Microbial characterization of an artisanal production of Robiola di Roccaverano cheese

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Microbial characterization of one Robiola di Roccaverano facility

Biolcati

Robiola di Roccaverano is an artisanal PDO cheese made with raw goat's milk, holding a high social and economic interest in the North-West Italy. In this study the viable microbiota of these cheese through the production steps of one dairy was monitored. Lactic acid bacteria, moulds and yeasts were isolated by classical microbiology and identified using molecular techniques. A quite biodiversity among the isolates was observed. Moreover, no pathogens were found in raw milk and cheese sample.

Microbial characterization of an artisanal production of Robiola di Roccaverano cheese

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ABSTRACT

Robiola di Roccaverano is a PDO (Protected Designation of Origin) soft cheese made with raw goat's milk, from the Piedmont region of Italy. The peculiarity of this cheese is that during the manufacturing process, a Natural Starter Culture (NSC) is added to raw milk. In this study, the viable microorganisms of technological interest, including Lactic Acid Bacteria (LAB) and fungal populations were examined in samples of raw milk, NSC, fresh and ripened cheese collected from one dairy by means of culture-dependent techniques. Firstly, the isolated colonies were analyzed by means of Random Amplify Polymorphic DNA-PCR (RAPD-PCR) and strains with similar fingerprint were clustered together. Further, representative isolates of each group were subjected to 16S rDNA or 26S rDNA sequencing. Finally, species-specific PCR was conducted to distinguish the *Lactococcus lactis* subspecies *lactis* and *cremoris*. Among the studied LAB, 13 RAPD-profiles were

obtained, corresponding to 9 different bacterial species or subspecies. Concerning mould and yeast isolates, 5 species were found which coincided with 5 RAPD-type. Observing the strains isolated in the study, *Lactococcus lactis* was the most prevalent species in raw milk and NSC samples. Instead, *Leuconostoc mesenteroides* was the predominant species identified in 5- and 15-days cheese isolates. Furthermore, while only these two species were detected in NSC, in raw milk and cheese *Enterococcus* and *Lactobacillus* genera were respectively found. Concerning the mould and yeast isolates, in NSC *Kluyveromices* spp. was mainly found, instead, in cheese samples representative species were *Geotrichum candidum* and *Yarrowia lipolytica*. Finally, the raw milk and cheese safety were evaluated and the samples complied with the standard required by the European Commission Regulation 2073/2005.

Key words: Robiola di Roccaverano, natural starter culture, lactic acid bacteria, fungal population

INTRODUCTION

Italy possesses an ancient tradition in the production of dairy products, with a wide variety of cheeses strongly related to their place of origin. Some of these typical products have received the Protected Designation of Origin (**PDO**) status, with the consequent strict production requirements that this entails. Among these, Robiola di Roccaverano cheese holds a high social and economic interest in the Piedmont region (North-West, Italy), and it is the only Italian goat's milk cheese that has been awarded with a PDO. The Robiola di Roccaverano is a soft and creamy cheese, made with at least 50% of raw goat's milk, with the possible addition of cow's or ewe's milk. Technical procedures establish that during the artisanal Robiola di Roccaverano production, Natural Starter Culture (**NSC**) obtained from back-slopping process must be used. In detail, fermented milk coming from previous fermentation process, is added, at room temperature, into fresh raw milk coming from two consecutive milking sessions (afternoon and morning). Curd, obtained with goat's or cow's rennet,

is overturned into a circular mold to allow the whey to release and after four days, the Robiola di Roccaverano can be consumed as fresh cheese, or allowed to ripen for up to 15 days (Gazzetta Ufficiale della Repubblica Italiana n. 160).

In traditional cheeses, such as Robiola di Roccaverano, the organoleptic features and quality of final products 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 NSC obtained by back-slopping process, rather than commercial starter, allow the development of an unique independent microbial community, adapted to the artisanal dairy environment (Smid et al., 2014; Bassi et al., 2015).

Microorganisms mainly involved in dairy production are Lactic Acid Bacteria (**LAB**) naturally present in raw milk for their ability to rapidly ferment lactose to lactate. Among this group, the most relevant to Robiola di Roccaverano are *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Enterococcus* genera. In the first steps of cheese production mesophilic Starter Lactic Acid Bacteria (**SLAB**), principally including *Lactococcus lactis* and *Leuconostoc* spp. are found. During ripening Non-Starter Lactic Acid Bacteria (**NSLAB**) mainly lactobacilli and pediococci are present (Wouters et al., 2002). Together with LAB, moulds 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, the detection and identification of microbial species in fermented products were determined using biochemical and phenotypic tests (Rossetti and Giraffa, 2005; Feligini et al., 2012). However, these methods alone are not optimal to study microbial communities in complex matrices since they lack specificity and, in some cases, do not permit the certain identification of species. In the last two decades, with the advent of molecular based techniques, the culture-dependent approaches to identify the microbial population have been improved. The most useful techniques are based on DNA-analysis, such as Restriction Fragment Length Polymorphisms-PCR (**RFLP-PCR**), 16S rDNA sequencing and Random Amplified Polymorphic DNA-PCR (**RAPD-PCR**), applied

