Oxoid Ltd.) at 31°C for 72 h in an anaerobic jar with an AnaeroGen 2.5 L (Oxoid Ltd.). Lactococci and streptococci were grown on M17 agar (Oxoid Ltd.) in aerobic conditions for 48 h at 31°C. Molds and yeasts were grown on OGYE agar (Oxoid Ltd.) at 25°C for 5 d.

For each sample, 5 LAB (2 from M17 and 3 from MRS culture media) and 3 mold and yeast colonies were randomly collected from each sample based on different morphology (shape and color). Approximatively 240 LAB and 144 molds and yeasts were collected. The confirmation of the colonies was performed using Gram staining and catalase test to discard non-lactic colonies. Gram-positive and catalase-negative strains were then inoculated in brain heart infusion broth (Oxoid Ltd.) and yeast extract peptone dextrose broth (Sigma-

Aldrich, St. Louis, MO). The growth conditions were as previously mentioned. Hereafter, isolates were stored in sterile glycerol (15% vol/vol) at −80°C until further analysis.

Safety Assessment

To evaluate the safety of raw goat milk and cheese samples, the presence of *Salmonella* spp. and *Listeria monocytogenes* was monitored according to ISO standard 6579-1:2017 (ISO, 2017a) and ISO standard 11290-1:2017 (ISO, 2017b), respectively, reported in European Commission regulation number 2073/2005 (European Commission, 2005). Moreover, the number of coagulase-positive staphylococci was evaluated at 37°C for 48 h on Baird-Parker RPF agar (Oxoid Ltd.) following ISO standard 6888-1:1999 (ISO, 1999).

Strain Identification Using Molecular Methods

After the isolation step, viable LAB, mold, and yeast colonies were characterized using molecular techniques (RAPD-PCR, 16S rDNA sequencing, 26S rDNA sequencing, and species-specific PCR) as described below. Before use, the bacterial and fungal colonies were grown overnight and lysed by microLysis-Plus (Microzone Ltd., Brightone, UK), and 1 µL of cell lysate was used for the following biomolecular analysis. Biodiversity of LAB was assessed using RAPD-PCR with D11344 primer (Andrighetto et al., 2002). The amplification conditions were as follow: initial step of 94°C for 2 min; 35 cycles of 94°C for 1 min, 42°C for 1 min, 72°C for 1 min 30 s; and a final step at 72°C for 10 min. Fungal strain diversity was analyzed with M13 primer for 35 cycles of 94°C for 1 min, 45°C for 20 s, ramp to 72°C at 0.5°C/s, and 72°C for 2 min (Suzzi et al., 2000).

The amplification products were separated by electrophoresis on 1.5% (wt/vol) gel using 0.5× Tris-borate-EDTA buffer at 5 V/cm for 3 h. The gels were treated with a solution of gel red 1× (Biotium Inc., Fremont, CA) for 45 min and captured with Image Master VDS (Pharmacia Biotech, Piscataway, NJ). The obtained profiles were grouped with Gel Compar II (Applied Maths, Sint-Martens-Latem, Belgium) for LAB and Gel Compar 4.0 for yeasts and molds using the Pearson product–moment correlation coefficient and unweighted pair group method with arithmetic mean cluster analysis. The fingerprint obtained was identified through comparison of each strain profile with a RAPD types database, present in Veneto Agricoltura laboratory (Thiene, PD, Italy).

Subsequently, representative isolates from the different RAPD-PCR clusters were subjected to DNA sequencing using a 3130 (4-capillary) genetic analyzer (Applied Biosystems, Foster City, CA). The V1-V3 region of the bacterial 16S rDNA was amplified using primer pairs P1 (Klijn et al., 1991) and P2 (Muyzer et al., 1993), whereas the D1/D2 domain of 26S rDNA of yeasts and molds was amplified following the protocol described by Kurtzman and Robnett (1998). The obtained sequences were aligned with the sequences available in public databases such as RDP Release 11 (https://rdp.cme.msu.edu/) and the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, due to the important number of *Lc. lactis* detected among LAB, a PCR was conducted to distinguish the 2 subspecies, *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* (Pu et al., 2002). Primer sequences are reported in Table 1.

RESULTS AND DISCUSSION

In this study, we investigated the viable microbiota dynamics occurring throughout the production of Robiola di Roccaverano cheese at a single facility, following the main steps of production (NC, milk, 5-d ripened cheese, and 15-d ripened cheese). A culture-based approach was used to achieve the aims of the study.

