J. Dairy Sci. 107:6541–6557
https://doi.org/10.3168/jds.2023-24049

Dynamic microbial and metabolic changes during Apulian Caciocavallo cheesemaking and ripening produced according to a standardized protocol

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ABSTRACT

The microbiota of a cheese play a critical role in influencing its sensory and physicochemical properties. In this study, traditional Apulian Caciocavallo cheeses coming from 4 different dairies in the same area and produced following standardized procedures were examined, as well as the different bulk milks and natural whey starter (NWS) 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 were carried out after salting and throughout the ripening time (2 mo) to investigate major shifts in the succession of the microbiota and flavor development. Culture-dependent and 16S rRNA metataxonomics results clearly clustered samples based on microbiota biodiversity related to the production dairy plant as a result of the use of different NWS or the intrinsic conditions of each production site. At the beginning of the ripening, cheeses were dominated by *Lactobacillus*, and in 2 dairies (Art and SdC), *Streptococcus* genera were associated with the NWS. The analysis allowed us to show that although the diversity of identified genera did not change significantly between the rind, underrind, 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 real-time PCR, also known as quantitative or qPCR, mainly differentiated the temporal adaptation of those species originating from bulk milks and those provided by NWS. The primary starters detected in NWS and cheeses contributed to 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 volatile free fatty acids during ripening, whereas cheeses displaying low abundances of *Streptococcus* and *Lactococcus* (dairy Del) had a lower total concentration of acetoin compared with Art and SdC. However, the subdominant strains and nonstarter lactic acid bacteria 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 only considering a delimited production area.

Key words: Caciocavallo cheese, bulk milk, natural whey starter, lactic acid bacteria, cheese volatilomics

INTRODUCTION

The microbial contribution to flavor is mainly determined by differences in protocols adopted during cheesemaking (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 subject 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 of inspecting microbial variations determined by manufacturing and ripening (Caridi et al., 2003; Korena et al., 2023). With the increasing advent of high-throughput sequencing analysis, investigations of the dairy microbiota have moved toward interest in defining and profiling 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 the same food.

Caciocavallo is a very popular typical pasta filata cheese manufactured from raw cow milk using natural

Received August 4, 2023.
Accepted March 12, 2024.
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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

whey or commercial thermophilic and mesophilic (e.g., *Lactococcus 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 semi-soft cheese varieties consumed typically. The term *pasta filata* refers to a process that consists in curd plasticization and stretching starting from whole milks (McMahon and Oberg, 2017). To obtain curd, protease activity is mainly provided by the addition of rennet. Also, during cheesemaking preliminarily phases, it is possible to add living microorganisms in form of natural whey starters (**NWS**) 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 NWS, 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 cheesemaking 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 2 mo-ripened Apulian Caciocavallo cheeses made following standardized procedures, as well as the related bulk milks and NWS, which were provided by 4 different dairies placed within a restricted area.

MATERIALS AND METHODS

Caciocavallo Production Process

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

Sample Collection and Processing

On the same day of production, aliquots of the milks and NWS used to produce the Caciocavallo samples were collected. The production was repeated 3 times to obtain biological replicates from each cheese factory. Samples of the same Caciocavallo batch were also collected at 1 d, 30 d, and 60 d of ripening. As previously detailed (Calasso et al., 2016), cheese samples were immediately processed to obtain aliquots from core, under-rind, and rind layers of the cheese (Supplemental Figure S1C). In details, an aliquot equal to 100 cm^2 was sampled from rind and under-rind 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., under-rind) was obtained by an additional scraping (<1 mm) process. Finally, 10 g of Caciocavallo core sites were collected. The samples were then immediately frozen at −20°C before further processing.

pH and Total Titratable Acidity Profiling

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

The total titratable acidity (**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 of 5 g from each bulk milk and NWS were mixed with 45 mL of physiological sterile saline solution (NaCl 0.9 g/L) in bags with 250-µm filters 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 purchased from Oxoid Ltd. (Basingstoke, Hampshire, England) according to UNI EN ISO 4833-1:2013 (ISO, 2013). Presumptive mesophilic and thermophilic lactic acid bacilli were enumerated on de Man-Rogosa-Sharpe 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 to 48 h (Calasso et al., 2016; Minervini et al., 2017). Submerged or surface compact or feathery, small, opaque, and white colonies were counted, and randomly representative colonies were used for Gram, catalase, and motility tests. 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) were counted according with manufacturer's instructions (Oxoid Ltd.). Randomly representative colonies were used for Gram, catalase, and motility tests. *Staphylococcus* coagulase-positive strains and *Enterobacteriaceae* were quantified after enrichment according to the official methods, ISO 6888-1:2021 (ISO, 2021) and ISO 21528-2:2017 (ISO, 2017), respectively. Enumeration of yeasts was assessed by means of medium wort agar (Oxoid Ltd.) after incubation of the plates for 5 d at 25°C. Colony morphology was evaluated (color, shape, and size).