singularly or in combination. RAPD-PCR has been widely used to study the dairy environment, allowing inter- and to some extent intra-species differentiation (Gala et al., 2008; Quigley et al., 2011; Randazzo et al., 2009; Rossetti and Giraffa, 2005; Soto del Rio et al., 2016). In contrast culture-independent methods are the ultimate tools to study the microbial community in dairy products; among these PCR-Denaturing Gradient Gel Electrophoresis (**PCR-DGGE**), PCR-Temporal Temperature Gradient Gel Electrophoresis (**PCR-TTGE**) and more recently High Throughput DNA Sequencing (**HTS**) are widely applied (Flórez and Mayo, 2006; Martín-Platero et al., 2009; Dalmasso et al., 2016). Therefore, these techniques do not allow the discrimination among live and dead microorganisms. Consequently, the combination of culture-dependent and -independent methods has been demonstrated to be the most reliable approach to study food-associated microbiota (Temmerman et al., 2004).

Several works concerning the investigation of the microbial populations involved in cheese manufacturing processes of high-value commercial 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). Indeed, in recent years, the research interest is being directed towards niche products, whose production has a historical and traditional relevance, 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 already investigated through classical microbiology and PCR-DGGE, in studies focusing on cheese coming from different dairies (Bonetta et al., 2008a,b).

The main objective of this study was to provide a characterization of LAB, moulds and yeasts biodiversity, through the analysis of the viable microbiota, involved during the production of Robiola di Roccaverano cheese. Culture-dependent techniques have been applied to samples of raw milk, NSC, fresh and ripened cheese. In addition, a safety assessment was conducted.

MATERIALS AND METHODS

Strains Isolation by Classical Microbiology

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

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

For the lactobacilli isolation, MRS agar (Oxoid Limited) at 31 °C for 72h in anaerobic jar with the AnaeroGen 2.5L (Oxoid Limited) was utilized; lactococci and streptococci were grown on M17 agar (Oxoid Limited) in aerobic conditions for 48h at 31°C. Moulds and yeasts were grown on OGYE agar (Oxoid Limited) at 25 °C for 5 days.

For each sample, 5 LAB respectively 2 from M17 and 3 from MRS culture media, and 3 mould and yeast colonies were randomly collected from each sample based on different morphology (shape and color). Approximatively a total of 240 LAB and 144 mould and yeast were collected. The confirmation of the colonies was performed by gram staining and catalase test in order to discard non-lactic colonies. Gram positive and catalase negative strains were then inoculated in Brain Heart Infusion broth (Oxoid Limited) and Yeast Extract Peptone Dextrose broth (Sigma-Aldrich, Missouri, US). The growth conditions were as previously mentioned. Hereafter, isolates were stored in sterile glycerol (15% v/v) at -80°C until the further analysis.

Safety Assessment

To evaluate the safety of raw goat's milk and cheese samples, the presence of *Salmonella* spp. and *Listeria monocytogenes* were monitored according to standard methods ISO 6579-1:2017 and ISO 11290-1: 2017 respectively, reported in the European Commission Regulation 2073/2005. Moreover, the number of Coagulase Positive Staphylococci (**CPS**) were evaluated at 37 °C for 48h on Baird-Parker RPF Agar (Oxoid Limited) following the standard method ISO 6888-1: 1999.

Strains Identification by Molecular Methods

After the isolation step, the characterization of viable LAB, moulds and yeasts colonies were performed by means of 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 Limited, Brightone, UK), and 1 µl of cell lysate was used for the following biomolecular analysis.

LAB biodiversity was assessed by 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 and 30 s and a final step at 72°C for 10 min. Fungal strains 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 $^{s-1}$, 72 °C for 2 min (Suzzi et al., 2000).

The amplification products were separated by electrophoresis on 1.5% (w/v) gel, using 0.5X TBE buffer, at 5V/cm for 3h. The gels were treated with a solution of Gel Red 1X (Biotium, Inc., California, US) for 45 min and captured with Image Master VDS (Pharmacia Biotech, New Jersey, US). The obtained profiles were grouped with Gel Compar II (Applied Maths, Sint-Martens-Latem, Belgio) for LAB, and Gel Compar 4.0 for yeasts and moulds, using the Pearson product moment correlation coefficient and UPGMA cluster analysis. A first identification of the fingerprint obtained was conducted through comparison of each strains profile with a RAPD-types database, present in Veneto Agricoltura laboratory (Thiene, PD).

Subsequently, representative isolates from the different RAPD-PCR clusters were subjected to DNA sequencing using the 3130 (4-capillary) Genetic Analyzer (Applied Biosystems, California, US). 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), while the D1/D2 domain of 26S rDNA of yeasts and moulds 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 National Centre for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Finally, since the important number of *L. lactis* detected among LAB a PCR reaction was conducted in order to distinguish the two subspecies *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* (Pu et al., 2002). Primers 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 (NSC, milk, 5-days and 15-days ripened cheese). A culture-based approach was utilized 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 mould and yeast colonies were confirmed and further analyzed. The distribution of the strains among the samples is reported in Supplementary Table S1.