The initial number of phenotypically detected isolates was reduced after the confirmation step. Finally, a total of 164 LAB and 101 mold and yeast colonies were confirmed and further analyzed. The distribution of the

Table 1. Primers used to identify lactic acid bacteria, molds, and yeasts with molecular methods

Primer	Sequence (5′-3′)	Molecular technique[1]	Reference
M13	GAGGGTGGCGGTTCT	RAPD-PCR	Andrighetto et al. (2002)
D11344	AGTGAATTCGCGGTGAGATGCCA	RAPD-PCR	Suzzi et al. (2000)
P1	GCGGCGTGCCTAATACATGC	16S rDNA sequencing	Klijn et al. (1991)
P2	ATTACCGCGGCTGCTGG	16S rDNA sequencing	Muyzer et al. (1993)
NL1	GCATATCAATAAGCGGAGGAAAAG	26S rDNA sequencing	Kurtzman and Robnett (1998)
NL4	GGTCCGTGTTTCAAGACGG	26S rDNA sequencing	Kurtzman and Robnett (1998)
LacreR	GGGATCATCTTTGAGTGAT	Species-specific PCR	Pu et al. (2002)
LacF	GTACTTGTACCGACTGGAT	Species-specific PCR	Pu et al. (2002)
CreF	GTACTTGTACCGACTGGAT	Species-specific PCR	Pu et al. (2002)

[1]RAPD = random amplified polymorphic DNA; rDNA = ribosomal DNA.

strains among the samples is reported in Supplemental Table S1 (https://doi.org/10.3168/jds.2019-17451).

Safety Assessment

The microbiological parameters of European Commission regulation number 2073/2005 (European Commission, 2005) were evaluated on raw milk and on 5- and 15-d ripened cheese. Safety parameters complied with the standard of law: *Salmonella* spp. and *Lis. monocytogenes* were absent in 25 g or 25 mL of product, and the total counts of coagulase-positive staphylococci were within the required limits (Supplemental Table S2; https://doi.org/10.3168/jds.2019-17451). These results agreed with a previous study that assessed the safety of Robiola di Roccaverano cheese (Bonetta et al., 2008a). It is worth noting that the absence of foodborne pathogens demonstrates that a high standard of hygiene can be met while maintaining the artisanal practices characteristic of the traditional cheese-making process. This is of particular importance considering that no thermal process is applied to the final product.

Alternatively, even if no analyses were performed to assess the role of LAB in the inhibition of pathogen growth, it could be supposed that the high number of LAB found in the analyzed samples (Supplemental Table S2; https://doi.org/10.3168/jds.2019-17451) was related to the absence of pathogenic bacteria. In fact, the ability of LAB isolates from artisanal cheese to inhibit the growth of undesirable bacteria has been assessed (Dal Bello et al., 2010; Ribeiro et al., 2016; Yoon et al., 2016). Lactic acid bacteria could exhibit an antagonistic role against pathogens through the production of antimicrobial substances such as bacteriocin, organic acid, and hydrogen peroxide. Consequently, the rapid acid production could lead to a reduction in pH and thus inhibit the growth of other species (Lindgren and Dobrogosz, 1990).

LAB Diversity in the Production Chain

Concerning the 164 LAB isolates, RAPD-PCR was able to distinguish 13 different clusters corresponding to 9 different bacterial species or subspecies (Figures 1 and 2). The number of strains isolated from each matrix and the species identified are reported in Table 2.

The most abundant isolated species was *Lc. lactis* (59%), grouped in 9 clusters (Figure 1). Among these isolates, species-specific PCR (Pu et al., 2002) highlighted that 32% were *Lc. lactis* ssp. *cremoris* and 26% were *Lc. lactis* ssp. *lactis* (Figure 3). This finding could be expected due to the culturing conditions used for the isolation. In addition, because thermal treatments on raw material are not permitted during the manu-

facturing process of Robiola di Roccaverano cheese, the growth of mesophilic flora may be promoted. The second most representative LAB found was *Leuconostoc mesenteroides* (29%), grouped in 2 clusters (Figures 2 and 3). Less abundant species included *Enterococcus faecalis* (4%) and *Lactobacillus plantarum* (4%; Figure 3).

Lactococcus lactis, the most abundant LAB isolated in this study through the culture-dependent approach used, is commonly found as a predominant microorganism in goat cheese. The main roles of *Lc. lactis* during the cheese-making process are acidification of the milk through production of lactic acid as well as a contribution to organoleptic features and the microbial quality of the final product (Ross et al., 2000). The high prevalence of this species has been reported in raw milk cheeses obtained without addition of commercial starter, such as Nicastrese, and in 2 highly appreciated farmhouse cheeses from Sierra de Aracena in southwest Spain (Martín-Platero et al., 2009; Pino et al., 2018). Moreover, *Lc. lactis* species were already detected in cheeses coming from different Robiola di Roccaverano dairies using a culture-independent approach (Bonetta et al., 2008b).

