The use of natural milk culture (NMC) represents a key factor in PDO Montasio cheeses, contributing to its distinctive sensory profile. The complex microbial ecosystem of NMCs is the result of heat treatment and incubation conditions, which can vary considerably among different production plants. In this study, the microbiota of NMCs collected from 10 PDO Montasio cheese dairies was investigated employing colony counts and metagenomic analysis. Furthermore, residual sugars, organic acids, and volatile profiles were quantitatively investigated. Results showed that *Streptococcus thermophilus* was the dominant species in all NMCs, and a subdominant population made of other streptococci and *L. salivarius* was also present. The incubation temperature appeared to be the main driver of biodiversity in NMCs. Metagenomics allowed us to evidence the presence of minor species involving safety (e.g., *Staph. aureus*) as well as possible functional aspects (Next Generation Probiotics). Statistical analysis based on residual sugars, organic acids, and volatiles’ content allowed to correlate the presence of specific microbial groups with metabolites of great technological and sensory relevance, which can contribute to giving value to the artisanal production procedures of NMCs and clarify their role in the creation of the characteristics of PDO Montasio cheese.

Keywords: natural milk cultures, PDO cheese, metagenomics, volatilome, metabolites

**INTRODUCTION**

Bacteria added to vat milk as acidifying agents for cheesemaking are defined and undefined (or natural) starters, depending on the way they are obtained. While defined starters are most frequently used because of their homogeneous performance which assures quality, stability, and convenience, natural starters are reproduced daily in dairy plants from the selection of the natural milk microbiota by applying a selective pressure through thermal treatment and incubation at a specific temperature range. These cultures are characterized by an undefined composition and great biodiversity, thus resulting in a highly variable performance in terms of biochemical and sensory features. In addition, they are more tolerant to phage infection due to their microbiological complexity, but they are also prone to contamination and microbiological hazards (Parente et al., 2017). For some European, mostly Italian, and South American cheeses, the use of natural starters is required by product specifications, to safeguard the role played by milk microbiota on the sensory characteristics, as well as to strengthen the connection between cheese and its production area, not always guaranteed by defined starters (Carraro et al., 2011; Gatti et al., 2014).

Natural cultures differ depending on the substrate used for preparation. Natural whey cultures (NWC) are obtained by incubating cheese whey (deproteinized or not) under specific temperature conditions, which selects mesophilic or thermophilic lactic acid bacteria (LAB) biotypes. NWCs are used for Parmigiano Reggiano PDO, Grana Padano PDO, stretched-curd Italian cheeses, and Pecorino, as well as for Brazilian Minas and Serra de Canastra cheese (Kamimura et al., 2019; Marino et al., 2019). Such cultures are dominated by *Streptococcus thermophilus* and thermophilic lactobacilli (*Lactobacillus helveticus*, *L. bulgaricus*, and *L. fermentum*), but sub-dominant populations of other LAB frequently occur (De Filippis et al., 2014). Very recently, an NWC was used to enrich raw milk with an indigenous microbiota, demonstrating that the use of this strategy enabled the same final pH to be obtained, albeit with an initial slowing of the curd acidification process (Bettera et al., 2023). Natural milk cultures (NMC) are instead obtained by incubating thermized or pasteurized milk at about 40–45°C until the desired acidity is obtained. Under these conditions, a hetero-

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*Corresponding author: Marilena Marino, Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, via Sondrio 2/A, 33100, Udine, Italy, marilena.marino@uniud.it
geneous thermophilic microbiota is selected. Although they are widely used for many Protected Designation of Origin (PDO) cheeses, much less is known about their composition than NWCs. A recent study carried out on artisanal NMC produced using backslopping (that is, inoculation of the culture in raw milk for up to 13 cycles) showed that cultures were dominated by *S. thermophilus* and *L. delbrueckii* ssp. *lactis*, with the presence of sub-dominant mesophilic LAB species (Parente et al., 2016). As for NMCs produced daily without backslopping, which is the case of many Italian PDO and TSG (Traditional Specialty Guaranteed) cheeses, only data obtained with culture-dependent techniques are available, which allows a partial view of the microbial complexity of this type of matrix (Marino et al., 2003).