Safety Assessment

The microbiological parameters of EC Regulation 2073/2005 were evaluated on raw milk, 5-days and 15-days ripened cheese. Safety parameters complied with the standard of law: *Salmonella* spp. and *Listeria monocytogenes* were absent in 25g or 25ml of product and the total counts of CPS were

within the required limits (Supplementary Table S2). These results agreed with a previous work published where the safety of Robiola di Roccaverano cheese was assessed (Bonetta et al., 2008a). It is worth noting that the absence of food-borne pathogens demonstrates that a high hygienic standard can be met while maintaining the artisanal practices characteristics 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 analysis have been performed in order to assess the role of LAB in the inhibition of pathogens growth, it could be supposed that the high number of LAB found in the analyzed samples (Table S2), 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 already assessed (Dal Bello et al., 2010; Ribeiro et al., 2016; Yoon et al., 2016). LAB 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 determine a reduction of 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 cluster, 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 *L. lactis* (59%), grouped in 9 different clusters (Figure 1). Among these isolates, species-specific PCR (Pu et al., 2002) highlighted that 32% were *L. lactis* ssp. *cremoris* and the 26% were *L. lactis* ssp. *lactis* (Figure 3). This finding could be expected due to the culturing conditions adopted for the isolation. In addition, since during the manufacturing process of Robiola di Roccaverano cheese, thermal treatments on raw material are not permitted, the mesophilic flora growth 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).

L. lactis, the most abundant LAB isolated in this study through the culture-dependent approach applied, is commonly found as a predominant microorganism in goat's cheese. The main roles of *L. lactis* during cheese-making process are acidification of the milk through production lactic acid, along with 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 already reported in raw milk cheeses obtained without addition of commercial starter, like Nicastrese and in two highly appreciated farmhouse cheeses located in Sierra de Arachena (south-west Spain) (Martín-Platero et al., 2009; Pino et al., 2018). Moreover, *L. lactis* species was already detected in cheeses coming from different Robiola di Roccaverano diaries by means of a culture-independent approach (Bonetta et al., 2008b).

As reported in Figure 3, milk's LAB community was different compared to the other matrices: it was dominated by *L. lactis*, and particularly by *L. lactis* subsp. *lactis*, although few isolates of *L. lactis* subsp. *cremoris* were also identified. Among the other identified LAB, the second most prevalent species was *E. faecalis* and only one strains belonged to *Enterococcus hirae*. The presence of enterococci was already reported in raw goat's milk (Colombo et al., 2010). Members of the *Enterococcus* genus are commonly found in the intestinal tracts of humans and, less frequently, animals, soil, water, plants, vegetable, birds and insects (Gelsomino et al., 2002). However, the genus *Enterococcus* was not found among the colonies isolated from NSC, 5- and 15- days ripened cheeses (Table 2).

In comparison with milk, the microbial colonies isolated from the indigenous NSC samples highlighted some differences in terms of the number of species and type of species/subspecies found. While the depth of sampling within each sample was limited, only two species were detected in the

NSC: *L. lactis* (92%), where *L. lactis* subsp. *cremoris* was the predominant subspecies (more than 80% of *L. lactis* strains), and *L. mesenteroides* (8%) (Figure 3).

While the microbial composition of NSC used in the production of Robiola di Roccaverano was not previously investigated, several studies have been conducted on undefined starter culture applied in cheese-manufacturing process by means of metagenomic approach (Erkus et al., 2013; Frantzen et al., 2018). For example, the microbial community of undefined Gouda cheese starter was investigated and the metagenome revealed that in this starter culture *L. lactis* was dominant followed by a small community of *L. 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 subjected to this study, seemed to be influenced by NSC: in fact, mainly *L. mesenteroides* and *L. lactis* strains have been isolated (Figure 3). However, *L. mesenteroides,* which was not detectable in milk samples and present in low number in NSC, was found with major prevalence in Robiola di Roccaverano cheese isolates, in both 5 days ripened and 15 days ripened cheese (Figure 3). This observation was expected since, as described by several authors, *L. 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 compound (acetaldehydes, diacetyl, acetoin) (McSweeney and Sousa, 2000; Settanni and Moschetti, 2010).

In conclusion, the short time of ripening of Robiola di Roccaverano (maximum 15 days), probably did not allow for the growth of other species: while NSLAB numbers increased over time, SLAB 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 *L. lactis* isolates decreased from NSC to cheese and whereas the number of *L. mesenteroides* isolates increased from NSC to cheese. However, this finding could also be related to the condition adopted for strain isolation.

Lb. plantarum was also isolated in fresh and ripened cheese (Figure 3): in fact, the presence of this species has been already reported in many cheese varieties. *L. plantarum* is a NSLAB and could be involved in maturation step, since it is able to utilize several types of metabolites as a nutrient sources, such as lactate, amino acids, ribose and N-acetylamino sugars (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 *L. lactis*, were grouped for similarity, as reported before, in 9 different clusters (Figure 1). Analyzing the distribution of the different biotypes, five of these belonged to *L. lactis* subsp. *lactis* (A, B, C, D, E) and four to *L. lactis* subsp. *cremoris* (G, F, H, I). Besides, a few biotypes were exclusive of some matrices: A, B and C biotypes for example were found only in milk, and F and H were reported only in NSC. However, D and G biotypes were ubiquitous along the dairy production chain. Among all 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.