As reported in Figure 3, the LAB community of milk was different compared with the other matrices. It was dominated by *Lc. lactis*, particularly by *Lc. lactis* ssp. *lactis*, although few isolates of *Lc. lactis* ssp. *cremoris* were also identified. Among the other identified LAB, the second most prevalent species was *Ent. faecalis*, and only 1 strain belonged to *Enterococcus hirae*. The presence of enterococci was already reported in raw goat milk (Colombo et al., 2010). Members of the *Enterococcus* genus are commonly found in the intestinal tracts of humans and, less frequently, in animals, soil, water, plants, vegetables, birds, and insects (Gelsomino et al., 2002). However, the genus *Enterococcus* was not found among the colonies isolated from NC and from 5- and 15-d ripened cheeses (Table 2).

Compared with milk, the microbial colonies isolated from the indigenous NC samples highlighted some differences in terms of the number of species and type of species or subspecies found. The depth of sampling within each sample was limited. Only 2 species were detected in the NC: *Lc. lactis* (92%), where *Lc. lactis* ssp. *cremoris* was the predominant subspecies (more than 80% of *Lc. lactis* strains), and *Leu. mesenteroides* (8%; Figure 3).

The microbial composition of NC used in the production of Robiola di Roccaverano has not been investigated previously; however, several studies have been conducted on undefined starter culture applied in the cheese-manufacturing process using the metagenomic approach (Erkus et al., 2013; Frantzen et al., 2018). For
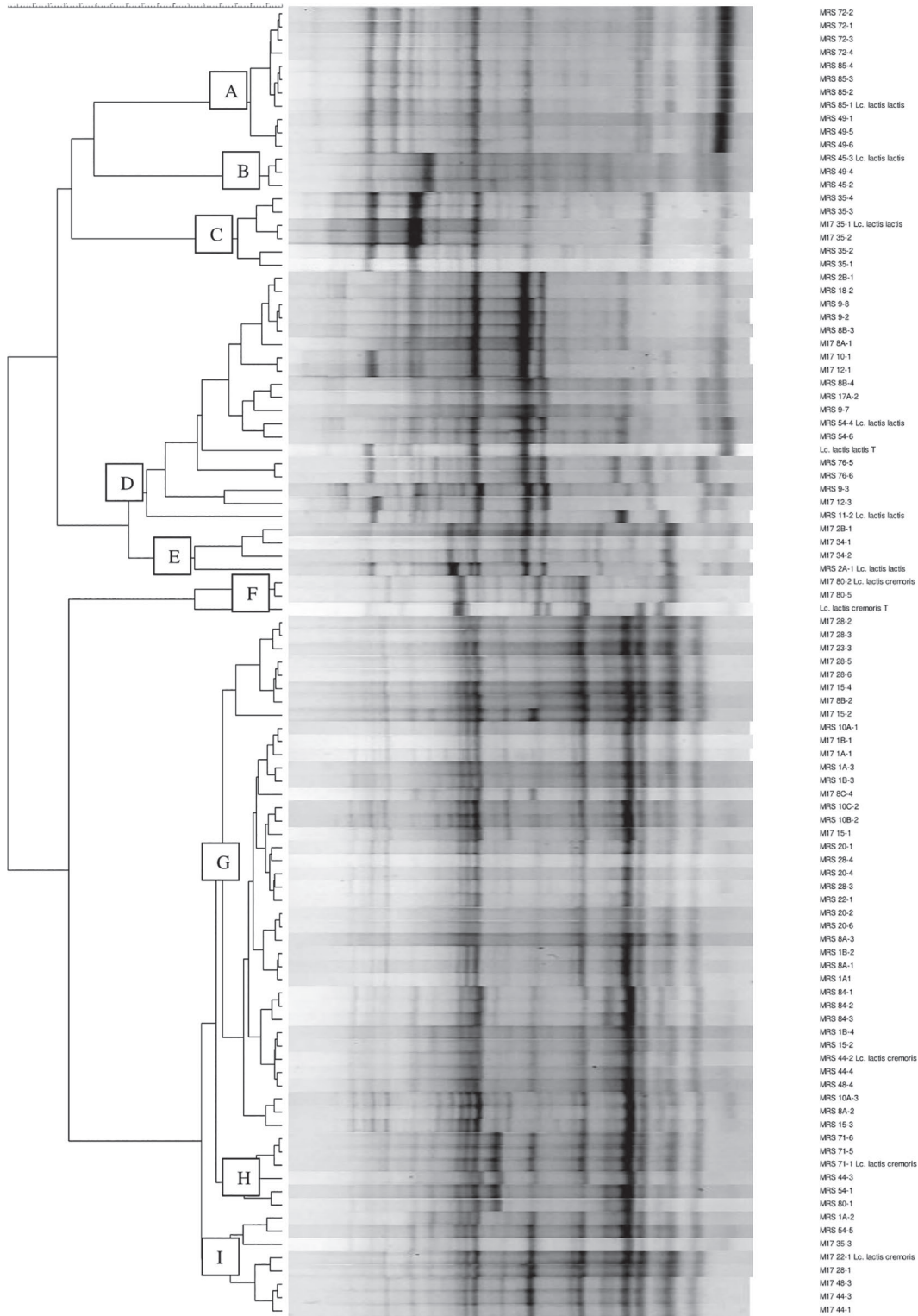
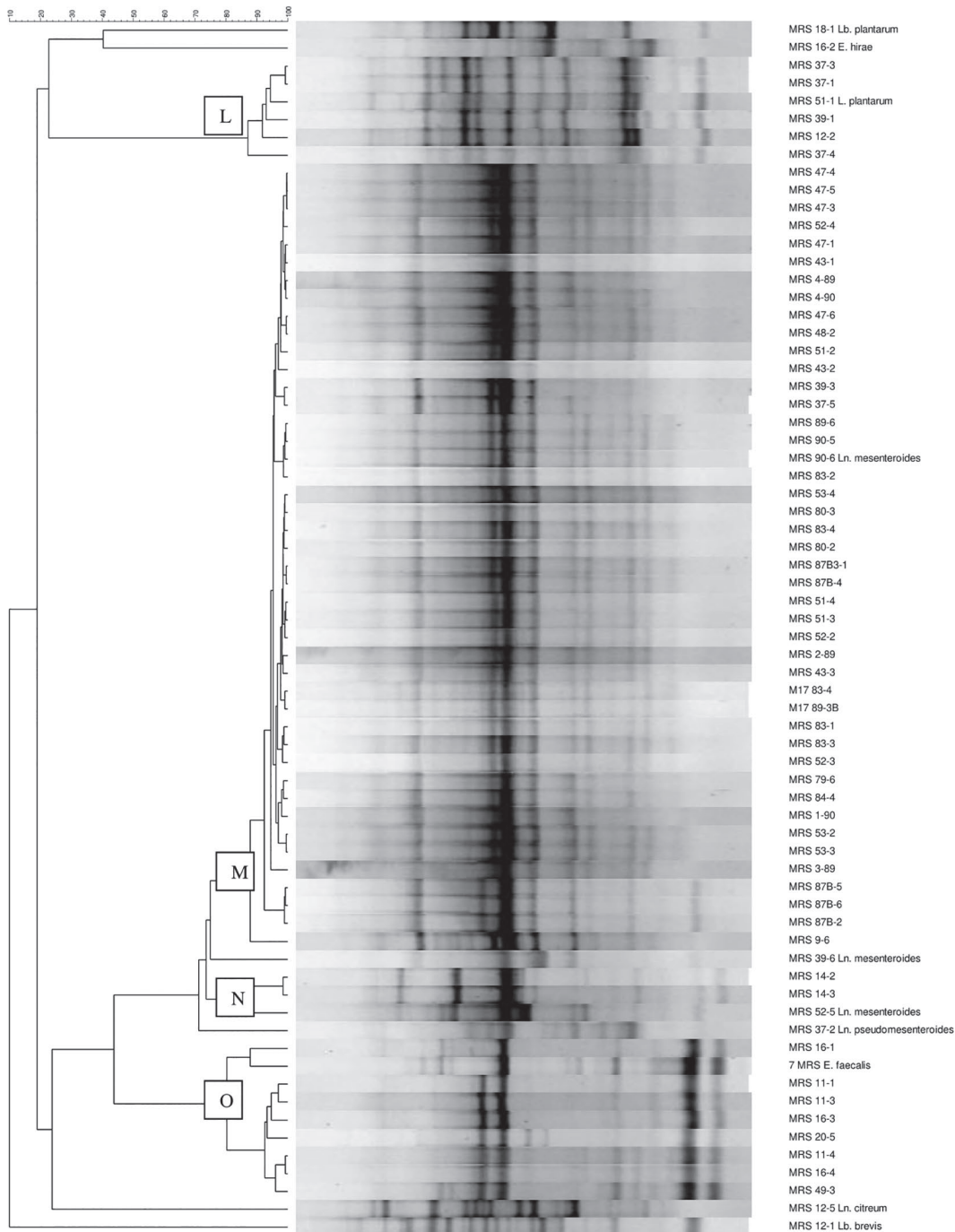**Figure 1.** Random amplified polymorphic DNA PCR cluster of isolates belonging to the *Lactococcus* genus analyzed with the D11344 primer.

example, the microbial community of undefined Gouda cheese starter was investigated, and the metagenome revealed that *Lc. lactis* was dominant in this starter culture, followed by a small community of *Leu. mesenteroides* (Erkus et al., 2013).