Montasio is a PDO semi-hard cheese, which is produced in North-East Italy using raw or thermized cow’s milk. The most traditional cheesemaking procedure uses a thermophilic NMC produced daily without backslopping (Innocente and Biasutti, 2013). During production, milk thermal treatment and incubation procedures select a different microbiota mainly composed of *S. thermophilus* and other thermophilic LAB at various abundance, as results from culture-dependent studies (Marino et al., 2008; Carraro et al., 2011). The use of culture-dependent techniques is not able to provide a complete picture of both the prevalent microorganisms and the marginal ones, although the latter may have an important role during both acidification and ripening, too. The use of NGS (Next Generation Techniques) techniques, on the other hand, allows for the highlighting of non-cultivable and sub-dominant populations, whose detection can help predict culture performance during cheesemaking (Zotta et al., 2022). Furthermore, the -omic approach allows for clarification of the correlation between food microbiota and environmental or matrix-related parameters, such as pH, aw, salt concentration, and temperature (De Filippis et al., 2016). Despite PDO Montasio cheese being largely studied from a chemical, rheological, and sensory point of view, only a rough knowledge regarding the microbiological characteristics of NMCs is available. Thus, in this study, a metagenomic approach was used to characterize the microbiota composition of different NMCs for PDO Montasio cheese. Moreover, measurements of physico-chemical features of NMCs were carried out and an integrative analysis of microbial composition and physico-chemical features was performed.

**MATERIALS AND METHODS**

**Sample collection**

Ten NMC samples, termed L1 to L10, were collected from different dairy plants in the production area of PDO Montasio cheese. For each NMC, data on thermal treatment (time and temperature) and incubation (time and temperature) conditions of milk were collected. The pH was determined immediately after sampling using a 5012T pH meter (Crisron, Barcelona, Spain).

**Microbiological analysis**

The samples were serially diluted in Maximum Recovery Diluent (Oxoid, Milan, Italy), and decimal dilutions pour-plated onto MRS agar (Oxoid) pH 5.4 for lactobacilli, Kanamycin Esculin Azide agar (Oxoid) for enterococci, and ST agar (Dave and Shah, 1996) for *S. thermophilus*. Plates were incubated at 37°C for 48 h under aerobic conditions (except for MRS, which was incubated anaerobically).

**DNA extraction, library preparation, and sequencing**

DNA was extracted using the DNeasy® PowerLyzer® PowerSoil® kit (Qiagen, Hilden, Germany). DNA was then used for preparing libraries according to the Illumina 16S Metagenomic Sequencing Library preparation protocol. The primers used to amplify the V3-V4 region were the following: 341F: CTACGGGNGGCWGCAG and 16S-805R: GACTACHVGGGTATCTAATCC. The resulting libraries were sequenced on a MiSeq instrument (Illumina, San Diego, CA) using 300-bp paired-end mode.

**Bioinformatics analysis**

After masking adapter sequences, reads were classified using Kraken2 (Wood et al., 2019) on the mini kraken2 database (https://benlangmead.github.io/aws-indexes/k2), including bacteria, viruses, archaea, and homo, when working at the species level, and on the Silva database (http://www.arb-silva.de/) when working at the genus level. Assignment to the species or genus level was further refined using Bracken (Lu et al., 2017). Both analyses were performed with default parameters except for the threshold for abundance estimation with bracken, which was set to 10. This means that, in each sample, abundance was estimated only for taxa covered by at least 10 reads; this was done to remove extremely rare taxa. Results were obtained as raw read counts and as relative abundance, expressed as a percentage of total reads. Diversity indices (ob-
served number of taxa, Simpson’s diversity index, and Shannon’s index) were estimated using raw read counts with the R package Vegan (https://CRAN.R-project.org/package = vegan). Code and data to reproduce metagenomics and integrative analysis are available at: https://github.com/genomeud/Montasio_NMC.

