The use of natural milk culture (NMC) represents a key factor in Protected Designation of Origin (PDO) Montasio cheese, contributing to its distinctive sensory profile. The complex microbial ecosystem of NMC is the result of heat treatment and incubation conditions, which can vary considerably among different production plants. In this study, the microbiota of NMC collected from 10 PDO Montasio cheese dairies was investigated by 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 NMC, and a subdominant population made of other streptococci and *Ligilactobacillus salivarius* was also present. The incubation temperature appeared to be the main driver of biodiversity in NMC. Metagenomics allowed us to evidence the presence of minor species involving safety (e.g., *Staphylococcus 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 PDO Montasio cheese.

**Key words:** natural milk cultures, Protected Designation of Origin cheese, metagenomics, volatilome, metabolites
ity is obtained. Under these conditions, a heterogeneous thermophilic microbiota is selected. Although they are widely used for many PDO cheeses, much less is known about their composition than NWC. 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 Strep. thermophilus and Lactobacillus delbrueckii ssp. lactis, with the presence of subdominant mesophilic LAB species (Parente et al., 2016). As for NMC produced daily without backslopping, which is the case of many Italian PDO and 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 produced in Northeast Italy using raw or thermized cow milk. The most traditional cheesemaking procedure uses a thermophilic NMC produced daily without backslopping (Innocente and Biasutti, 2013). During production, milk thermal treatment and inoculation procedures select a different microbiota mainly composed of Strep. 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 next-generation techniques, however, allows for the highlighting of noncultivable and subdominant populations, in which 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, water activity, 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 NMC is available. Thus, in this study, a metagenomic approach was used to characterize the microbiota composition of different NMC for PDO Montasio cheese. Moreover, measurements of physico-chemical features of NMC 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 (Crisom, Barcelona, Spain).

Microbiological Analysis

The samples were serially diluted in Maximum Recovery Diluent (Oxoid, Milan, Italy), and decimal dilutions pour-plated onto de Man, Rogosa, and Sharpe (MRS) agar (Oxoid) pH 5.4 for lactobacilli, Kanamycin Esculin Azide agar (Oxoid) for enterococci, and Strep. thermophilus agar (Dave and Shah, 1996). 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

A DNeasy PowerLyzer PowerSoil kit (Qiagen, Hilden, Germany) was used to extract DNA. The 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 (observed 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 NMC were quantified following the method proposed by Vénica et al. (2014) with slight modifications. Briefly, samples were diluted 1:10 with 25 mM H$_2$SO$_4$ and homogenized for 5 min. The suspension was then centrifuged for 20 min at 15,000 $\times$ g and 4°C. Sugars and organic acids were separated by using a binary LC 250 HPLC (Perkin Elmer, Waltham, MA) equipped with an Aminex HPX-87H column (300 mm × 7.8 mm × 9 $\mu$m; BioRad, Hercules, CA), an automated 7125 NS injector (Rheodyne, Rohnert Park, CA), a 20-$\mu$L 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 $\mu$L sample loop, and an external water bath set at 65°C.

In this study, NMC 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 and 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. Sample L6 was quite different from other NMC, because a lower temperature (35°C) was used with a longer incubation time. The 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

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thermal treatment</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>Time (min)</td>
</tr>
<tr>
<td>L1</td>
<td>66</td>
<td>1</td>
</tr>
<tr>
<td>L2</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>L3</td>
<td>65</td>
<td>5</td>
</tr>
<tr>
<td>L4</td>
<td>66</td>
<td>20 s</td>
</tr>
<tr>
<td>L5</td>
<td>68</td>
<td>20 s</td>
</tr>
<tr>
<td>L6</td>
<td>62.5</td>
<td>1</td>
</tr>
<tr>
<td>L7</td>
<td>68</td>
<td>20 s</td>
</tr>
<tr>
<td>L8</td>
<td>68</td>
<td>5</td>
</tr>
<tr>
<td>L9</td>
<td>65</td>
<td>5</td>
</tr>
<tr>
<td>L10</td>
<td>65</td>
<td>2</td>
</tr>
</tbody>
</table>

$^1$Values are mean ± SD.

RESULTS AND DISCUSSION

In this study, NMC 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 and 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. Sample L6 was quite different from other NMC, because a lower temperature (35°C) was used with a longer incubation time. The 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). Samples L3 and L10 showed the highest pH (5.18 and 5.76, respectively).