Despite the limited number of analyzed colonies, the composition of the viable microbial population of Robiola di Roccaverano cheese (both fresh and ripened) coming from the facility in this study seemed to be influenced by NC. In fact, mainly *Leu. mesenteroides*

Figure 2. Random amplified polymorphic DNA PCR cluster of isolates belonging to non-*Lactococcus* genera analyzed with the D11344 primer.

Table 2. Quantity and species of lactic acid bacteria (LAB) and fungal strains identified using random amplified polymorphic DNA PCR, 16S and 26S ribosomal DNA sequencing, and species-specific PCR

Species identified	Milk	NC[1]	5-d cheese	15-d cheese
LAB population				
Lactococcus lactis ssp. *cremoris*	5	41	7	1
Lactococcus lactis ssp. *lactis*	24	5	9	5
Leuconostoc mesenteroides	0	4	22	22
Enterococcus faecalis	8	0	0	0
Lactobacillus plantarum	0	0	1	6
Leuconostoc pseudomesenteroides	0	0	0	1
Lactobacillus brevis	0	0	1	0
Leuconostoc citreum	0	0	1	0
Enterococcus hirae	1	0	0	0
Total isolates	38	50	41	35
Fungal population				
Geotrichum candidum	0	3	13	22
Kluyveromyces marxianus	0	1	5	1
Saturnispora silvae	0	0	8	9
Yarrowia lipolytica	0	0	15	9
Kluyveromyces lactis	0	8	2	0
Candida pararugosa	1	1	0	0
Rhodotorula mucilaginosa	1	0	0	0
Meyerozyma guilliermondii	0	1	0	0
Trichosporon coremiiforme	0	0	1	0
Total isolates	2	14	44	41

[1]Natural starter culture.

and *Lc. lactis* strains were isolated (Figure 3). However, *Leu. mesenteroides*, which was not detectable in milk samples and present in low numbers in NC, was found with major prevalence in Robiola di Roccaverano cheese isolates in both 5- and 15-d ripened cheese (Figure 3). This observation was expected because, as described by several authors, *Leu. mesenteroides* has a double role of SLAB and NSLAB: it usually grows poorly in milk, but it is particularly involved in the cheese-ripening process because it produces aroma compounds (acetaldehydes, diacetyl, acetoin; McSweeney and Sousa, 2000; Settanni and Moschetti, 2010). In conclusion, the short time of ripening of Robiola di Roccaverano (maximum 15 d) probably did not allow for the growth of other species: NSLAB numbers increased over time, whereas SLAB numbers decreased (Settanni and Moschetti, 2010).

Even if the culturing approach applied did not permit a quantitative analysis, it was possible to observe that the number of *Lc. lactis* isolates decreased from NC to cheese, whereas the number of *Leu. mesenteroides* isolates increased from NC to cheese. However, this finding could also be related to the condition adopted for strain isolation.

Lactobacillus plantarum was also isolated in fresh and ripened cheese (Figure 3). In fact, the presence of this species has been reported in many cheese varieties. *Lactobacillus plantarum* is an NSLAB and could be involved in the maturation step because it is able to utilize several types of metabolites, such as lactate, amino acids, ribose, and *N*-acetylamino sugars, as nu-

trient sources (Pisano et al., 2006; Martín-Platero et al., 2009; Pino et al., 2018).

An in-depth analysis of RAPD-PCR output showed different biotypes of each species (Table 3). The 97 profiles identified as *Lc. lactis* were grouped for similarity, as reported before, in 9 different clusters (Figure 1). Analyzing the distribution of the different biotypes, 5 of these belonged to *Lc. lactis* ssp. *lactis* (A, B, C, D, E) and 4 belonged to *Lc. lactis* ssp. *cremoris* (F, G, H, I). A few biotypes were exclusive of some matrices; for example, A, B, and C biotypes were found only in milk, and F and H were reported only in NC. However, D and G biotypes were ubiquitous along the dairy production chain. Among all of the isolates, profile G was the most common, representative of 37 isolates. From the strains analyzed in this study, it could be supposed that some biotypes present only in milk (A, B, C) were lost through the production chain. On the contrary, biotypes D, E, G, and I persisted during the steps of the manufacturing process, suggesting their adaptability to the dairy environment along the year of production sampled.