L. 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 two biotypes were distinguished (M and N), where the most representative was the cluster M. Some authors conducted a study in which *L. mesenteroides* strains were efficiently genotyped and subspecies discriminated by means of different RAPD-PCR protocol (Zàrate and Cardell, 2002). Thus, the limited number of *L. mesenteroides* biotypes reported in the present study, could be due to a true lack in species diversity or to an inefficiency of the short arbitrary primer used to discriminate intra-species 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 *L. lactis*. On the contrary, cheese samples displayed poor diversity with only 3 biotypes in fresh cheese and 2 in

ripened cheese. The NSC, in contrast with the low species variability, showed a medium diversity in terms of biotypes (5) found.

Fungal Diversity in the Production Chain

The viable fungal population biodiversity has been performed analyzing the colonies by 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-type corresponding to an equal number of identified species (Figure 4). No information was obtained for milk fungal population because of the limited number of moulds and yeasts isolated (Table S2); this issue could be ascribed to a true lack of 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). *G. candidum* is commonly found in various habitats like soil, grass, silage, plant, fruits, feeding stuffs, insects, humans, other mammals and in dairy products. In fact, besides its naturally presence in low numbers in milk, it is an important component of soft cheese made with different types of raw milk. *G. 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 commercialized 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 has been already reported through DGGE approach (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). *Y. lypolitica* 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 by means of HTS approach. (Mounier et al., 2009; Ceugniez et al., 2015).

In conclusion, among the 101 isolates, the third most abundant yeast identified, was *Saturnispora sylvae*, which has been already reported in Roccaverano cheese (Bonetta et al. 2008b).

In the NSC and cheese samples, few colonies of *Kluyveromyces lactis* and *Kluyveromyces marxianus* were also identified among the isolates (Figure 5). These results were expected since *K. marxianus* could be a component of whey and milk starter: in fact, this organism is able to utilize lactose as source of carbon (Binetti et al., 2013). Furthermore *Kluyveromyces* spp. has been commonly 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 species level, since RAPD-PCR with primer M13 did not allow the detection of strains biodiversity.

CONCLUSION

Regardless of the limitation of the culture-dependent methods, this study represents an insight to the diversity of the viable LAB and fungal population present in a Robiola di Roccaverano production. Many of the isolated microorganisms belonged to species that are known to be involved in dairy process for their technological potential. It is clear how further studies could be necessary to better describe the entire microbiota involved in the manufacturing process of Robiola di Roccaverano cheese. In addition, a comparison with other Robiola di Roccaverano facilities could be performed in order to assess if the technical polices adopted determine some common characteristics amongst diaries.

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491 Table 1. Primers used to identify LAB, moulds and yeasts by molecular methods.

Primer	Sequence (5'-3')	Molecular technique	References
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

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505 Table 2. Quantity and species of LAB and fungal strains identified by means of RAPD-PCR, 16S

506 DNA sequencing, 26 rDNA sequencing and species-specific PCR.

Species identified	Milk	NSC[1]	5 Days Cheese	15 Days Cheese
	LAB[2] populations			
Lactococcus lactis subsp. cremoris	5	41	7	1
Lactococcus lactis subsp. 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
Tot. Isolates	38	50	41	35
	Fungal populations			
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
Tot. Isolates	2	14	44	41

507 [1]NSC= natural starter culture

508 [2]LAB= lactic acid bacteria

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Table 3. *L. lactis* and *L. mesenteroides* biotypes determined by RAPD-PCR and number of isolates for each biotype.

	Milk	NSC[1]	5 Days Cheese	15 Days Cheese
L. lactis subsp.lactis	**A**11, **B**3, **C**6, **D**2, **E**2	**D**3, **E**2	**D**8, **E**1	**D**5
L. lactis subsp. cremoris	**G**4, **I**1	**F**2, **G**28, **H**6, **I**5	**G**6, **I**1	**G**1
L. mesenteroides	**-**	**M**4	**M**21, **N**1	**M**20**, N**2

[1]NSC= natural starter culture

Biotypes are showed in bold and the number of isolates for each biotype are indicated by the number following the bold letter

Supplementary Table S1: Distribution of isolates among the different samples analyzed

Matrix	LAB[1] isolates	Fungal isolates
Milk	**49**(5), **72**(4), **85** (4), **45**(2), **35**(7), **2**(3), **11**(4), **20**(5), **16**(4)	**2**(1), **49**(1)
NSC[2]	**54**(4), **10**(5), **34**(2), **28**(7), **44**(5), **48**(3), **84**(4), **1**(8), **80**(5), **15**(4), **71**(3)	**71**(2), **44**(1), **48**(3), **80**(5), **28**(1), **40**(2)
5-days cheese	**8**(8), **17**(1), **76**(2), **12**(5), **22**(3), **47**(5), **52**(4), **83**(5), **87**(5), **43**(3)	**38**(3), **47**(3), **52**(2), **76**(4), **83**(4), **87**(4), **8**(4), **22**(3), **33**(3), **12**(2), **17**(3)
15-days cheese	**9**(4), **18**(2), **23**(1), **37**(5), **39**(3), **51**(4), **90**(5), **89**(5), **53**(3), **14**(2), **79**(1)	**89**(4), **90**(2), **14**(2), **18**(3), **37**(4), **39**(3), **53**(4), **66**(5), **79**(4), **23**(2), **51**(5), **65**(3)

[1]LAB= Lactic Acid Bacteria

[2]NSC= Natural Starter Culture

Among the brackets are reported the numbers of isolates belonging to each sample. The corresponding ID samples are indicated by the number in bold before the bracket.