DNA Extraction and 16S rRNA Gene Sequencing

Aliquots (10 g) of milk, NWS, and cheeses were homogenized in 1:10 ratios with physiological sterile saline solution for 5 min, centrifuged (1,000 × *g*, 5 min, 4°C), and supernatants were then recovered to carry out an additional centrifugation step (5,000 × *g*, 15 min, 4°C). Pellets were resuspended in 0.5 mL of a physiological sterile saline solution to extract the total DNA by using the FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to the manufacturer's instructions (Calasso et al., 2016). The quality and concentration of total extracted DNA were evaluated spectrophotometrically (NanoDrop ND-1000, Thermo Fisher Scientific Inc.). The metagenomic analyses were carried out at Genomix4life (a spin-off of the University of Salerno, Fisciano, Italy) by using the Illumina MiSeq platform. In detail, primers 28F (forward: 5′-GAGTTTGATCNTGGCTCAG-3′) and 388R (reverse: TGCTGCCTCCCGTAGGAGT) were used to amplify the V1 to V3 hypervariable regions of the 16S rRNA gene and to analyze the diversity inside the bacteria domain. The 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 the16S rRNA amplification and library preparation were added without including samples. Libraries were quantified using a Qubit fluorometer (Invitrogen Co., Carlsbad, CA) and pooled, including the Phix Control Library (Illumina Inc.), to an equimolar amount (4 n*M* 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 (version 2020.8; https://view.qiime2.org) microbiome platform. Paired demultiplexed 16S rRNA sequences of amplicons were denoised by using the q2-deblur QIIME plugin (https://github.com/qiime2/q2-deblur). Taxonomy was inferred by using the QIIME-compatible database Silva v.138 SSU. The α-diversity metrics of Shannon's entropy were also computed by using the QIIME2 platform (Chao and Bunge, 2002).

Real-Time PCR

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., *Fructilactobacillus* (*Fl.*) *sanfranciscensis*, *Lactobacillus* (*L.*) *acidophilus*, *L. delbrueckii* ssp. *bulgaricus*, *Lacticaseibacillus* (*Lc.*) *casei*, *Lc. paracasei*, *Lc. rhamnosus*, *Lentilactobacillus* (*Ll.*) *hilgardii*, *Lentilactobacillus* (*Ll.*) *buchneri*, *Ll. parabuchneri*, *Lactiplantibacillus* (*Lp.*) *plantarum*, *Lp. pentosus*, *Limosilactobacillus* (*Ls.*) *fermentum*, *Ls. reuteri*, *Latilactobacillus* (*Lt.*) *curvatus*, *Lt. sakei*, *Levilactobacillus* (*Lv.*) *brevis* on collected samples of the bulk milks, NWS, and the core, under-rind, and rind of the 4 different Apulian Caciocavallo cheeses at the beginning (d 1) and at the end (d 60) of the ripening. To better inspect the taxa profile, 3 further species of lactic acid bacteria, i.e., *Weissella* (*W.*) *cibaria*, *Pediococcus* (*P.*) *pentosaceus*, *Streptococcus* (*S.*) *thermophilus*, as well as the *Leuconostoc* genus, were also included for the qPCR-based investigation. The primer specificity was verified by PCR on different strains of the same targeted lactic acid bacteria (**LAB**) species by sub-

sequent sequencing and checking in BLAST (v. 2.15.0, 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 (Supplemental Table S1, see Notes). The qPCR reactions were carried out using an Applied Biosystems 7300 Real-Time PCR System. According with optimal dilutions of DNA samples chosen to ensure the absence of PCR inhibitors, the total reaction mix (25 µL) contained 12.5 µL of SYBR Green Mix (#1725271, Bio-Rad Laboratories S.r.l., Milano, Italy), 0.1 µL of 0.2 µ*M* primer, 11.4 µL of DNase- and RNase-free water (Qiagen S.r.l., Milano, Italy), and 1 µL of template (40 ng). Each reaction was performed in duplicate. The amplification setup accounted for 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 5 s, appropriate annealing temperature (see Supplemental Table S1) for 30 s, and a single phase at 72°C for 35 s. The PCR amplicon melting curve analysis started at a temperature of 60°C and increased by 1°C every 5 s until 95°C (Kwok et al., 2014). For species quantification, a standard curve was built by using the related reference strain. The qPCR was performed with serially diluted bacterial genomic DNA extracted from pure cell cultures, and the respective gene copy number (**CopyN**) was calculated. The CopyN and the logarithms considered the DNA concentration and amplicon length, whereas the standard curve was obtained by interpolating the cycle threshold and the logarithm of the DNA copy number (CopyNLog).

