Dynamic microbial and metabolic changes during Apulian Caciocavallo cheese-making and ripening produced according to a standardized protocol

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ABSTRACT

The cheese microbiota plays a critical role in influencing its sensory and physicochemical properties. In this study, traditional Apulian Caciocavallo cheese coming from 4 different dairies in the same area and produced following standardized procedures have been examined, as well as the different bulk milks and natural whey starter cultures used. Moreover, considering the cheese wheels as the blocks of Caciocavallo cheeses as whole, these were characterized at different layers (i.e., core, under-rind, and rind) of the block using a multi-omics approach. In addition to physical-chemical characterization, culturomics, quantitative PCR, metagenomics, and metabolomics analysis, have been carried out post-salting and throughout ripening time (2 mo) to investigate the major shifts in the succession of the microbiota and flavor development. Culture-dependent and 16S rRNA metataxonomics results clearly clustered samples based on the microbiota biodiversity related to the production dairy plant as the result of the use of different NWS or intrinsic conditions of each production site. At the beginning of the ripening, cheeses were dominated by the Lactobacillus and, in 2 dairies (Art and SdC), Streptococcus genera associated with the NWS. The analysis allowed us to show that, although the diversity of identified genera did not change significantly between the rind, under-rind and core fractions of the same samples, there was an evolution in the relative abundance and absolute quantification, modifying and differentiating profiles during ripening. The qPCR mainly differentiated the temporal adaptation of those species originating from bulk milks and those provided by NWSs. The primary starter detected in NWS and cheese reassured the high relative concentration of 1-butanol, 2-butanol, 2-heptanol, 2-butanone, acetoin, delta-dodecalactone, hexanoic acid ethyl ester, octanoic acid ethyl ester, and VFFA during ripening, while cheeses displaying low abundances of Streptococcus and Lactococcus (dairy Def) have a lower total concentration of acetoin compared with Art and SdC. However, the sub-dominant strains and NSLAB present in cheeses are responsible for the production of secondary metabolites belonging to the chemical classes of ketones, alcohols, and organic acids, reaffirming the importance and relevance of autochthonous strains of each dairy plant although considering a delimited production area. Keywords: Caciocavallo cheese, bulk milks, natural whey starters, lactic acid bacteria, cheese volatilomics

INTRODUCTION

The microbial contribution to flavor is mainly determined by differences in protocols adopted during the cheese making (Gobbetti et al., 2015; Korena et al., 2023). Interest in understanding the origin and composition of microbiota in ripened cheeses and, therefore, the understanding concerning roles provided by different microorganisms with respect to flavor development and diversification between different cheeses is an ongoing field of research (Tilocca et al., 2020). In previous studies, researchers examined results derived by means of traditional methods (e.g., culture-based approaches) with the purpose to inspect microbial variations determined by manufacturing and ripening (Caridi et al., 2003; Korena et al., 2023). With the increasing advent of the high throughput sequencing analysis, the investigation of the dairy microbiota moved toward the interest to define and profile the microbiota harbored in various niches (De Pasquale et al., 2014; Ding et al., 2020) and, therefore, employing a holistic conceptualization of the biomolecules responsible for the structure, functions, and dynamics of a given consortium of microorganisms inhabiting different layers of a same food.

Caciocavallo is a very popular typical pasta filata cheese manufactured from raw cow’s milk using natural whey or commercial thermophilic and mesophilic (e.g., Lc. lactis) cultures (Calasso et al., 2016). In the
northern Mediterranean area (e.g., Italy, Greece, and Balkans), pasta-filata cheeses are a group of soft or semisoft cheese varieties consumed typically. By using the term pasta-filata, it means a process that consists in curd plasticization and stretching starting from whole milks (McMahon and Oberg, 2017). To obtain curd, the protease activity is mainly provided by the addition rennet. Also, during cheese-making preliminarily phases, it is possible to add living microorganisms in form of natural whey cultures/starters (NWSs) obtained from previous manufactures according to traditional back-slopping procedures (Zheng et al., 2021). Thus, pasta-filata cheeses undergo a texturization after soaking of the acidified curd in hot water with the aim to achieve the optimal plastic consistency (Zheng et al., 2021). Time and temperature for making pasta-filata cheeses are both pivotal parameters for the dairy microbiota development. Moreover, the process is significantly influenced by the ripening time, which allows for the succession of different microbial dynamics (Caridi et al., 2003; Korena et al., 2023). Another factor influencing the cheese microbiota is the use of commercial starters rather than NWSs, a practice that facilitates the prediction of future flavors in dairy products, enhancing reproducibility, but reducing variations that may distinguish local products and their typical flavors.

During the manufacture of pasta-filata cheeses, starter cultures synthesize lactic acid as adjuvant to demineralize and transform the curd before to be stretched in hot water at the optimal pH (McMahon and Oberg, 2017; Zheng et al., 2021). Therefore, used starters provide a significant contribution to drive the entire microbial biomass in young curd.

Although all stages of Caciocavallo cheese-processing are manual, methods and tools for cheese making can account for artisanal and traditional procedures, with quality and flavor development implications linked to each cheesemaking site (Zhao et al., 2021). In fact, the quality of artisanal cheeses is intimately bound up with the territory of production and its traditions. Creating such specific conditions simultaneously accounting for the relationships occurring between soil climatic characteristics, native genetic variations, and anthropic components, it would be extremely difficult to reproduce a similar environment elsewhere. As a natural consequence, the uniqueness of the historical and cultural environment is also a concern aiming at safeguarding these traditional products (Parasecoli, 2017).

Based on these considerations, to explore major shifts in the succession of microbiota and flavor development through the application of a multi-omics approach incorporating culturomics, quantitative PCR, metagenomics, and metabolomics, and physical-chemical analysis, the current research examined traditional mo-ripened Apulian Caciocavallo cheeses made following standardized procedures, as well as the related bulk milks and NWSs, which were provided by 4 different dairies placed within a restricted area.