**Quantification of sugars and organic acids**

Lactose, glucose, galactose, and organic acids (citric, lactic, and formic) from NMCs were quantified following the method proposed by Vénica et al. (2014) with slight modifications. Briefly, samples were diluted 1:10 with 25 mM H₂SO₄ and homogenized for 5 min. The suspension was then centrifuged for 20 min at 15,000 × g and 4°C. Sugars and organic acids were separated by using a binary LC 250 HPLC (Perkin Elmer, Waltham MA, USA) equipped with a 300 mm x 7.8 mm, 9 µm Aminex HPX-87H column (Biorad, Hercules CA, USA), an automated 7125 NS injector (Rheodyne, Rohnert Park CA, USA), a 20 µL sample loop, and an external water bath set at 65°C. The elution flow rate was set at 0.7 mL/min and 25 mM H₂SO₄ was used as the mobile phase. Organic acids and sugars were revealed with a 2550 UV detector (Varian Chromatography Systems, Palo Alto CA, USA) set at 210 nm, and a refractive index RID-10A detector (Shimadzu Corporation, Kyoto, Japan), respectively. Quantification was performed by preparing standard solutions at different concentrations for external calibration.

**Quantification of volatiles**

A solid-phase microextraction (SPME) coupled to a gas chromatography-mass spectrometry (GC-MS) system was used to assess volatile compounds in NMCs (Innocente et al., 2013). For sample preparation, 5 g of NMC were weighed in a 20 mL vial and sealed. Samples equilibration was performed at 40°C for 30 min using an HT2800T autosampler (HTA s.r.l., Brescia, Italy), then a 2 cm × 50/30 µm Stableflex 24Ga divinylbenzene/carboxen/poly-dimethylsiloxane coated SPME fiber (Supelco, Bellefonte PA, USA) was exposed for 30 min. Volatile compounds were separated and identified by using a QP2020 NX GC-MS (Shimadzu Corporation) equipped with a DB-WAX capillary column 30 m in length, 0.25 mm internal diameter, and 0.25 µm film thickness (Agilent Technologies, Santa Clara CA, USA). After the fiber desorption at 250°C for 3 min under splitless conditions, the following conditions were adopted: 1 mL/min helium flow rate; the interface, source, and quadrupole temperature set at 250, 175, and 150°C, respectively. The temperature program was set initially at 50°C for 5 min, followed by the first ramp at 10°C/min to 230°C, the temperature was kept steady for 10 min, then a second ramp at 10°C/min to 240°C for 10 min. The mass spectrometry range from 25 to 350 m/z for the analysis in full scan mode. Volatile compounds were identified by spectra comparison using the NIST/EPA/NIH 20 Mass Spectral Library (John Wiley & Sons Inc., Hoboken NJ, USA) and Kovat’s Retention Index (RI) from the literature (https://webbook.nist.gov/chemistry/). Data were expressed as absolute areas of the obtained peaks measured in the headspace of each NMC.

**Statistical analysis**

Results from pH measurement are expressed as mean ± standard deviation of at least 2 analytical replicates (n ≥ 2). The correlation between relative abundance and chemical and physical parameters was measured by Spearman’s correlation coefficient and plotted using the corrplot package (https://github.com/taiyun/corrplot). Only data on bacterial abundance and parameters with less than 50% missing data were used for correlation analysis. The clustering of samples based on the volatile compound area was obtained via principal component analysis (PCA) and hierarchical clustering using OriginPro 9 software (OriginLab, Northampton, MA, USA). For these statistical elaborations, data were log10[x]-transformed and scaled, while the heatmap with dendrogram was performed using Euclidean distances and Ward’s minimum variance as a clustering method.