Leuconostoc mesenteroides, the second most abundant species found through the isolation process, showed a low level of diversity. In fact, as reported in Table 3, only 2 biotypes were distinguished (M and N), where the most representative was cluster M. Some authors conducted a study in which *Leu. mesenteroides* strains were efficiently genotyped and subspecies were discriminated by means of different RAPD-PCR
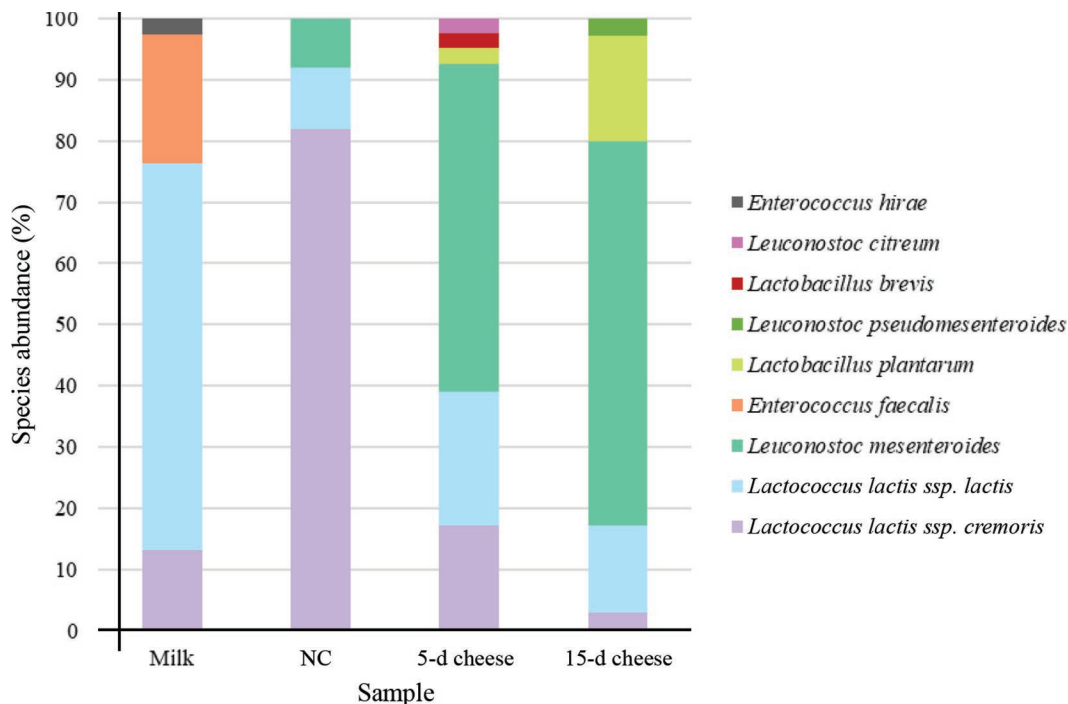
Figure 3. Abundance of lactic acid bacteria isolated along the production chain and identified using random amplified polymorphic DNA PCR, 16S ribosomal DNA sequences, and species-specific PCR. NC = natural starter culture.

protocol (Perez et al., 2002). Thus, the limited number of *Leu. mesenteroides* biotypes reported in the present study could be due to a true lack of species diversity or to an inefficiency of the short arbitrary primer used to discriminate intraspecies differences of the isolates.

Generally, from the viable microbiota analyzed in this study, the variability in terms of biotypes showed that milk samples were the richest matrices, showing 7 different biotypes of *Lc. lactis*. On the contrary, cheese samples displayed poor diversity, with only 3 biotypes in fresh cheese and 2 in ripened cheese. In contrast, the NC showed medium diversity in terms of number of biotypes (5) found.

Fungal Diversity in the Production Chain

The biodiversity of the viable fungal population was analyzed using RAPD-PCR. The fingerprints obtained were grouped by similarity, and the representative isolates were sequenced. The number of strains isolated from each matrix and the species identified are reported in Table 2. The results showed 9 different RAPD types corresponding to an equal number of identified species (Figure 4). No information was obtained for the milk fungal population because of the limited number of molds and yeasts isolated (Supplemental Table S2; https://doi.org/10.3168/jds.2019-17451). This issue could be ascribed to a true lack of a fungal community in milk or to the low specificity of the culturing methods applied.