**MATERIALS AND METHODS**

**Caciocavallo production process**

Caciocavallo cheeses were manufactured at an artisanal-type (labeled as SdC and Cur) and semi-industrial-type (labeled as Art and Del) dairy plants located in the province of Bari, Southern Italy, representative of the production area that, after being selected according to production specifications, were considered eligible to be included (Supp. Fig. S1A and B). In first instance, dairies have been trained by formed personnel of this research group with the aim to standardize the workflow while an equal production protocol was provided (Supp. Fig. S1). Except for the workflow, no further information has been given during the training time about aims and scopes of the present research.

**Sample collection and processing**

The same day of the production, aliquots of used milks (M) and of natural whey starters (NWS) used to produce the Caciocavallo samples have been collected. The production was repeated 3 times to obtain biological replicates from each cheese factory. Samples of the same Caciocavallo batch have been also collected at different ripening times, specifically at one day (T1), 30 d (T30), and 60 d (T60). As previously detailed (Calasso et al., 2016), cheese-samples were immediately processed to obtain aliquots from core (C), under-rind (UR), and rind (R) layers of the cheese (Supplementary Figure S1C). In details, an aliquot equal to 100 cm² was sampled from R- and UR-sites of the cheese-wheel. The rind was scraped for a maximum of 1 mm of the whole cheese-wheel. The underlying layer (i.e., UR) was obtained by an additional scraping (<1 mm) process. Finally, 10 g of Caciocavallo-core sites were collected. Then, samples were immediately frozen at −20°C before to be further processed.

**pH and total titratable acidity (TTA) profiling**

The pH of bulk milks, NWS, and cheese fractions were determined by an ultrabasic-10 pH meter (Denver Instrument Company; Arvada, CO, USA) equipped with a food penetration probe.

The TTA was expressed as the volume (mL) of NaOH solution (0.1 M) added in samples to reach a pH value equal to 8.3 (Rizzello et al., 2014).
**Viable microbiota**

Aliquots equal to 5 g from each bulk milk and NWSs were mixed with 45 mL of physiological sterile saline solution (NaCl 0.9 g/L) in bags with filter (250 µm) and used to carry out plate counting after 10-fold serial dilutions as previously detailed (Calasso et al., 2016). The total mesophilic aerobic counts based on the pour plate technique after 48 h at 30°C were carried out on Plate count agar (PCA) purchased from Oxoid Ltd. (Basingstoke, Hampshire, England) according to UNI EN ISO 4833–1:2013. Presumptive mesophilic and thermophilic lactic acid bacilli were enumerated on de Man, Rogosa, and Sharpe (MRS) medium (Oxoid Ltd.) supplemented with cycloheximide (0.1% [wt/vol]) (Oxoid Ltd.) and incubated aerobically for 48 h at 30°C and 37°C, respectively. Presumptive mesophilic and thermophilic lactic acid cocci were enumerated on M17 agar media (Oxoid Ltd.) supplemented with 5 g/L D-lactose (Oxoid Ltd.) supplemented with cycloheximide (0.1% [wt/vol]) and incubated aerobically at 30 and 37°C for 24–48 h (Calasso et al., 2016; Minervini et al., 2017). Submerged or surface compact or featherly, small, opaque, and white colonies are counted, and randomly representative colonies were used for Gram, catalase, and motility test. Presumptive enterococci counts were determined by plating spread evenly over the agar surface of Slanetz and Bartley agar medium (Oxoid Ltd.) followed by incubation at 37°C for 48 h, after which typical colonies (pink or dark red, with a narrow whitish border) are counted (Nordic Committee on Food Analysis, 1968) and randomly representative colonies were used for Gram, catalase, and motility test. *Staphylococcus* coagulase-positive strains and *Enterobacteriaceae* were quantified after enrichment by respecting the official methods, ISO 6888–1:2021 and ISO 21528–2:2017, respectively. Enumeration of yeasts was assessed by means of the medium Wort agar (Oxoid Ltd.) after incubation of 5 d at 25°C of the plates. Colony morphology was evaluated (color, shape, and size).

**DNA extraction and 16S rRNA gene sequencing**

Aliquots (10 g) of milk, natural whey starters, and cheeses were homogenized in ratios 1:10 with physiological sterile saline solution for 5 min, centrifuged (1000 × g, 5 min, 4°C), and supernatants were then recovered to carry out an additional centrifugation step (5000 × g, 15 min, 4°C). Pellets were resuspended in 0.5 mL physiological sterile saline solution to extract the total DNA by using the FastDNA Spin Kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer’s instructions (Calasso et al., 2016).

Quality and concentration of total extracted DNA was evaluated spectrophotometrically (NanoDrop ND-1000, Thermo Fisher Scientific, Inc.). The metagenomic analyses were carried out at Genomix4Life (spin-off of the University of Salerno, Fisciano, Italy) by using the Illumina MiSeq platform. In detail, primers 28F (forward: 5’-GAGTTTGATCCTGGCTCAG-3’) and 388R (reverse: TGCTGGCCCTCCGTAGGAGT) were used to amplify the V1-V3 hypervariable regions of the 16S rRNA gene and to analyze the diversity inside the Bacteria domain. PCR reactions were carried out following internal protocols of Genomix4Life, which were in accordance with Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA). A negative control is included to assess potential contamination; hence, all the reagents necessary for the 16S rRNA amplification and library preparation have been added without including samples. Libraries were quantified using a Qubit fluorometer (Invitrogen Co., Carlsbad, CA) and pooled, including the Phix Control Library, to an equimolar amount (4 nM of final concentration). The fasta files including raw sequences underwent a quality control check with FastQC. Raw sequences (reads) of 16S rRNA amplicons were analyzed in QIIME2 (https://10.1308/s41587-019-0209-9; accessed online in May 2023) microbiome platform (version 2020.8). Paired demultiplexed 16S rRNA sequences of amplicons have been denoised by using q2-deblur QiIME plugin (https://github.com/qiime2/q2-deblur; accessed online 12/2021). Taxonomy has been inferred by using the QIIME-compatible database Silva v.138 SSU. Alpha diversity metrics of Shannon’s entropy was also computed by using the QIIME2 platform (Chao and Bunge, 2002).