Cheese Volatile Organic Compound Profiling

Cheese volatilome was obtained according with previously conducted procedures (Celano et al., 2022b). In detail, 4-g aliquots from each Caciocavallo cheese (as a whole) were obtained by grating and placed in 20-mL glass vials, and 10 µL of internal standard solution (2-octanol) corresponding to 10 mg/kg was added. Vials were sealed with polytetrafluoroethylene-coated silicone rubber septa (20 mm diameter; Supelco, Bellefonte, PA). 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 divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco) 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 volatile organic compounds (**VOC**) were desorbed in splitless mode (3 min at 220°C) and analyzed through a Clarus 680 (Perkin Elmer) GC system equipped with a capillary Rtx-Wax column (30 m × 0.25 mm i.d., 0.25-µm film thickness; Restek, Bellefonte, PA). 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 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 interval of 34 to 350. Each chromatogram was analyzed for peak identification using the National Institute of Standard and Technology 2008 library (NIST, 2019). 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 were expressed as mean ± SD or percentage, as appropriate. Continuous variables were subjected to one-way ANOVA. For metataxonomics (16S rRNA) data, sample β-diversity was inspected by principal coordinates analysis considering the Bray-Curtis index as distance method, and the comparisons accounted for permutational ANOVA analysis. For the afore-mentioned sample comparisons, significant differences were explained by $P < 0.05$. In the clustering heatmap visualization, CopyN 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. In addition, normalized data of VOC 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, CA).

RESULTS

Bulk Milks

Bulk milks were collected from dairies on 3 consecutive days and corresponded to the milks that were specifically used to produce the Caciocavallo cheeses we analyzed. Before profiling the microbiota, the bulk milks were analyzed for pH and TTA, and the values ranged between 6.6 and 6.7 (± 0.05) and between 2 and 2.5 (± 0.2) mL of 0.1 *M* NaOH, respectively. The bulk milk viable microbiota were then profiled (Table 1). The total mesophilic aerobes (**TMA**) ranged between 5.4 and 4.1 log cfu/mL, observed in the SdC and Art samples, respectively. The comparison of SdC against Art achieved the significance threshold ($P < 0.05$), whereas Cur and Del samples exhibited intermediate TMA densities. Presump-

Table 1. Viable bacterial counts (log cfu/mL ± SD) in bulk milks of 4 different Caciocavallo cheese-producing dairy companies (Art, SdC, Cur, and Del); bulk milks were sampled on 3 consecutive days

Item	Art	SdC	Cur	Del
Total mesophilic aerobes	4.1 ± 0.1^{c}	5.4 ± 0.2^{a}	4.9 ± 0.2^{b}	5.1 ± 0.2^{ab}
Mesophilic lactic acid bacilli	3.2 ± 0.1^{c}	4.4 ± 0.1^{a}	3.8 ± 0.1^{b}	4.4 ± 0.1^{a}
Thermophilic lactic acid bacilli	3.2 ± 0.2^{c}	4.4 ± 0.1^{a}	3.6 ± 0.1^{b}	4.6 ± 0.1^{a}
Mesophilic lactic acid cocci	4.1 ± 0.1^{b}	5.3 ± 0.1^{a}	4.2 ± 0.2^{b}	5.5 ± 0.2^{a}
Thermophilic lactic acid cocci	4.3 ± 0.1^{b}	6.1 ± 0.3^{a}	4.6 ± 0.2^{b}	4.9 ± 0.1^{a}
Enterococci	1.0 ± 0.1^{d}	3.4 ± 0.2^{b}	2.8 ± 0.1^{c}	4.9 ± 0.2^{a}
Staphylococci	2.5 ± 0.1^{c}	3.3 ± 0.1^{a}	3.4 ± 0.1^{a}	3.0 ± 0.1^{b}
Enterobacteriaceae	2.9 ± 0.2^{c}	3.8 ± 0.1^{a}	3.5 ± 0.2^{b}	3.3 ± 0.3^{bc}
Yeasts	1.3 ± 0.2^{d}	1.7 ± 0.1^{c}	2.8 ± 0.1^{b}	3.5 ± 0.1^{a}