**RESULTS AND DISCUSSION**

In this study, NMCs were collected from 10 different PDO Montasio cheese plants. No standardized protocol exists for NMC production, except for heat treatment and thermophilic incubation temperature. For this reason, the times and temperatures of incubation and heat treatment differed across dairies (Table 1). Milk was heated in a wide range of time/temperature conditions corresponding to thermization, which safeguards a part of the autochthonous microbiota (Innocente et al., 2014). Milk was then incubated in a narrow range of temperatures (42–45°C) for 4 to 8 h, allowing thermophilic LAB to grow rapidly. L6 was quite different from other NMCs since a lower temperature (35°C) was used together with a longer incubation time. pH ranged from 4.33 to 5.76. The lowest values were found in samples L8 and L9, in which milk was incubated for the longest time intervals, i.e., 6.5 and 8 h, respectively. L3 and L10 showed the highest pH (5.18 and 5.76, respectively).
**Bacterial counts**

Almost all NMCs showed over $10^8$ cfu/g of *St. thermophilus* (Table 2). Lactobacilli and enterococci were not always detected, and counts ranged 3.28–5.64 log cfu/g and 1.30–3.30 log cfu/g, respectively. The sporadic presence of enterococci is probably attributable to their ability to tolerate stresses, including heat treatment, and to grow up to 45°C. Their presence in raw milk cheeses can be meaningful due to their lipolytic and proteolytic activity and volatiles’ production (Marino et al., 2003). Differently from NMCs for stretched cheese and NWCs, lactobacilli were present in a limited concentration (Zotta et al., 2022). It is reasonable that raw milk species are mainly mesophilic, and therefore badly suffer heat treatment and incubation at high temperatures.

**Metagenomics**

While being very useful in routine practice, traditional bacterial counts can’t provide a complete portrait of complex microbial communities. Thus, metagenomic analysis was used to dissect the bacterial biodiversity of the NMCs. The number of species identified in each sample ranged from 24 to 44, and the Shannon and Simpson indices were quite low in most NMCs, indicating low biodiversity, except for samples L3, L5, and L6 (Table 3). Shannon’s index ranged from 0.31 to 1.30 (median 0.52), and Simpson’s from 0.09 to 0.59 (0.15). Diversity indices were negatively correlated ($P < 0.05$) with incubation temperature (Figure 1). This means that when incubating at temperatures ranging from 35 to 45°C, higher temperatures favor the predominance of a limited number of microbial species. In support of this, pH increased as biodiversity indices increased, although without statistical significance. The incubation temperature would therefore be a main driver of biodiversity in NMCs. Treatment temperature was inversely correlated, albeit not significantly, with diversity indexes. On the contrary, a weak positive correlation was observed for the time of treatment and incubation, albeit without statistical significance. We can conclude that milk treatments at lower temperatures for longer times are favorable to the biodiversity of NMCs.

According to viable counts, in all samples, *St. thermophilus* was predominant (59.5–95.1%; Figure 2). *St. thermophilus* is the most metabolically active species after 60 d of PDO Montasio ripening and is still present in high concentrations for up to 150 d (Marino et al., 2003; Carraro et al., 2011). A subdominant population made of other *Streptococcus* (2.6–5.3%) and *L. salivarius* was also detected. Streptococci are commensal of mucosal animal surfaces, and tolerate heat and incubation conditions of NMCs (Ribeiro et al., 2018). Some of them (*Streptococcus agalactiae* and *S. dysgalactiae*) might suggest the use of mastic milk (Wyder et al., 2011). Lactobacilli of dairy interest (*L. helveticus*, *L. delbrueckii*) and other LAB (*Lactococcus garviae* and *Weissella paramesenteroides*) were detected in a low prevalence (<0.05%) only in some NMCs (Table S1), which highlights the major difference between NMC and NWC, where thermophilic lactobacilli represent the prevalent LAB group (Sola et al., 2022). Metagenomic data highlighted the occurrence, in some NMCs, of some *Streptococcus* species, which might be due to the use of mastic milk in some cases.