**Real Time PCR (qPCR)**

According with previous investigations carried out for NWS and cheese profiling (Bottari et al., 2013), 16 species of lactobacilli were here quantified by Real Time PCR (qPCR), i.e., *Fruictilactobacillus* (FL) sanfranciscensis, *Lactobacillus* (L.) acidophilus, *L. delbrueckii* ssp. bulgaricus, *Lacticaseibacillus* (Lc.) casei, *Lc. paracasei*, *Lc. rhamnosus*, *Lentilactobacillus* (Ll.) hilgardii, *Lentilactobacillus* (Ll.) buchneri, *Ll. parabuchneri*, *Lacticiplantibacillus* (Lp.) plantarum, *Lp. pentosus*, *Limosilactobacillus* (Ls.) fermentum, *Ls. reuteri*, *Lactobacillus* (L.) curvatus, *L. sakei*, *Levilactobacillus* (Lv.) brevis on bulk milks, natural whey starter, and core, under-rind, and rind of the 4 different Apulian Caciocavallo cheese at the beginning (T1) and at the end (T60) of the ripening, collected samples. To better inspect the taxa profile, further 3 species of lactic acid bacteria, i.e., *Weissella* (W.) cibaria, *Pediococcus*...
(P.) pentosaceus, Streptococcus (S.) thermophilus, and Leuconostoc genus were also included for the qPCR-based investigation. The primer specificity was verified by PCR on different strains of the same targeted LAB species by subsequent sequencing and checking in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the resulting qPCR amplicons. Each qPCR design was assessed for cross-amplification against all other LAB species selected in this study (Supp. Tab. S1). qPCR reactions were carried out by Applied Biosystems 7300 Real-Time PCR System. According with qPCR reactions were carried out by Applied Biosystems 7300 Real-Time PCR System. According with qPCR reactions were carried out by Applied Biosystems 7300 Real-Time PCR System. According with qPCR reactions were carried out by Applied Biosystems 7300 Real-Time PCR System.

Cheese volatile organic compound (VOC) profiling

Cheese volatilome was obtained according with previously conducted procedures (Celano et al., 2022b). In details, aliquots (4 g) from each Caciocavallo-cheese (as a whole) were obtained by grating, placed in 20 mL glass vials, and added with 10 µL of internal standard solution (2-octanol) corresponding to 10 ppm. Vials were sealed with polytetrafluoroethylene-coated silicone rubber septa (20 mm diameter) (Supelco, Bellefonte, PA, USA). The best extraction efficiency was obtained as described by Salum et al., 2017. After sample equilibration (10 min at 54.75°C), a conditioned 50/30 µm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) was exposed for 60 min. The temperature was kept constant during analysis, and the vials were maintained on a heater plate (CTC Analytics, Zwingen, Switzerland) of a CombiPAL system injector autosampler (CTC Analytics). The extracted VOC were desorbed in splitless mode (3 min at 220°C) and analyzed through a Clarus 680 (Perkin Elmer) gas-chromatography (GC) system equipped with a capillary Rtx-WAX column (30 m x 0.25 mm i.d., 0.25 µm film thickness) (Restek, Bellfonte, PA, USA). The column temperature was set initially at 35°C for 8 min, then increased to 60°C at 4°C min−1, to 160°C at 6°C min−1, and finally to 200°C at 20°C min−1 and held for 15 min. Helium was used as the carrier gas at flow rate of 1 mL min−1. A single quadrupole mass spectrometer (MS) Clarus SQ 8C (Perkin Elmer) was coupled to the GC system. The source and transfer line temperatures were kept at 250 and 230°C, respectively. Electron ionization masses were recorded at 70 eV in the mass-to-charge ratio ([m/z] interval 34–350. Each chromatogram was analyzed for peak identification using the NIST (National Institute of Standard and Technology) 2008 library. A peak area threshold of 1,000,000 and at least 85% probability of match were used for dentification, followed by visual inspection of the fragment patterns when required. The concentrations of VOC were calculated in ratio to the internal standard and expressed as mg/kg of cheese.

Statistical analyses

Data have been expressed as mean ± standard deviation (SD) or percentage, as appropriate. Continuous variables were subjected to one-way ANOVA (ANOVA). For metatransomics (16S rRNA) data, sample β-diversity was inspected by Principal Coordinates Analysis (PCoA) considering Bray-Curtis Index as distance method while the comparisons accounted for permutational ANOVA (PERMANOVA) analysis. For the afore-mentioned sample comparisons, significant differences were explained by a p-values (< 0.05). In the Clustering Heatmap Visualization, CN-values from qPCR were initially normalized and the resulting Z-scores were used to proceed with the sample clustering was based on Euclidean distance and Ward’s algorithm. Also, normalized data of volatile compounds (VOCs) were used for Heatmap analysis and subjected to a hierarchical clustering based on Euclidean distance and Ward's metrics using the statistical software Statistica 12.5 (TIBCO Software Inc., Palo Alto, USA).

Bulk milks

Bulk milks were collected from dairies on 3 consecutive days and corresponded to those milks that were specifically used to produce the here analyzed Caciocavallo cheeses. Before to profile the microbiota, bulk milks were analyzed for pH and TTA and values ranged between 6.6 and 6.7 (±0.05) and 2–2.5 (±0.2) mL of 0.1 M NaOH, respectively. Bulk milk viable microbiota
was then profiled (Tab. 1). Total mesophilic aerobes (TMA) ranged between 5.4 and 4.1 log cfu/mL densities observed in SdC and Art samples, respectively. The comparison of SdC against Art achieved the significance threshold ($P < 0.05$), while Cur and Del samples exhibited intermediate TMA densities. Presumptive viable lactobacilli, lactic acid cocci, and enterococci densities reported similar ratios than those explained by TMA counts since even all these microbial groups were found at the highest density in SdC and lowest in Art. In all samples, presumptive staphylococci were around 3 log (min to max: 2.5–3.4 log) cfu/mL. The highest Enterobacteriaceae density was found in SdC milks (3.8 log cfu/mL), while yeasts mainly featured Del milks with densities closer to 3.5 log cfu/mL.