[a–d]Within the same row, different superscript letters show a significant difference ($P < 0.05$; one-way ANOVA test).

tive viable lactobacilli, lactic acid cocci, and enterococci densities reported similar ratios to those explained by TMA counts, as 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 cfu/mL (minimum to maximum: 2.5–3.4 log cfu/mL). The highest *Enterobacteriaceae* density was found in SdC milks (3.8 log cfu/mL), whereas 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 sequencing data. Accounting for Shannon's value entropy, the α-diversity differed between samples. Both Art and SdC reported the highest α-diversity values, whereas Cur had the lowest (Figure 1A). The Del milks showed intermediate α-diversity and an absence of significant differences compared with others because 1 out of its 3 collected bulk milks featured more heterogeneous microbiota. In addition, our samples could 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 in revealing how both samples reporting high Shannon's values (i.e., Art and SdC) also featured 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*, whereas the latter had a considerable abundance of *Lactobacillus*.

Natural Whey Starters

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

The 16S gene sequencing profiling showed the absence of differences for Shannon's index between NWS (data not shown). At genus level, the major difference involved *Lactobacillus* and *Streptococcus* genera. Although the former taxon featured Art, Cur, and Del NWS almost for the totality of the microbiota, the latter was the most representative for SdC samples (Supplemental Figure S2, see Notes).

Caciocavallo Cheeses

From each out of the 4 dairies that were part of the present study, Caciocavallo cheeses were collected at different time points (i.e., 1, 30, and 60 d after the cheesemaking) and the profiling also considered differences driven by different sites and 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 detail, Cur core samples showed the highest pH values 1 d after cheesemaking, 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 samples underwent a partial deacidification. At 1 and 30 d after cheesemaking, 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 values did not differ. After cheesemaking (1 d), no differences were found between the pH values of the under-rind (Figure 2B) and rind (Figure 2C) samples. Differences between these cheese wheel sites were mainly observed at 30 d of ripening, when Art and Cur samples exhibited