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556 Supplementary Table S2. Enumeration of coagulase positive staphylococci, Lactobacilli, Lactococci,

557 Mould and Yeast. All results were expressed as Log CFU/mL or Log CFU/g.

Matrices	CPS[1]	Lactococci	Lactobacilli	M&Y[2]
milk	3,8	3,8	3,7	2,5
milk	3,1	3,9	3,3	3,4
milk	2,3	2,5	2,0	2,5
milk	3,0	3,4	3,5	<2,0
milk	3,7	3,6	3,6	<2,0
milk	3,5	3,8	3,5	<2,0
milk	3,4	4,2	3,4	<2,0
milk	3,8	3,7	3,3	<2,0
milk	3,1	3,2	3,0	<2,0
NSC[3]	-	9,4	8,9	3,5
NSC	-	9,4	9,1	6,5
NSC	-	8,7	8,3	9,5
NSC	-	8,9	8,5	3,6
NSC	-	8,1	6,4	4,0
NSC	-	9,1	8,1	2,9
NSC	-	9,0	8,9	3,8
NSC	-	9,1	9,7	2,5
NSC	-	8,3	8,3	2,4
NSC	-	9,2	8,6	2,4
NSC	-	9,0	7,5	2,6
5 days cheese	<2,0	6,5	5,8	5,8
5 days cheese	<2,0	7,9	6,8	6,7
5 days cheese	2,3	7,5	8,3	7,1
5 days cheese	2,8	7,7	7,3	7,2
5 days cheese	2,9	8,1	7,4	7,6
5 days cheese	2,4	7,8	8,2	8,2
5 days cheese	2,5	8,1	8,5	7,6
5 days cheese	2,0	8,2	7,4	7,8
5 days cheese	2,3	8,4	3,8	7,6
5 days cheese	<2,0	8,2	7,9	7,8
15 days cheese	3,1	8,3	8,0	8,5
15 days cheese	2,5	7,9	7,6	8,2
15 days cheese	2,6	8,7	8,3	8,0
15 days cheese	2,0	9,2	8,7	7,4
15 days cheese	3,6	8,4	7,9	7,8
15 days cheese	3,1	8,3	8,4	7,8
15 days cheese	3,2	8,4	8,1	8,0
15 days cheese	2,6	8,3	8,4	8,0
15 days cheese	2,5	8,3	8,0	7,9
15 days cheese	<2,0	8,3	8,1	7,6