The discrepancy in microbiota composition between samples was further confirmed by 16S rRNA gene-seq data. Accounting for Shannon’s value entropy, the α-diversity differed between samples. Both Art and SdC reported the highest α-diversity values while Cur showed the lowest (Figure 1A). Del milks showed intermediate α-diversity with the absence of significant differences compared with others due to one out of its the 3 collected bulk milks that was featured by a more heterogenous microbiota. Besides, our samples can be allocated in 2 different groups distinguished by high (i.e., Art and SdC) and low Shannon’s values (i.e., Cur and Del).

The metataxonomics-based inspection (Figure 1B) was useful to reveal how both samples reporting high Shannon’s values (i.e., Art and SdC) were also featured by a similar and heterogeneous microbiota. However, the major difference explained by the 16S rRNA gene-based inspection involved Cur and Del bulk milks, because the former mainly accounted for a considerable abundance of Acinetobacter while the latter of Lactobacillus.

### Natural whey starters

Before to characterize the Caciocavallo cheese microbiota, we also examined the used NWSs. As observed in the related bulk milks, the highest density of TMA was found in SdC NWS while the lowest in Art (Tab. 2). In line with TMA, presumptive viable lactobacilli, lactococci, and enterococci were also at highest densities in SdC while at lowest in Art NWS. Both presumptive viable staphylococci and Enterobacteriaceae were lower than 1 log cfu/mL in all NWSs. Although based on different data, viable yeast densities were aligned with those found in bulk milks. In detail, Cur and Del NWSs had the highest yeast density whereas SdC displayed the lowest.

The 16S gene-seq profiling showed the absence of differences for Shannon’s index between NWSs (data not shown). At genus level, the major difference involved Lactobacillus and Streptococcus genera. While the former taxon featured Art, Cur, and Del NWSs almost for the totality of the microbiota, the latter was the most representative for SdC samples (Supp. Fig. S2).

### Caciocavallo cheeses

From each out of the 4 dairies being a part of the present study, Caciocavallo cheeses have been collected at different time points - i.e., 1, 30, and 60 d/s after the cheese-making – and the profiling also considered differences driven by different sites/levels of the cheese-wheel sampling (i.e., core, under-rind, and rind of the Caciocavallo cheese).

Tendencies in pH and TTA values almost overlapped in core, rind, and under-rind samples of the same cheese-wheel (Figure 2). In details, Cur core samples showed the highest pH values 1 d after cheese-making and these significantly differed ($P < 0.05$) compared with SdC and Art core samples (Figure 2A). After 30 d of ripening, a significant acidification was observed in Cur, Del and SdC core samples, whereas Art core

### Table 1. Viable bacterial counts (log cfu/mL) in bulk milks of four different Caciocavallo cheese-producing dairy companies (Art, SdC, Cur, Del). Bulk milks were samples on three consecutive days

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<th>Art</th>
<th>SdC</th>
<th>Cur</th>
<th>Del</th>
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<tr>
<td>total mesophilic aerobes</td>
<td>4.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.1 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>mesophilic lactic acid bacilli</td>
<td>3.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>thermophilic lactic acid bacilli</td>
<td>3.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>mesophilic lactic acid cocci</td>
<td>4.1 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>thermophilic lactic acid cocci</td>
<td>4.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>enterococci</td>
<td>1.0 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.4 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>staphylococci</td>
<td>2.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.4 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Enterobacteriaceae</td>
<td>2.9 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.8 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>yeasts</td>
<td>1.3 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.7 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.8 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a-d</sup> Within the same row, different superscript letters show a significant difference ($P < 0.05$; one-way ANOVA test).
samples underwent to a partial deacidification. At 1 and 30 d/s after cheese-making, values of TTA had an opposite trend than pH. At 60 d of ripening, all core samples showed intermediate pH values compared with those previously found, and these did not differ themselves. After cheese making (1 d), no differences were found between pH values of both under-rind (Figure 2B) and rind (Figure 2C) samples. Differences between these sites of cheese-wheel were mainly observed at 30 d of ripening when Art and Cur samples exhibited the highest pH values. However, further 30 d of ripening (i.e., 60 d) filled this gap between samples for both sites.

Figure 1. Boxplots showing the biodiversity measured as the Shannon index (panel A) and microbiota composition at genus level (panel B) in bulk milks from 4 different Apulian Caciocavallo cheese-producing dairy companies (i.e., Art, SdC, Cur, Del). Bulk milks were provided on the same day of the Caciocavallo cheese-making. In panel A, different letters indicate significant differences (P < 0.05; one-way ANOVA test).
Cur found unchanged at 1 (Figure 3A), 30 (Figure 3B) and samples – supported by variance distances – have been 60 d of ripening (Figure 3). In fact, differences between cheese microbiota under a dependent way during all <0.001) contribution of the dairy in shape the Caciocavallo microbiota β-diversity confirmed the significant (P supported for the ANOVA, cheese differentiation based allowed for the 

The 16S metataxonomic microbiota inspection allowed for the Caciocavallo cheese differentiation based on the making-company. Accounting for the ANOVA, the microbiota β-diversity confirmed the significant (P < 0.001) contribution of the dairy in shape the Caciocavallo cheese microbiota under a dependent way during all 60 d of ripening (Figure 3). In fact, differences between samples – supported by variance distances – have been found unchanged at 1 (Figure 3A), 30 (Figure 3B) and 60 d (Figure 3C). The sample plot pointed out how Cur and Del cheese microbiota almost overlapped, while Art and SdC samples significantly differed. This difference partially duplicated that suggested by Shannon’s index of the related bulk milks, which differentiated both Cur and Del samples to Art and SdC. No differences were found between core, under-rind, or rind microbiota of the same cheese, exception made for SdC cheese. In Art, Cur, and Del cheeses the different sites of the same cheese-wheel were close placed themselves into the multivariate plot and this scenario remained unvaried despite considering the 3 different ripening time points. At d 1, under rind samples from SdC were separated from both rind and core based on the first principal component that explains 97% of the variance. Based on variance evaluation, a similar microbiota depiction was also found at d 30 and 60.