Considering the fungal population isolated, the yeast *Geotrichum candidum* was the most abundant species (37%; Figure 5). *Geotrichum candidum* is commonly found in various habitats such as soil, grass, silage, plants, fruits, feed stuffs, insects, humans, other mammals, and dairy products. In fact, besides its natural

Table 3. *Lactococcus lactis* and *Leuconostoc mesenteroides* biotypes determined using random amplified polymorphic DNA PCR and number of isolates for each biotype[1]

Species	Milk	NC[2]	5-d cheese	15-d cheese
Lactococcus lactis ssp. *lactis*	A11, B3, C6, D2, E2	D3, E2	D8, E1	D5
Lactococcus lactis ssp. *cremoris*	G4, I1	F2, G28, H6, I5	G6, I1	G1
Leuconostoc mesenteroides	—	M4	M21, N1	M20, N2

[1]Letters indicate biotypes A to N; numbers following letter = number of isolates for each biotype.
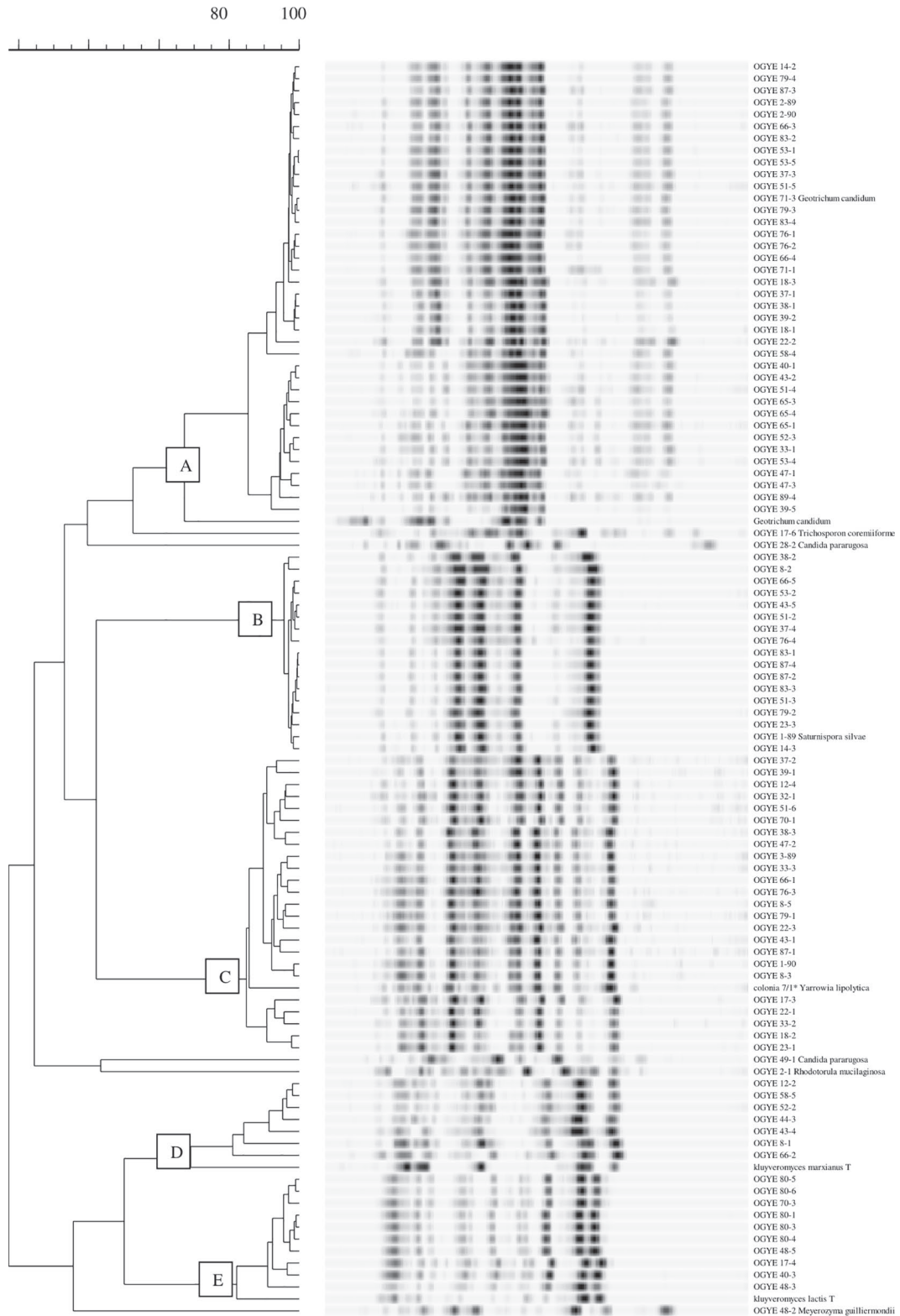[2]Natural starter culture.

Figure 4. Random amplified polymorphic DNA PCR cluster of mold and yeast isolates analyzed with the M13 primer.

presence in low numbers in milk, it is an important component of soft cheese made with different types of raw milk. *Geotrichum candidum* can produce several enzymes involved in the breakdown of protein and fat; therefore, it is responsible for the production of important aroma compounds. Due to these properties, it is sold as a selected starter to be used in cheese ripening (Boutrou and Guéguen, 2005). Moreover, the presence of *Geotrichum* spp. in Robiola di Roccaverano cheese through the DGGE approach has been previously reported (Bonetta et al., 2008b).