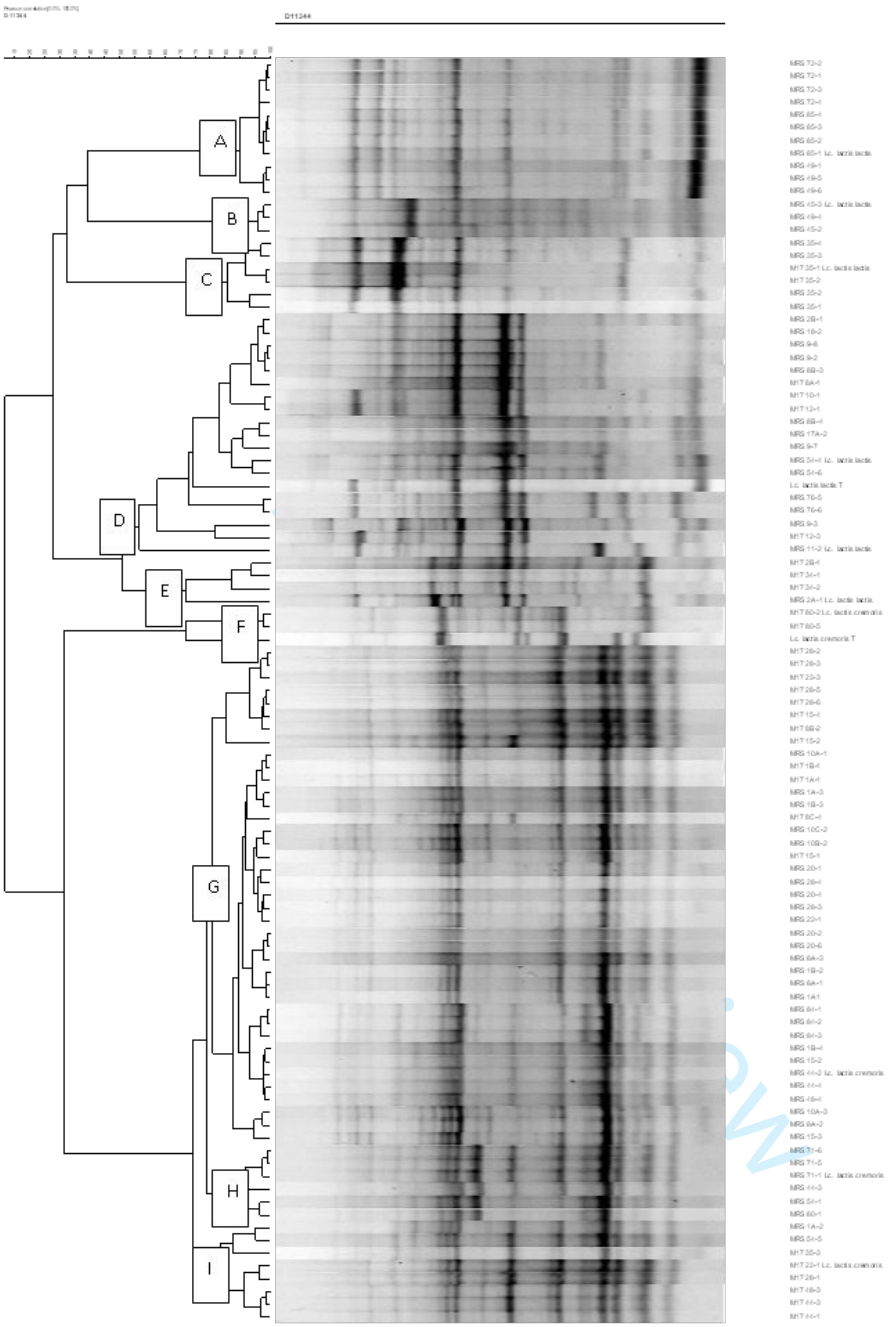
15 days cheese	<2,0	8,4	8,0	7,6

[1] CPS= Coagulase positive staphylococci

[2]M&Y= Mould and Yeast

[3]NSC= Natural Starter Culture

<2 correspond to the limit of detection of the method



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563 Biolcati Figure 1.

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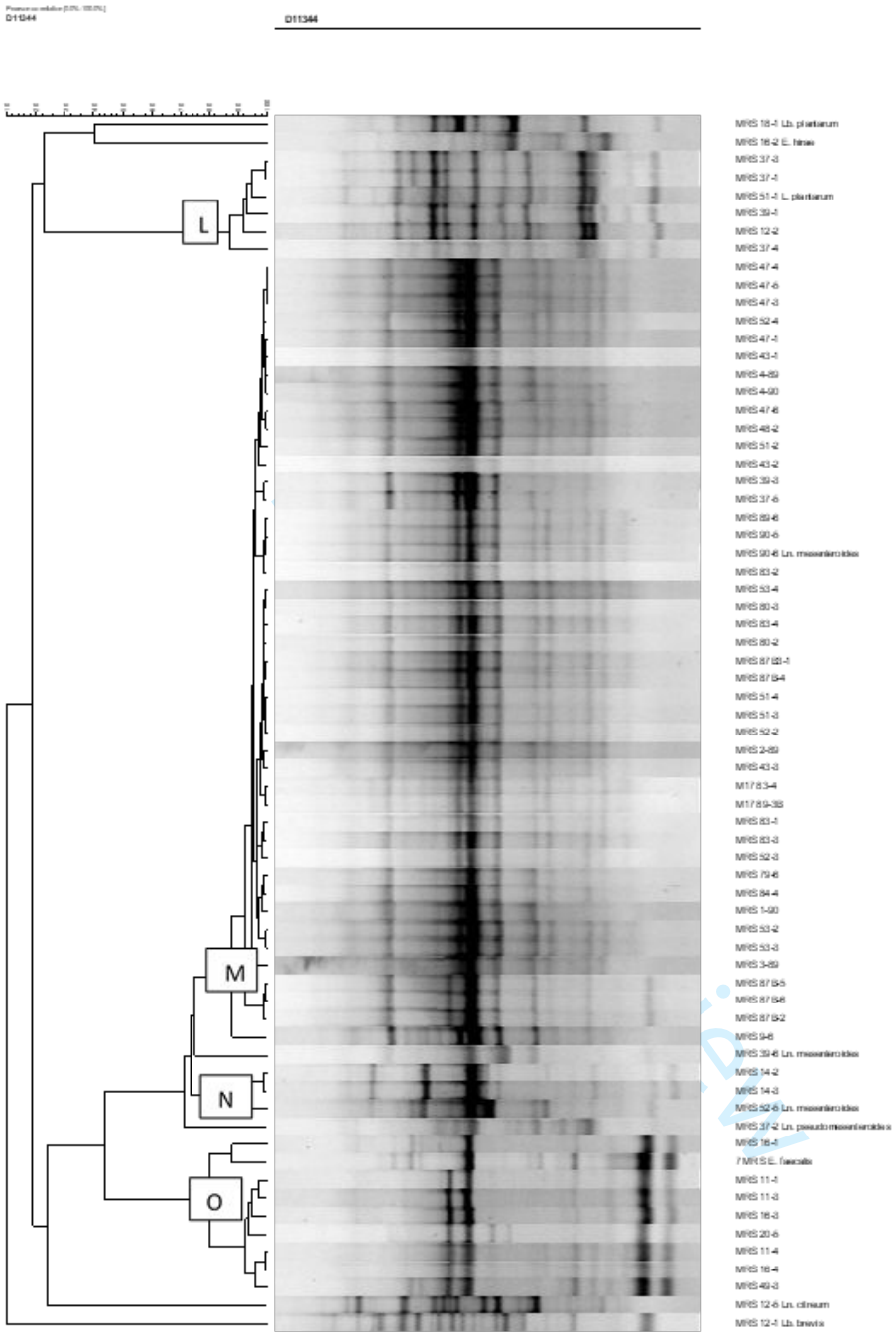