**Bacterial Counts**

Almost all NMC showed over $10^8$ cfu/g of *Strep. thermophilus* (Table 2). Lactobacilli and enterococci were not always detected, and counts ranged from 3.28 to 5.64 log cfu/g and 1.30 to 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 NMC for stretched cheese and NWC, lactobacilli were present in a limited concentration (Zotta et al., 2022). It is reasonable to assume that the predominant species in raw milk are mesophilic and, as a result, they are highly susceptible to heat treatment and incubation at high temperatures.

**Metagenomics**

Although 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 NMC. The number of species identified in each sample ranged from 24 to 44, and the Shannon and Simpson indices were quite low in most NMC, 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 NMC. 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 NMC.

According to viable counts, in all samples, *Strep. thermophilus* was predominant (59.5%–95.1%; Figure 2). *Streptococcus 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* spp. (2.6%–5.3%) and *Ligilactobacillus salivarius* was also detected. Streptococci are commensal of mucosal animal surfaces, and tolerate heat and incubation conditions of NMCs (Ribeiro Júnior et al., 2018). Some of them (*Streptococcus agalactiae* and *Streptococcus 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 NMC (Supplemental Table S1, see Notes), 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 NMC, 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. Next-generation probiotics have already been identified in dairy cow milk (Savin et al., 2019); consequently, milk and NMC might present a promising avenue for the exploration of novel strains that hold potential for probiotic applications.

**Table 2.** Microbial counts (log cfu/mL) in 10 samples (L1–L10) of NMC

<table>
<thead>
<tr>
<th>Item</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><em>Streptococcus thermophilus</em></td>
<td>9.33</td>
<td>8.92</td>
<td>7.07</td>
<td>8.33</td>
<td>8.27</td>
<td>9.03</td>
<td>8.76</td>
<td>8.28</td>
<td>8.18</td>
<td>8.11</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>5.64</td>
<td>ND</td>
<td>ND</td>
<td>4.18</td>
<td>3.30</td>
<td>ND</td>
<td>ND</td>
<td>4.83</td>
<td>3.28</td>
<td>4.18</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 in 10 samples (L1–L10) of NMC

<table>
<thead>
<tr>
<th>Item</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>Species (n)</td>
<td>40</td>
<td>43</td>
<td>44</td>
<td>28</td>
<td>42</td>
<td>43</td>
<td>24</td>
<td>31</td>
<td>24</td>
<td>35</td>
</tr>
<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>
In sample 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 NMC for PDO Montasio cheese; indeed, the milk is usually processed just after production. Kurthia has a nonfermentative metabolism and doesn’t contribute to milk acidification, which may concur with the high pH of L3. The NMC 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 NMC incubation, partially contributing to the acidification of milk. However, such sporeformers are potential spoilage microorganisms producing enzymes and acids that may lead to off-flavors (Lacorte et al., 2022). Occasionally, the presence of Bacillus cereus and Staphylococcus aureus has been highlighted in the NMC characterized by the greatest Shannon index (i.e., in L3, L5, and L6; Supplemental Table S1, see Notes), suggesting that NMC with high biodiversity may contribute to the spread of pathogens in the dairy supply chain. In the latest EFSA report, Staph. aureus and B. cereus were responsible for 178 and 7 foodborne outbreaks in Europe, respectively (EFSA and ECDC, 2022). Sample L8 contained 4% Acinetobacter baumannii, which has already been found as contaminant species in milk and in NWC. 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, because 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 NMC (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. Samples L1, L4, L5, and L6 displayed the highest concentrations (over 3 g/100 g), whereas the remaining samples had concentrations lower than 2 g/100 g. The percentage of species other than Strept. thermophilus in samples L5 and L6 was quite high; in particular, many Bacillus and Anoxybacillus species were detected. The high residual lactose in such samples might be because spore-former bacilli only rarely metabolize this disaccharide (Logan and Vos et al., 2011).

Glucose and galactose are usually present in low concentrations in cow milk (about 0.01 g/100 g; Ohlsson et al., 2017). During lactic fermentation, lactose is hydrolyzed into glucose and galactose. Streptococcus thermophilus, the prevalent microbial species in NMC, 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/100 g). In contrast, in the presence of an excess of lactose, Strep. 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 NMC, 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 NMC 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 relevant from a technological perspective because it prevents pathogens and spoilage microorganisms’ contamination in raw or thermized milk cheeses, such as PDO Montasio. In addition,
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 cow milk) results from the synthesis of fatty acids in the mammary gland during galactopoiesis (Akkerman et al., 2019). In most NMC, 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 *Strep. thermophilus*. Fermentation of citrates is scarcely performed by this species, being more widespread among lactococci, *Leuconostoc*, and mesophilic lactobacilli (Vénica et al., 2014). In AA deficiency, some strains of *Strep. 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 NMC 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 NMC is probably due to *Strep. thermophilus*, which can produce formate in high quantities. Despite *Strep. 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 NMC are, this metabolite promotes proto-cooperation between lactic cocci and rods (Roux et al., 2022). In cheese, formic acid could stimulate lactobacilli not only during acidification but also through ripening, when *Lacticaseibacillus 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 com-