By inspecting the microbiota at the genus level (Figure 4), Cur and Del samples exhibited Lactobacillus as dominant taxon accounting for a relative abundance higher than 90%. It should be mentioned that both Cur and Del reported the low richness in the related bulk milks, a condition that might have facilitated the colonization by lactobacilli (basonym Lactobacillus) of the related NWSs in both cases (Cur and Del). The few differences concerned their satellite microbiota. In Cur samples, Acinetobacter, which was the major colonizer of used Cur bulk milks, was also found in cheeses for all sampled times. In Del samples, instead, the satellite microbiota changed according with time because Aeromonas was found in samples after 1 d, Escherichia-Shigella and Acinetobacter were observed after 30 d, while no taxon exhibited a homogeneous detection among samples ripened for 60 d. In line with 16S based profiling of the NWSs, SdC samples reported the highest abundance of Streptococcus in cheeses while the Lactobacillus abundance varied according with the sampling time and the cheese level. The satellite microbiota of SdC samples was made up of Lactococcus and Acinetobacter after 1 d, Lactococcus, Acinetobacter and Staphylococcus after 30 d, and Acinetobacter and Leuconostoc, Weissella after 60 d. Intermediate relative abundances of Lactobacillus and Streptococcus featured the Art cheese microbiota, while Lactococcus was constantly detected during ripening in minor relative abundance.

### Dynamic microbial changes

Given the limited species differentiation provided by 16S rRNA gene sequencing in our samples, we conducted quantitative PCR (qPCR) to genotype lactic acid bacteria (LAB) subtaxa. This approach allowed us to assess the dynamic changes in LAB subpopulations within bulk milks, non-starter cultures (NWS), and the corresponding Caciocavallo cheese samples also considering differences at the beginning and conclusion of the 60-d cheese ripening time (Figure 5). In NWS, 4 different species did not provide amplification, i.e., L. acidophilus, Lc. rhamnosus, Ll. hilgardii, Lt. curvatus (Supp. Fig. S3).

In Art samples (Figure 5A), all species included in clusters A1-A3 were lactobacilli deriving from bulk milks, among which, those included in the cluster A3 reported lowest CNs in the related NWSs. Exception made for the subcluster A4.2 encompassing S. thermophilus and Leuconostoc spp., taxa that equally colonized the related bulk milks and NWSs, the microbial cluster A4 including various species of lactobacilli, P. pentosaceus and W. cibaria showing high CN in NWSs while very low in bulk milks. In cheeses, the clusters A1

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**Table 2.** Viable bacterial counts (log cfu/mL) in natural whey starter (NWS) of four different Caciocavallo cheese-producing dairies (Art, SdC, Cur, Del). NWSs were provided on the same day of Caciocavallo making.

<table>
<thead>
<tr>
<th></th>
<th>Art</th>
<th>SdC</th>
<th>Cur</th>
<th>Del</th>
</tr>
</thead>
<tbody>
<tr>
<td>total aerobic mesophilic</td>
<td>3.7 ± 0.1a</td>
<td>6.6 ± 0.2a</td>
<td>6.0 ± 0.2b</td>
<td>5.5 ± 0.3c</td>
</tr>
<tr>
<td>mesophilic lactic acid bacilli</td>
<td>3.7 ± 0.1b</td>
<td>6.3 ± 0.1a</td>
<td>4.7 ± 0.2b</td>
<td>4.7 ± 0.2a</td>
</tr>
<tr>
<td>thermophilic lactic acid bacilli</td>
<td>4.3 ± 0.2b</td>
<td>6.5 ± 0.2a</td>
<td>5.2 ± 0.1b</td>
<td>5.0 ± 0.1a</td>
</tr>
<tr>
<td>mesophilic lactic acid cocci</td>
<td>3.2 ± 0.2a</td>
<td>8.6 ± 0.2b</td>
<td>5.1 ± 0.1b</td>
<td>5.3 ± 0.2a</td>
</tr>
<tr>
<td>thermophilic lactic acid cocci</td>
<td>3.2 ± 0.1b</td>
<td>5.7 ± 0.1b</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
</tr>
<tr>
<td>enterococci</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
</tr>
<tr>
<td>staphylococci</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
<td>&lt;1a</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>3.2 ± 0.2e</td>
<td>1.8 ± 0.1c</td>
<td>4.0 ± 0.1b</td>
<td>4.1 ± 0.1c</td>
</tr>
<tr>
<td>yeasts</td>
<td>3.2 ± 0.2a</td>
<td>1.8 ± 0.1c</td>
<td>4.0 ± 0.1b</td>
<td>4.1 ± 0.1c</td>
</tr>
</tbody>
</table>

*Within the same row, different superscript letters indicate a significant difference (P < 0.05; one-way ANOVA test).*
and A2 did not report clear tendencies in colonizing specific sites. The microbial cluster A3 was detected starting from 1-d-samples and its CN increased in cheeses profiled at 60 d. *Lt. sakei*, which was purged out from the clustering, was detected at high CN in NWSs, rind and under rind sites at 1 d, and only in the under rind of the cheese-wheel ripened for 60 d. Species of the subcluster A4.1 reported unvaried CN from 1 d after the cheese-making until 60 d, whereas *S. thermophilus* and *Leuconostoc* spp. reported an equal detection after 1 d in all the 3 sites of the cheese-wheel while their CN were low in *Caciocavallo*-core microbiota at 60 d of ripening.

In *Cur* samples (Figure 5B), *Lt. sakei* and *Ls. reuteri* produced amplicons from the related NWSs; however, these species were purged out from the clustering due to their controversial detection in cheeses. Species included in the cluster B1 were found in NWSs while at lower CN in the related bulk milks and exhibited clear tendencies in colonizing rind and under rind sites of the cheese-wheel during 60 d of ripening. *L. acidophilus* showed similar CNs in both milks and NWSs whereas showed controversial tendencies in cheeses during ripening. The microbial cluster B2 – including 2 lactobacilli (*Ls. fermentum* and *Lp. plantarum*), *S. thermophilus* and *Leuconostoc* spp. – featured both bulk milks and NWSs reporting a predilection to colonize the cheese-wheel after 1 d from making. In fact, the B2-cluster CN decreased after 60 d. Grouping 5 species of lactobacilli, the cluster B3 displayed a high CN in bulk milks while low in NWSs and these lactobacilli persisted in cheeses from 1 d to 60 d of ripening.