### Table 1. Milk heat treatment and incubation conditions of NMCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thermal treatment</th>
<th>Incubation</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>66°C 1 min</td>
<td>44°C 5.5 h</td>
<td>5.18 ± 0.01</td>
</tr>
<tr>
<td>L2</td>
<td>65°C 10 min</td>
<td>45°C 6 h</td>
<td>4.83 ± 0.04</td>
</tr>
<tr>
<td>L3</td>
<td>65°C 5 min</td>
<td>42°C 5 h</td>
<td>5.76 ± 0.05</td>
</tr>
<tr>
<td>L4</td>
<td>66°C 20 s</td>
<td>44°C 4 h</td>
<td>4.73 ± 0.02</td>
</tr>
<tr>
<td>L5</td>
<td>68°C 20 s</td>
<td>42°C 6 h</td>
<td>4.91 ± 0.06</td>
</tr>
<tr>
<td>L6</td>
<td>62.5°C 1 min</td>
<td>35°C 8 h</td>
<td>4.56 ± 0.12</td>
</tr>
<tr>
<td>L7</td>
<td>68°C 20 s</td>
<td>43°C 5 h</td>
<td>4.80 ± 0.04</td>
</tr>
<tr>
<td>L8</td>
<td>68°C 5 min</td>
<td>42°C 6.5 h</td>
<td>4.33 ± 0.01</td>
</tr>
<tr>
<td>L9</td>
<td>65°C 5 min</td>
<td>44°C 8 h</td>
<td>4.36 ± 0.02</td>
</tr>
<tr>
<td>L10</td>
<td>65°C 2 min</td>
<td>45°C 5 h</td>
<td>5.57 ± 0.05</td>
</tr>
</tbody>
</table>

### Table 2. Microbial counts (Log cfu/mL) within NMCs

<table>
<thead>
<tr>
<th>St. thermophilus</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>L6</th>
<th>L7</th>
<th>L8</th>
<th>L9</th>
<th>L10</th>
</tr>
</thead>
<tbody>
<tr>
<td>lactobacilli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enterococci</td>
<td>ND</td>
<td>ND</td>
<td>3.30</td>
<td>2.00</td>
<td>ND</td>
<td>ND</td>
<td>1.30</td>
<td>2.70</td>
<td>ND</td>
<td>2.65</td>
</tr>
</tbody>
</table>
| ND = not detected (<10 cfu/mL).

### Table 3. Diversity indices within NMCs

<table>
<thead>
<tr>
<th>N. species</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>L6</th>
<th>L7</th>
<th>L8</th>
<th>L9</th>
<th>L10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simpson</td>
<td>0.15</td>
<td>0.14</td>
<td>0.41</td>
<td>0.12</td>
<td>0.27</td>
<td>0.59</td>
<td>0.09</td>
<td>0.19</td>
<td>0.09</td>
<td>0.14</td>
</tr>
<tr>
<td>Shannon</td>
<td>0.54</td>
<td>0.50</td>
<td>1.00</td>
<td>0.39</td>
<td>0.81</td>
<td>1.30</td>
<td>0.31</td>
<td>0.59</td>
<td>0.32</td>
<td>0.48</td>
</tr>
</tbody>
</table>
of species referring to Next Generation Probiotics, such as *Bacteroides thetaiotaomicron*, *Eubacterium hallii*, and *Akkermansia muciniphila*, which were here first detected in natural cultures for cheesemaking. NGPs have already been identified in dairy cow milk (Savin et al., 2019), consequently, milk and NMCs might present a promising avenue for the exploration of novel strains that hold potential for probiotic applications.