![Microbiota composition at the species level in NMC from 10 different dairy plants in the production area of PDO Montasio cheese. Only the 15 most abundant species are represented.](image-url)
pounds through fermentative, proteolytic, and lipolytic microbial activities. In this perspective, NMC can play a central role in preserving the peculiar flavor of artisanal cheeses (Marilley and Casey, 2004). In the NMC under study, 35 volatiles were detected, including acids, alcohols, ketones, aldehydes, and sulfur compounds (Supplemental Table S2, see Notes). Principal component analysis was performed to describe the global differences in the volatilome of NMC (Figure 4a). The 2 PCA axes described 75.12% of the total variability, with principal component (PC)1 and PC2 accounting for 39.63% and 35.49% of the entire variability, respectively. Principal component 1 was positively associated with alcohols and ketones and negatively related to aldehydes. In contrast, PC2 was positively correlated to aldehydes, acids, and ketones and negatively to 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, NMC 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, and 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 those between 2 and 6 mainly generate from lactose fermentation and proteolysis. They can also derive from ketones and aldehydes oxidation. These compounds are relevant 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, whereas 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 Strep. 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 AA metabolism (McSweeney and Sousa, 2000). Group IV showed the highest concentrations of most alcohols, with ethanol, 1-hexanol, and 1,2-butandiol as the most representative. Within group IV, L6 was characterized by a high presence of Anoxybacillus gonensis and Anoxybacillus 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 Bacillus 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 butty aroma to cheese. Ketones derive mainly from lactose or citrate catabolism by LAB, but they can also be a product of AA catabolism (McSweeney and Sousa, 2000). The abundance of *Strep. thermophilus* mostly determined the production of 2,3-butanedione, 2,3-pentanedione, and acetoin in the headspace of NMC. Aldehydes and S-compounds were the least detected volatiles. Aldehydes originate from AA and don’t accumulate in cheese, as 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-demethylase, which cuts the bond between carbon and sulfur (McSweeney and Sousa, 2000).

*S. thermophilus* abundance was negatively correlated with glucose concentration (Figure 5); this result is expected because *Strep. thermophilus* was the most abundant species in all samples, and it metabolizes only the glucose moiety of lactose (Sørensen et al., 2016). *Streptococcus thermophilus* abundance was positively correlated with the concentration of benzoic acid. The production of benzoic acid was documented by *Strep. 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, *Strep. thermophilus* was also negatively correlated with 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 nonstarter LAB, which contribute to the aromatic profile and texture of the product by their proteolytic and lipolytic activities (Gatti et al., 2014).

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**Figure 4.** Principal component analysis of the main classes of volatile compounds in NMC (a) and hierarchical clustering with a heatmap representation based on Euclidean distances and Ward’s minimum variance (b). Colors ranging from brown to green indicate low to high abundance of the 35 volatile compounds detected.

**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 (*P* < 0.05) are plotted.
CONCLUSIONS

In this study, a description of the microbiota present in NMC 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 NMC from different farms and the different raw materials used, it was possible to define a core microbial population, consisting of Strep. thermophilus, and a subdominant population made up of other streptococci and L. salivarius, ensured by the application of high incubation temperatures. The NMC 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, characterized 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 Strep. thermophilus, suggesting that several additional factors, in addition to the abundance of the dominant microbial species, play a role in the physicochemical features of NMC.

NOTES

The authors thank Consorzio Tutela Formaggio Montasio (Codroipo, UD, Italy), which supported the research activities within the project “Strengthening the typicality and improving the sustainability of the production chain of PDO Montasio cheese.” 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/genomeud/Montasio_NMC). Supplemental material for this article is available at https://doi.org/10.17632/g8392fw2k4.1. No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board. The authors have not stated any conflicts of interest.

Abbreviations used: L1 to L10 = NMC samples; LAB = lactic acid bacteria; MRS = de Man, Rogosa, and Sharpe; NMC = natural milk culture; NWC = natural whey cultures; PC1 and PC2 = principal component 1 and 2, respectively; PCA = principal component analysis; PDO = Protected Designation of Origin.

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