Two microbial clusters (C1 and C2) were found processing *Del* samples (Figure 5C), whereas 4 LAB species (i.e., *P. pentosaceus*, *Ls. fermentum*, *L. acidophilus*, *Leuconostoc* spp.) were purged out from the clustering. The C1 cluster included those species showing low CN in bulk milks while being detected with high CN in NWSs. All species allocated in C1 were found in cheeses from 1 d until 60 d showing the highest CN.
in the under-rind sites 1 d after the cheese-making. The C2 cluster included LAB that originated from bulk milks, and, on average, these were found in all Caciocavallo samples during ripening with some and specific exception. In details, *Lc. rhamnosus, Lc. casei, and Ll. hilgardii* exhibited the highest CN in under-rind sites at both 1 d and 60 d after cheese-making. The opposite was provided by *W. cibaria* amplicons being lower in under-rind while higher in other sites than *Lc. rhamnosus, Lc. casei*, and *Ll. hilgardii*. The other C2 members (*Lt. curvatus, Ls. reuteri, L. delbruecki, and S. thermophilus*) did not provide specific evidence in discriminate sites or time of ripening.

In SdC samples (Figure 5D), LAB were grouped within 3 different clusters, i.e., D1, D2, and D3, based on their CN detection in NWSs, bulk milks, or both (bulk milk and NWSs), respectively. Based on the Caciocavallo ripening time, species of the cluster D1 can be further distinguished in those exhibiting a higher CN 1 d after cheese making (i.e., *P. pentosaceus, Lp. pentosus, S. thermophilus, Fl. sanfranciscensis, W. cibaria* and *Ls. fermentum*) than those reporting higher CNs at 60 d (i.e., *Lt. sakei, Lv. brevis, Lc. paracasei, Lc. buchneri* and *Lc. parabuchneri*).

**Dynamic volatile organic compound (VOC) changes**

In Caciocavallo-cheeses, dynamic volatile organic compound (VOC) changes were inspected by processing samples collected at different ripening times (i.e., 1, 30, and 60 d after cheese-making). The metabolite relative concentrations were normalized, and a clustering analysis was run. Variables (i.e., VOCs) resulted to be grouped in 6 different clusters (i.e., A-F; Figure 6), among which, VOCs of the clusters A (i.e., n-Propyl acetate, 2-Heptanone), B (i.e., Diacetyl, Hexanal), and C (i.e., Acetoin; 3-methyl-1-Butanol; Nonanal; 2-ethyl-1-Hexanol; Ethanol) were mostly representative for samples made 1 d before. At 1 d, acetoin was lowest in all Del samples, the same whose NWSs provided the lowest *S. thermophilus* CN (Suppl. Fig. S3). The cluster C and the cluster D grouped most of the secondary metabolites, including alcohols (3-methyl-1-Butanol, 2-ethyl-1-Hexanol, Ethanol, 2-Heptanal, 2-Butanol), aldehydes (Nonanal), lactones (delta-Dodecalactone), ketones (Acetoin, 2-Butanone) and organic acids (Acetate) deriving from lactic acid, residual bioavailable lactose, and free amino acid fermentation. As an example of the cluster C, ethanol was found in samples during the entire ripening time, from 1 to 60 d. The cluster-D metabolites, instead, mainly featured SdC samples at 30 d and Art samples at 60 d. The clusters E, including 2 esters and 1-Butanol, showed positive heats in few samples, was the most representative of
**DISCUSSION**

*Caciocavallo* cheese is one of the most typical Italian *pasta-filata* cheese and, within this niche of products, it has a great popularity (De Pasquale et al., 2014). As a fermented dairy food, cheese reports diverse to microbiological and sensorial quality features which are determined by multiple factors such as the milk sources, manufacturing process, biotechnological parameters – including the use of starters – and facility-specific and environmental microbiome (Gobbetti et al., 2018).

In an effort to elucidate the influence of microbiota from bulk milks and non-starter cultures (NWSs) on shaping microbial communities and the development of volatile off-flavors in cheese, we selected *Caciocavallo Pugliese* cheese as our model system. The cheese was produced according to a standardized protocol shared among 4 distinct Apulian dairy industries situated within the same geographical production area. The strategy of reducing distances between cheese-diaries was conditioned by previous evidence discussing how local tradi-

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**Figure 4.** Metataxonomics (16S rRNA gene sequencing) microbiota at genus level from 4 different Apulian *Caciocavallo* samples (*Art, SdC, Cur, Del*) profiled at different time points (i.e., 1, 30, and 60 d/s after making) and 3 different sites/levels of the cheese-wheel (i.e., core, under-rind, and rind).
tions influenced the cheese-making processes leading to a different cheese microbiota development (Uzun et al., 2020). Since no differences have been found for both pH and TTA between bulk milks specifically used to produce the analyzed Caciocavallo cheeses when grouped based on cheesemaking site, the here adopted strategy to limit the geographical location (Bonizzi et al., 2009) and seasonality (Celano et al., 2022a) allowed to reduce the organic acid variability between samples. In line with previous studies accounting for different milk sampling from a restricted geographical area (Fricker et al., 2011; Hahne et al., 2019), mesophilic aerobic bacteria, lactobacilli, cocci and thermophilic cocci were the most abundant cultivable bacteria found on bulk milks, especially in Art and SdC samples. As previous explained, a great attention has been paid to reduce inter-sample variability. However, various co-factors can affect milk microbiota, such as grazing animal feeding systems (Hagi et al., 2010), bedding (Murphy et al., 2019) and milking procedures (Du et al., 2020) and, probably, these may have had an impact on the viable microbiota community.

The 16S rRNA targeted metagenomics data were different and allow to allocate bulk milk samples in 2 different groups distinguished by high (i.e., Art and SdC) and low Shannon’s values (i.e., Cur and Del). Cheeses were screened at different time points (i.e., 1 and 60 d/s after the cheese-making) and considering different sites/levels of the cheese-wheel (i.e., core, C; under-rind, UR; and rind, R). The color-bar ranged between green (lowest Z-scores) to red (highest Z-scores). As shown in each panel, after setting the clustering threshold, variables (LAB species) that were purged out from the clustering were marked with red lines.