In L3, the genus *Kurthia* covered 14.9% of the identified sequences. It is a psychrotrophic genus, which suggests the use of refrigerated milk. It is an unusual practice in the production of NMCs for PDO Montasio cheese, indeed the milk is usually processed just after production. *Kurthia* has a non-fermentative metabolism and doesn’t contribute to milk acidification, which may concur with the high pH of L3. NMCs with the highest Shannon index values were associated with a high prevalence of spore-former bacilli belonging to genera *Anoxybacillus*, *Bacillus*, and *Rummeliibacillus*. They can survive thermal treatment and grow during NMCs incubation, partially contributing to the acidification of milk. However, they are potential spoilage microorganisms producing enzymes and acids which may lead to off-flavors (Lacorte et al., 2022). Occasionally, the presence of *Bacillus cereus* and *Staphylococcus aureus* has been highlighted in the NMCs characterized by the greatest Shannon index, i.e., in L3, L5, and L6 (Table S1), suggesting that NMCs with high biodiversity may contribute to the spread of pathogens in the dairy supply chain. In the latest EFSA report, *S. aureus* and *B. cereus* were responsible for 178 and 7 foodborne outbreaks in Europe, respectively (EFSA, 2022). L8 contained 4% *Acinetobacter baumannii*, which has already been found as contaminant species in milk and in NWCs. Given its ubiquitous character, it can derive from several sources, including teat surface, air, dust, or milking parlor. It can tolerate heat and adapt to different environments, however, it is rarely detected in cheese since during ripening it is affected by competition with other microorganisms (Gurung et al., 2013).

**Residual sugars and organic acids**

Residual sugars and organic acids were determined in NMCs (Figure 3). All samples revealed a lower lactose concentration than raw milk due to microbial metabolism, the median value being 1.79 g/100 g. L1, L4, L5, and L6 displayed the highest concentrations (over 3 g/100 g), while the remaining samples had concentrations lower than 2 g/100 g. The percentage of species other than *St. thermophilus* in samples L5 and L6 was quite high, in particular, many *Bacillus* and *Anoxybacillus* were detected. The high residual lactose in such samples might be because spore-former bacilli only rarely metabolize this disaccharide (Vos et al., 2011).

Glucose and galactose are usually present in low concentrations in cows’ milk (about 0.01 g/100 g) (Ohlsson et al., 2017). During lactic fermentation, lactose is hydrolyzed into glucose and galactose. *St. thermophilus*, the prevalent microbial species in NMCs, metabolizes glucose through the homolactic pathway, which results in a large amount of lactic acid and ATP. Indeed, glucose was found in very low amounts (median value 0.08 g/100g). On the other hand, in the presence of an excess of lactose, *St. thermophilus* transfers galactose outside the cell through an antiport mechanism (Sangwan et al., 2015). The presence of galactose concentrations between 0.29 and 0.8 g/100 g (median value 0.43 g/100 g) is probably due to the metabolization by other microorganisms (e.g., *L. salivarius*).

As for lactic acid, which was the most abundant organic acid in NMCs, the median value was 1.30 g/100 g. No significant correlation was observed between pH and the lactic acid amount (Spearman’s rho −0.54, p-value 0.11). For instance, L1 displayed a relatively high
pH (Table 1), despite having an amount of lactic acid similar to other NMCs with a lower pH (e.g., L2). This is probably due to the contribution of all the organic acids to the pH. The high amount of lactate produced is significant from a technological perspective since it prevents pathogens and spoilage microorganisms’ contamination in raw or thermized milk cheeses, such as PDO Montasio. Additionally, lactic acid contributes to the sensory features of the final product (Silva et al., 2020). The citric acid (0.1–0.2 g/100 g in cows’ milk) results from the synthesis of fatty acids in the mammary gland during galactopoiesis (Akkerman et al., 2019). In most NMCs, citric acid appeared almost unchanged in comparison to milk, ranging from 0.09 to 0.26 g/100 g, probably due to the large prevalence of *St. thermophilus*. Fermentation of citrates is scarcely performed by this species, being more widespread among lactococci, *Leuconostoc*, and mesophilic lactobacilli (Vénica et al., 2014). In amino acid deficiency, some strains of *St. thermophilus* can even use small amounts of citrate to supply the need, such as glutamate (Pastink et al., 2009). *Kurthia* can also metabolize citrates contributing to their reduction, as observed in L3, where *Kurthia* represented almost 15% of the total microbiota. As for formic acid, the median value in NMCs was 0.33 g/100 g. It is quite higher than the usual level in milk, which is about 0.05 g/100 g (Álvarez-Martín et al., 2008). The increased concentration from milk to NMCs is probably due to *St. thermophilus*, which can produce formate in significant quantities. Despite *St. thermophilus* metabolism being primarily homolactic, a part of pyruvate is converted to formate, as a result of pyruvate-formate lyase activity. In complex microbial communities, as NMCs are, this metabolite promotes proto-cooperation between lactic cocci and rods (Roux et al., 2022). In cheese, formic acid could stimulate lactobacilli not only