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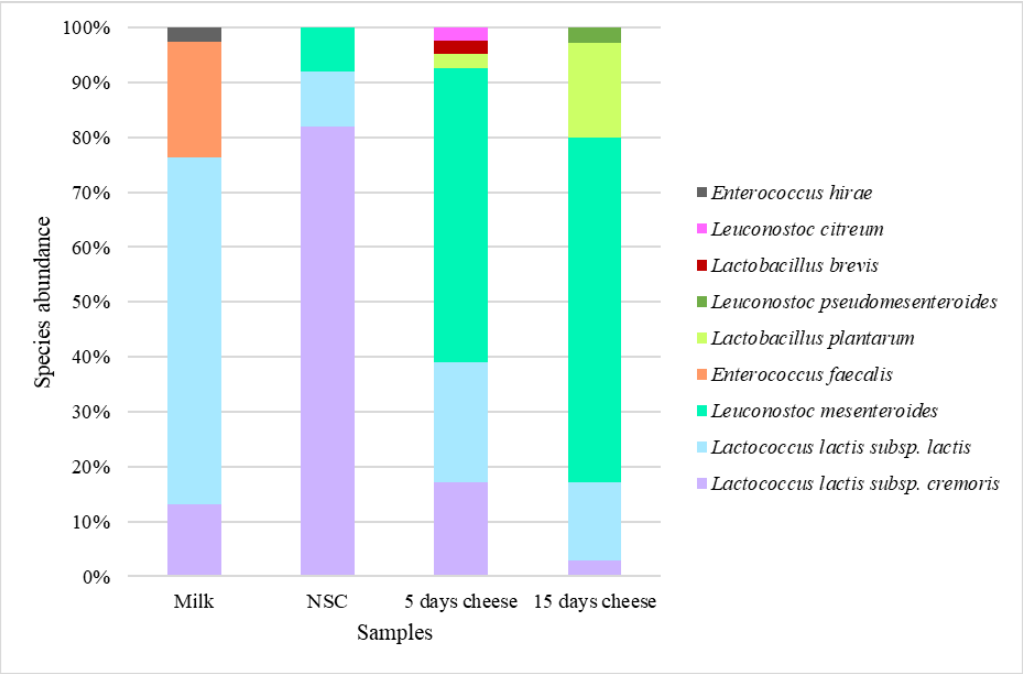
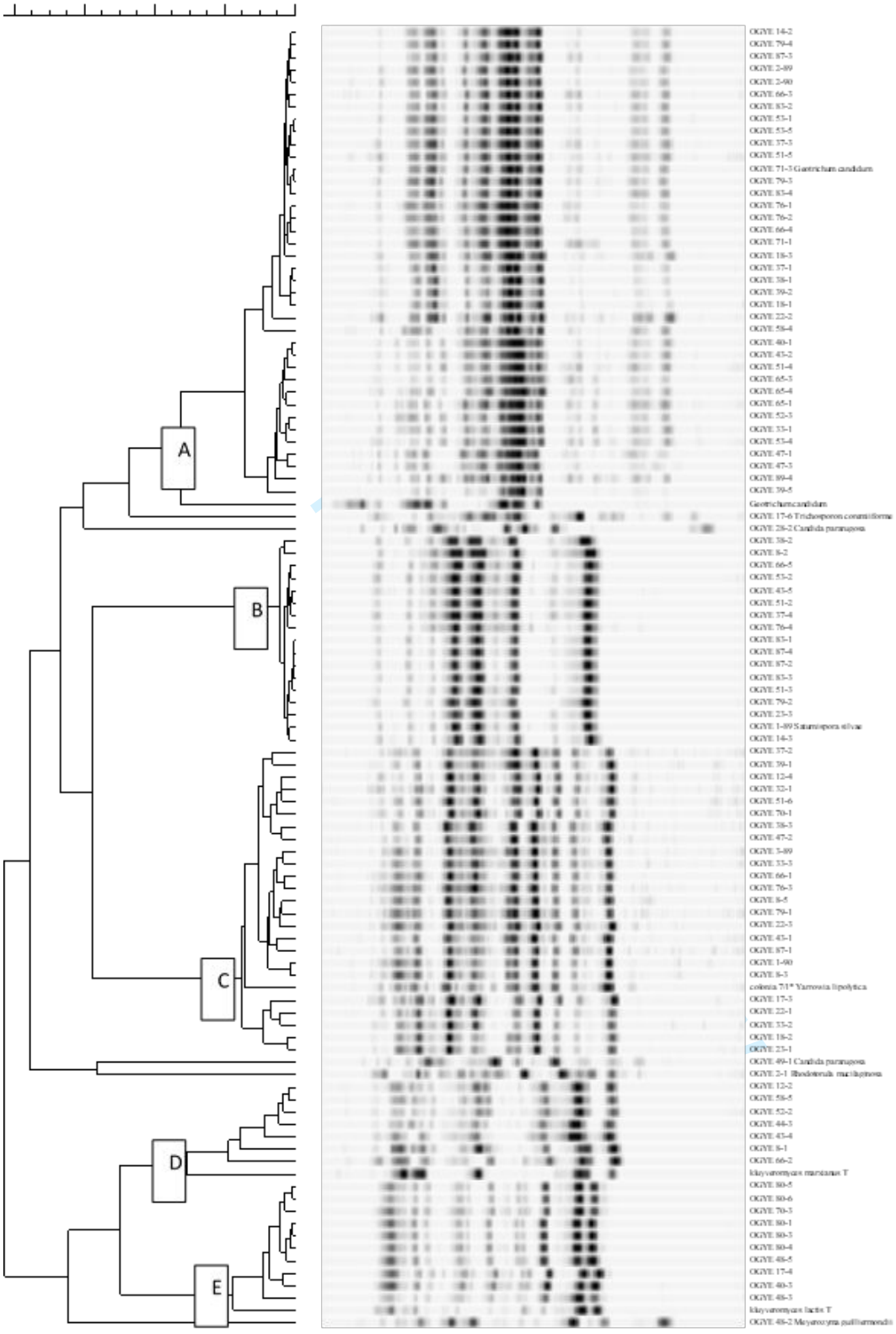
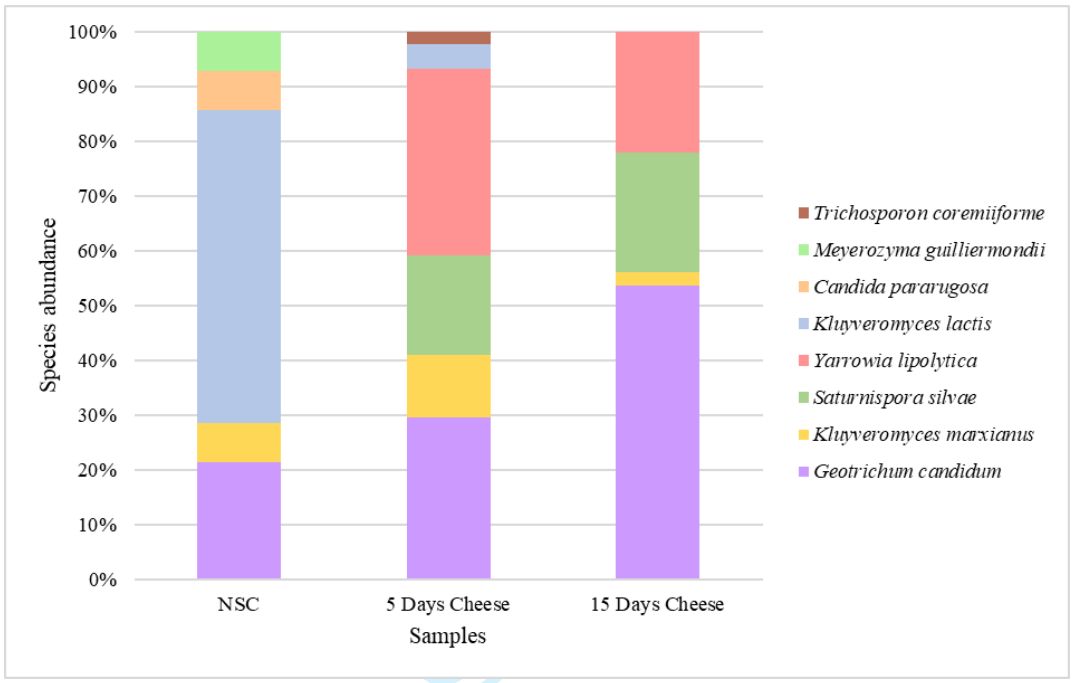
566 Biolcati Figure 2.



Biolcati Figure 3.



Biolcati Figure 4.



Biolcati Figure 5.

Figure 1. RAPD-PCR cluster of isolates belonging to *Lactoccoccus* genus analyzed with D11344 primer.

Figure 2. RAPD-PCR cluster of isolates belonging to non-*Lactoccoccus* genus analyzed with D11344 primer.

Figure 3. Abundance of LAB isolated along the production chain and identified by RAPD-PCR, 16S rDNA sequences and species-specific PCR.

Figure 4. RAPD-PCR cluster of moulds and yeasts isolates analyzed with M13 primer.

Figure 5. Abundance of fungal population isolated along the production chain and identified by RAPD-PCR and 26S rDNA sequences.