**Figure 5.** Based on copy number (CN) Z-scores, dynamic microbial evolution heatmap with clustering (Euclidean distance) of 20 different lactic acid bacteria (lactobacilli (n. 16), *Pediococcus pentosaceus*, *Weissella cibaria*, and *Streptococcus thermophilus*, and *Leuconostoc spp.*) found in bulk milks (M), natural whey starter (NWS), and cheeses provided by 4 different Apulian Caciocavallo cheese-producing dairy companies (Art, SdC, Cur, and Del; panel A, B, C, and D, respectively). Cheeses were screened at different time points (i.e., 1 and 60 d/s after the cheese-making) and considering different sites/levels of the cheese-wheel (i.e., core, C; under-rind, UR; and rind, R). The color-bar ranged between green (lowest Z-scores) to red (highest Z-scores). As shown in each panel, after setting the clustering threshold, variables (LAB species) that were purged out from the clustering were marked with red lines.
representative colonizer of Apulian milk core microbiota (Celano et al., 2022a). In Cur and Del bulk milks a considerable abundance of Acinetobacter and Lactobacillus was observed, respectively. This large difference might be explained by a different environmental contamination level by psychrotrophic bacteria before undergoing bulk tank storage (Hahne et al., 2019). Subsequently, both temperature and storage time can have greatly influenced the bulk milk microbiota composition favoring psychrotrophic bacterium growth, as supported by previous studies on Acinetobacter or Pseudomonas (Doyle et al., 2017; Parente et al., 2020). Although Lactobacillus also includes psychrotolerant strains (Von Neubeck et al., 2015), no attention has been paid to LAB due to their large incidence in milks and wide knowledge about their pretechnological activities during cheese making and ripening (Quigley et al., 2013). As observed in the related bulk milks, the highest viable lactobacilli, lactococci, and enterococci microbial density was found in SdC NWS while the lowest in Art. Viable staphylococci and Enterobacteriaceae were lower than 1 log cfu/mL in all NWSs as the result of the

Figure 6. Based on normalized relative concentrations (Z-scores), volatile organic compound (VOC) dynamic changes collected from Caciocavallo-cheeses produced by 4 different Apulian dairy companies (Art, Cur, Del, and SdC) and screened at different time points (1, 30, and 60 d/s after the cheese-making). The color-bar ranged between white (lowest Z-score) to blue (highest Z-score).
occurred acidification process (Da Silva Duarte et al., 2020). Although based on different data, viable yeast densities were aligned with those found in bulk milks. In detail, \textit{Cur} and \textit{Del} NWSs had the highest yeast density whereas \textit{SdC} displayed the lowest, a result that needs to be further evaluated due to the evidence about the role of yeasts in supporting typical flavor development in cheeses (Martini et al., 2021). The 16S rRNA gene-seq profile showed the absence of differences for Shannon’s index between NWSs. As also discussed by previous studies exploring difference between NWSs used for \textit{Caciocavallo} cheese making (Ercolini et al., 2008; Pogačić et al., 2013), the major difference here observed at the genus level involved \textit{Lactobacillus} and \textit{Streptococcus}. In fact, while the former taxon was the most representative genus for \textit{Art}, \textit{Cur}, and \textit{Del} NWSs, \textit{Streptococcus} was found as the major colonizer of \textit{SdC} samples.

Due to the low differentiation between species supported by 16S rDNA gene-seq in our samples, we carried out also a qPCR to genotyping LAB subtaxa in NWSs and to evaluate their dynamic changes during ripening. Overall, the qPCR analysis pointed out how NWSs harbored various LAB species, such as \textit{S. thermophilus}, \textit{Lc. paracasei}, \textit{L. sakei}, \textit{Ll. buchneri}, \textit{Ll. parabuchneri}, \textit{Fl. sanfranciscensis}, \textit{Lv. brevis}, and \textit{P. pentosaceus}. These results are in line with the study conducted by Ercolini et al. (Ercolini et al., 2008) that investigated the microbiota of NWSs used for the \textit{Caciocavallo Silano} PDO cheese production. Due to the detection of some species-specific LAB discriminating NWSs, authors concluded that microbiota diversity was independent from the geographical origin. Various SLAB, e.g., \textit{L. acidophilus}, \textit{Lc. casei}, \textit{Lc. paracasei}, \textit{Lc. rhamnosus}, \textit{Lt. curvatus}, \textit{Lp. pentosus}, \textit{Ls. reuteri}, \textit{Ls. fermentum}, \textit{Ll. buchneri}, \textit{Lt. sakei}, and \textit{Lv. brevis} specie can be indentied as the most frequent NWS colonizers since all these species demonstrated their ability to adapt to adverse abiotic factors such as nutrient scarcity, low pH, and fairly high temperatures during manufacturing (Quigley et al., 2013).