The second most prevalent identified species was *Yarrowia lipolytica*, a ubiquitous yeast that naturally grows on cheese surfaces. Due to its strong proteolytic and lipolytic capacities, it usually plays a role in cheese aroma formation and in texture development (Suzzi et al., 2001). *Yarrowia lipolytica* is one of the most common species occurring in blue cheese (Gkatzionis et al., 2013), but it was also found in Tomme d'Orchies and Livarot in studies conducted using a high-throughput DNA sequencing approach (Mounier et al., 2009; Ceugniez et al., 2015). Last, among the 101 isolates, the third most abundant yeast identified was *Saturnispora sylvae*, which has previously been reported in Roccaverano cheese (Bonetta et al., 2008b).

In the NC and cheese samples, a few colonies of *Kluyveromyces lactis* and *Kluyveromyces marxianus* were also identified among the isolates (Figure 5). These results were expected because *K. marxianus* could be a component of whey and milk starter; in fact, this organism is able to utilize lactose as a source of carbon (Binetti et al., 2013). Furthermore, *Kluyveromyces* spp. commonly has been found in dairy products, and recently its probiotic potential was assessed in isolates from Fiore Sardo cheese (Fadda et al., 2004). No different biotypes were found for each fungal isolate identified at the species level because RAPD-PCR with primer M13 did not allow the detection of strain biodiversity.

CONCLUSIONS

Regardless of the limitations of the culture-dependent methods, this study provides insight into the diversity of the viable LAB and fungal population present in the production of Robiola di Roccaverano. Many of the isolated microorganisms belonged to species that are known to be involved in the dairy process for their technological potential. Further studies could be necessary to better describe the entire microbiota involved in the manufacturing process of Robiola di Roccaverano cheese. In addition, comparison with other Robiola di Roccaverano facilities could be performed to assess whether the technical policies used determine some common characteristics among dairies.
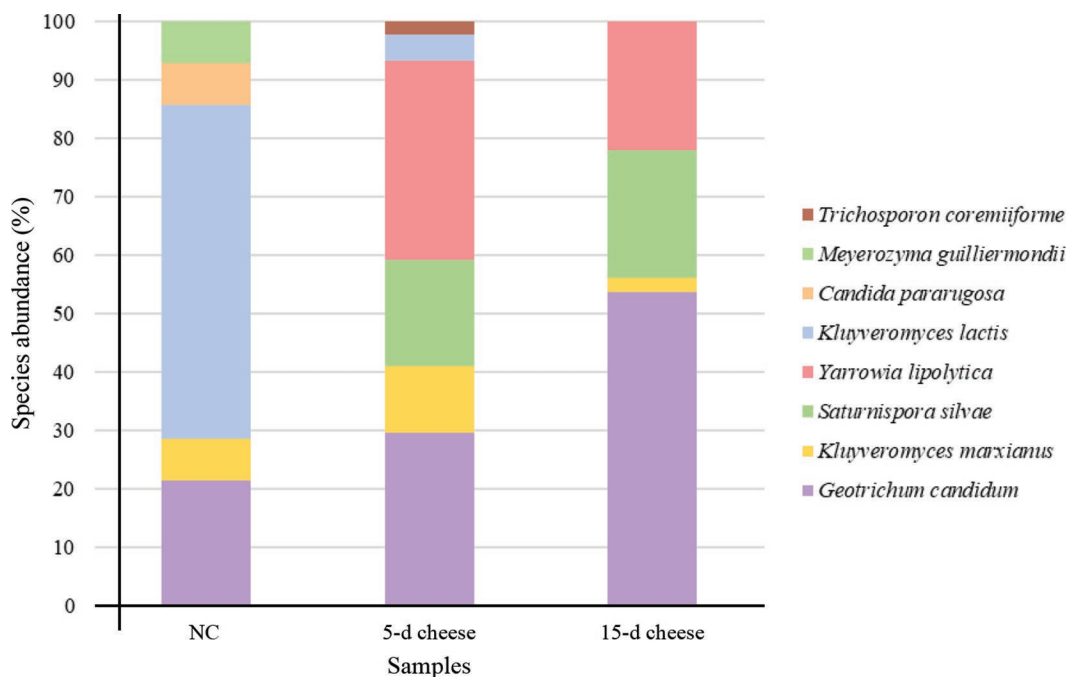

Figure 5. Abundance of fungal population isolated along the production chain and identified using random amplified polymorphic DNA PCR and 26S ribosomal DNA sequences. NC = natural starter culture.

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