![Figure 2. Relative abundance of the 15 most abundant species in NMCs](image-url)
during acidification but also through ripening, when \textit{Lb. casei} spreads (Marino et al., 2003).

The volatilome of cheese is the result of complex biochemical reactions occurring during ripening. Volatiles originate from lactose, proteins, lipids, and to a lesser extent citrate. These can be converted into volatile compounds through fermentative, proteolytic, and lipolytic microbial activities. In this perspective, NMCs can play a central role in preserving the peculiar flavor of artisanal cheeses (Marilley and Casey, 2004). In the NMCs under study, 35 volatiles were detected, including acids, alcohols, ketones, aldehydes, and sulfur compounds (Table S2). PCA was performed to describe the global differences in the volatilome of NMCs (Figure 4a). The 2 PCA axes described 75.12% of the total variability, with PC1 and PC2 accounting for 39.63 and 35.49% of the entire variability, respectively. PC1 was positively associated with alcohols and ketones, and negatively related with aldehydes. PC2 was instead positively correlated to aldehydes, acids, and ketones and negatively with S-compounds.

A clustering analysis was also performed, and the results are shown with a heatmap representation and dendrograms (Figure 4b). According to the PCA biplot and hierarchical analysis, NMCs were grouped into 4 leading clusters. Groups I and II were characterized by a very low prevalence of aldehydes. Acetaldehyde and hexanal were missing in both groups, whereas nonanal was only present in group I. Group III included L4 and L9, which were positively associated with S-compounds, and, to a lesser extent ketones and alcohols. Group IV (L1, L2, L6, and L8) was positively associated with acids, ketones, aldehydes, and alcohols, while negatively with S-compounds.

Overall, the most abundant class of volatiles was represented by acids, the main being acetic, butanoic, hexanoic, and octanoic acids. Generally, acids between 4 and 20 carbon atoms develop from lipolysis, whereas between 2 and 6 mainly generate from lactose fermentation and proteolysis. They can also derive from ketones and aldehydes oxidation. These compounds are significant contributors to the aroma of a wide variety of hard and semi-hard cheeses. Nevertheless, if in excess they can contribute negatively to cheese flavor (Innocente et al., 2013). Group IV showed the highest production of acids, while group II was characterized by lower concentrations of acids produced compared with the other samples. However, acetic acid was widely produced in all samples, probably mainly due to the metabolic activities of \textit{St. thermophilus} (Morandi et al., 2019), which was the most abundant microorganism detected in all samples. The second most abundant class was alcohols. Primary alcohols develop from the reduction of the corresponding aldehydes throughout lactose or amino acid metabolism (McSweeney and Sousa, 2000). Group IV showed the highest concentrations of most alcohols, with ethanol, 1-hexanol, and 1,2-butanediol as the most representative. Within group IV, L6 was characterized by a significant presence of \textit{A. gonensis} and \textit{A. flavithermus}, which are capable of metabolizing lactose leading to the formation of alcohols, in particular, ethanol (Zhao et al., 2018). An analogous observation can be made about \textit{B. subtilis}, 5% in L5. Although present in small numbers compared with acids, the concentration
of the different ketones within the samples was high. Acetoin, 2,3-butanedione, 2,3-pentanedione, acetone, and 2-butanone were the most detected. Ketones are common key aroma constituents of most dairy products, in particular acetoin and 2,3-butanedione, which can confer a typical buttery aroma to cheese. Ketones derive mainly from lactose or citrate catabolism by lactic acid bacteria, but they can also be a product of amino acid catabolism (McSweeney and Sousa, 2000). The abundance of St. thermophilus mostly determined the production of 2,3-butanedione, 2,3-pentanedione, and acetoin in the headspace of NMCs. Aldehydes and S-compounds were the least detected volatiles. Aldehydes originate from amino acids and don’t accumulate in cheese since they are rapidly converted into the corresponding alcohols or acids, through reduction or oxidation reactions, respectively. As for sulfur compounds, they derive from methionine degradation by methionine-demethlase, which cuts the bond between carbon and sulfur (McSweeney and Sousa, 2000).