From each out of the 4 dairies plants, \textit{Caciocavallo} cheeses have been collected at different time points –1, 30, and 60 d/s after the cheese-making – and the profiling also considered differences driven by different sites of the cheese-block (core, under-rind, and rind of the \textit{Caciocavallo} cheese). The pH and TTA values in the 3 fractions were similar within the same product. The partial decadification observed in \textit{Art} and \textit{Cur} cheeses during the first 30 d of ripening than their produced at \textit{Del} and \textit{SdC} may be due to an accumulating microbial density from milk storage or during the manufacturing process and for microbial interactions (Calasso et al., 2016). At the end of ripening all cheeses showed similar values for all sampled fractions. Comparing \textit{Caciocavallo} from the same ripening time, samples across the 4 facilities showed relatively higher microbiota \( \beta \)-diversity \((P < 0.001)\) and, as the PCA has pointed out, no changes throughout the ripening process were assessed. Interestingly, according to Shannon’s index of the related bulk milks, very low bacterial diversity was determined between cheeses produced in the \textit{Cur} and \textit{Del} dairy plants compared with \textit{Art} and \textit{SdC} samples. Lactic acid-producing bacteria belonging to the genera \textit{Lactobacillus}, \textit{Streptococcus} and \textit{Lactococcus} were found in \textit{Caciocavallo} samples. These genera play knowing important roles in flavor formation and nutrient composition by using carbohydrates, especially lactose, proteins, fats, and peptides, originating short peptides, free amino acids, SCFAs and aromatic compounds. As expected, bacterial diversity detected at the genus level was found as a function of the site of production or the ripening process (Viljoen et al., 2003; Rea et al., 2007). \textit{Cur} and \textit{Del} samples which showed the low richness in the related bulk milks, exhibited \textit{Lactobacillus} as dominant taxon. In turn, the low richness of the dairy bulk milk might have facilitated the colonization by lactobacilli (basonym \textit{Lactobacillus}) in the related NWS that were used to acidify the curd. Under the conditions of the dairy industries of this study, we can speculate that the capacity of curd contamination by \textit{Lactobacillus} resident in milk and, especially, in NWS was markedly higher than that highlighted for \textit{Streptococcus}. As previously shown, \textit{Streptococcus} strains were usually detected during the manufacture of traditional \textit{Caciocavallo} type cheese (Calasso et al., 2016). In line with 16S based profiling of the NWSs, \textit{SdC} samples reported the highest abundance of \textit{Streptococcus} with ripening-time and spatial variations. \textit{Streptococcus} genus was also found in \textit{Caciocavallo} cheese produced at \textit{Art} facility, although it was not detected in the related NWS. As previously shown, the colonization may be due to commercial starter cultures that are used to produce other varieties of cheeses in the dairy plant and persistent in the equipment (Settanni et al., 2012). This species plays pivotal roles during both curd forming and cheese ripening, and its persistence in cheeses can be influenced by biotic factors. In fact, was previously demonstrated how \textit{S. thermophilus} synergistically interacts with \textit{L. delbrueckii} ssp. \textit{bulgaricus} (Calasso et al., 2016), both species originating from milks as we assessed by qPCR analysis.

\textit{Lactococcus} ssp., traditionally utilized in the crafting of mesophilic lactic starter cultures for \textit{Caciocavallo Pugliese} cheese production (Calasso et al., 2016), consistently appeared in both \textit{Art} and \textit{SdC} samples, despite its regularly detectable presence as reported in earlier studies (De Pasquale et al., 2014). Among
LAB, *Lactococcus* has been extensively studied for its role in the citrate to diacetyl/acetoin pathway (Lo et al., 2018), implicated in the desirable development of a butyryl aroma in cheeses (Curioni and Bosset, 2002). In accordance with this observation, *Del* samples, in which we detected the lowest levels of *Lactococcus* and *S. thermophilus*, exhibited the lowest acetoin concentration after 1 d of ripening. By contrast, *Cur* samples also exhibited a low abundance of *Lactococcus* and *S. thermophilus* despite a discrete relative concentration of diacetyl and acetoin was assessed in cheeses until 30 d of ripening. With this respect, it should be also considered the concentration of the precursor citrate in the related milk that, as previously demonstrated, can varied according to different factors including the animal feeding (Dunshea et al., 2019). Moreover, Oliveira and coworkers (Oliveira et al., 2012) also suggested that differences in acetoin concentration in dairy products can also be attributed to interactions between *S. thermophilus* and other SLAB influencing the expression of α-acetolactate synthase by *S. thermophilus* and other SLAB, which we detected the lowest levels of *Lactococcus* and *Lactobacillus* during ripening. These findings suggest the existence of a core microbiota that has adapted to the cheese surface (Irlinger et al., 2015). Considering the distribution of microbial communities between the rind and core of several cheeses (O'Sullivan et al., 2015), in our study, exception made for *SdC Caciocavallo* samples, no significant differences in the microbial populations present in the respective cores, under-rind and rinds were detected being comparable through ripening. These findings suggest the existence of a core microbiota that has adapted to the cheese surface (Irlinger et al., 2015). Considering the distribution of microbial communities between the rind, under-rind and core, we can suppose a role of the abiotic characteristics of the product, including pH, in *SdC* cheese samples (Sheehan et al., 2009). In line with previous studies showing how Gram-positive LAB can be distributed in the core more than onto the cheese surface (O'Sullivan et al., 2015), in *SdC* samples we observed higher abundance of *Lactobacillus* in the core than in the under-rind and rind throughout ripening, probably due to their preference for a micro-anaerobic environment. By contrast, streptococci, present in the core, under-rind and rind throughout ripening in both *SdC* and *Art* cheeses, were found at higher percentages in the rind probably due to
the oxygen concentration present at or near the surface of the cheese in contrast to the more anaerobic core (Monfredini et al., 2012).

CONCLUSIONS

Microbial biodiversity of milk is considerably higher than its derivatives and it resulted mainly guided by the native microbiota present in the NWS during the cheese manufacturing process and ripening. Moreover, although cheeses were produced with an accurately standardized protocol, the distinct clustering of samples based on microbiota biodiversity provided evidence on how cheese-specific features were influenced by means of milk and NWS differences as well as intrinsic conditions unique to each production site. Therefore, the holistic vision of all these components has demonstrated how the resulting microbiota led to the cheese-specific sensory characteristics during ripening. In fact, it was also noted that although there was a predominance of the same major VOCs among samples, minor compounds, crucial for the sample characterization and differentiation, differed. Even spatial variations between the core, under-rind, and rind of the cheese-blocks cannot be a generalized concept since the abundance and taxonomy of microorganisms reflected the combination of different factors, including milk colonizers and NWSs, as well as biotic and abiotic variables featuring the cheese-dairy environment, which are not predictable a priori and standardizable. In light of these considerations, further studies employing metatranscriptomics are necessary to verify the effect of the dominant and subdominant microbiota of raw milk on the properties of cheeses while studying the taxon activity according to different parts of the same cheese-block.

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Conflict of interest Authors declare no conflict of interest.

REFERENCES


Da Silva Duarte, V., M. Carlot, S. Pakroo, A. Tarrah, A. Lombardi, H. Santiago, V. Corich, and A. Giaromini. 2020. Comparative evaluation of cheese whey microbial composition from four Italian cheese factories by viable counts and 16S rRNA gene amplicon sequen-
Vacca et al.: Dynamic microbial and metabolic...