Streptococcus abundance was negatively correlated with glucose concentration (Figure 5); this result is expected since St. thermophilus was the most abundant species in all samples and it metabolizes only the glucose moiety of lactose (Sørensen et al., 2016). St. thermophilus abundance was positively correlated with the concentration of benzoic acid. The production of benzoic acid was documented by St. thermophilus in milk (Han et al., 2016). The antimicrobial feature of benzoic acid could to some extent influence the microbial kinetics in cheese (Yu et al., 2016). However, St. thermophilus was also negatively correlated to isovaleric acid, which is considered a desired volatile compound due to its strong cheese aroma (Helinck et al., 2004). Lactobacillus abundance was positively correlated with pH and various volatile compounds (acetoin, 1-hexanol, and 1-nonanol). Lactobacilli, particularly those belonging to Lacticaseibacillus spp., mainly arise from raw milk, and despite being initially present in low concentration in cheese, they reach high abundance in ripened cheese (Bottari et al., 2018). Such behavior is typical of non-starter lactic acid bacteria (NSLAB), that significantly contribute to the aromatic profile and texture of the product by their proteolytic and lipolytic activities (Gatti et al., 2014).

CONCLUSIONS

In this study, a description of the microbiota present in NMCs for PDO Montasio cheese is provided, using an integrated approach based on culture-dependent and -independent methods, together with quantification of residual sugars and organic acids and characterization of volatiles in the headspace. Ten different samples were studied, to fully observe the extent of the variability across different producers. Despite the origin of NMCs from different farms and the different raw materials used, it was possible to define a core microbial population, consisting of St. thermophilus, and a subdominant population made up of other streptococci and L. salivarius, ensured by the application of high incubation temperatures. NMCs revealed different sugars, organic acids, and volatile compositions, which can be attributed to the heterogeneous microbial community selected by heat treatment and incubation of milk. We can assume that this microbiota, character-
ized by distinct metabolic pathways, would be able to exert them also inside the cheese, affecting its sensory profile. Quantitative data on sugars, and acids and clustering analyses based on volatile compounds didn’t differentiate samples having different abundance of St. thermophilus, suggesting that several additional factors, besides the abundance of the dominant microbial species, play a role in the physicochemical features of NMCs.

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Declaration of competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability The sequencing reads used in this study are available in Sequence Read Archive under the accession PRJNA916949 (https://www.ncbi.nlm.nih.gov/sra/PRJNA916949). Scripts, functions, and data used in the present work are released on GitHub under the GNU General Public License v3.0: (https://github.com/genomenuid/Montasio_NMC).

REFERENCES


Figure 5. Correlation between bacterial genus abundance (cyan), sugars and pH (red), and volatile compounds (orange). The size of bacterial nodes is scaled according to bacterial relative abundance. Purple lines indicate a positive correlation; black lines indicate a negative correlation. Only significant correlations are plotted.
Rossi et al.: Microbial diversity of natural milk cultures


ORCIDS

Anna Rossi: https://orcid.org/0000-0003-2648-3613
Fabio Marroni: https://orcid.org/0000-0002-1556-5907
Nicolo Renoldi: https://orcid.org/0000-0003-4327-4768
Giulia Di Filippo: https://orcid.org/0000-0002-0579-6947
Elisabetta Gover: https://orcid.org/0000-0004-1331-2439
Marilena Marino: https://orcid.org/0000-0001-7443-3472
Nadia Innocente: https://orcid.org/0000-0003-2141-4663