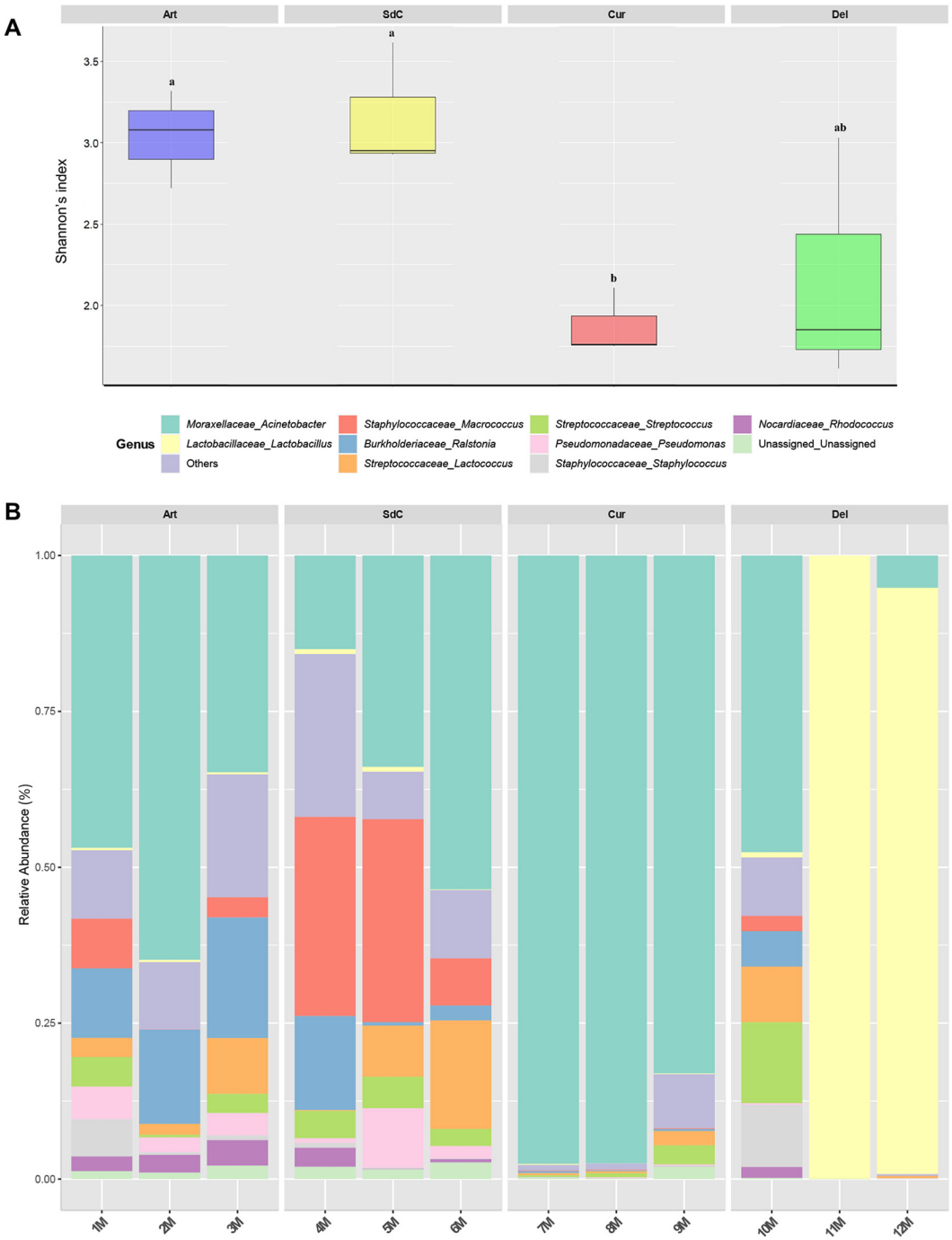

Figure 1. Box plots showing the biodiversity measured as the Shannon index (panel A) and microbiota composition at the genus level (panel B) in bulk milks from 4 different Apulian Caciocavallo cheese-producing dairy companies (i.e., Art, SdC, Cur, and Del). Bulk milks were provided on the same day of the Caciocavallo cheesemaking. In panel A, different letters indicate significant differences ($P < 0.05$; one-way ANOVA test). The lower edge of the box represents the 25th percentile, the upper edge represents the 75th percentile, and the whiskers represent the 5th and 95th percentiles. The median is represented by a black midline within the box.

the highest pH values. However, an additional 30 d of ripening (i.e., 60 d total) filled this gap between samples for both sites.

The 16S metataxonomic microbiota inspection allowed for 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 shaping the Caciocavallo cheese microbiota in a dependent way during all 60 d of ripening (Figure 3). In fact, differences between

Table 2. Viable bacterial counts (log cfu/mL ± SD) in the natural whey starter (NWS) of 4 different Caciocavallo cheese-producing dairies (Art, SdC, Cur, and Del); NWS were provided on the same day of Caciocavallo making

Item	Art	SdC	Cur	Del
Total aerobic mesophilic	3.7 ± 0.1^{d}	6.6 ± 0.2^{a}	6.0 ± 0.2^{b}	5.5 ± 0.3^{c}
Mesophilic lactic acid bacilli	3.7 ± 0.1^{c}	6.3 ± 0.1^{a}	4.7 ± 0.2^{b}	4.7 ± 0.2^{b}
Thermophilic lactic acid bacilli	4.3 ± 0.2^{c}	6.5 ± 0.2^{a}	5.2 ± 0.1^{b}	5.0 ± 0.1^{b}
Mesophilic lactic acid cocci	3.2 ± 0.2^{d}	8.5 ± 0.1^{a}	4.9 ± 0.1^{c}	5.6 ± 0.2^{b}
Thermophilic lactic acid cocci	3.2 ± 0.1^{c}	8.6 ± 0.2^{a}	5.1 ± 0.1^{b}	5.3 ± 0.2^{b}
Enterococci	$<1^{c}$	5.7 ± 0.1^{a}	$<1^{c}$	1.0 ± 0.1^{b}
Staphylococci	$<1^{a}$	$<1^{a}$	$<1^{a}$	$<1^{a}$
Enterobacteriaceae	$<1^{a}$	$<1^{a}$	$<1^{a}$	$<1^{a}$
Yeasts	3.2 ± 0.2^{b}	1.8 ± 0.1^{c}	4.0 ± 0.1^{a}	4.1 ± 0.1^{a}

[a–d]Within the same row, different superscript letters indicate a significant difference ($P < 0.05$; one-way ANOVA test).

samples (supported by variance distances) were 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, whereas the Art and SdC samples significantly differed. This difference partially duplicated that suggested by the Shannon's index of the related bulk milks, which differentiated both Cur and Del samples from Art and SdC. No differences were found between core, under-rind, or rind microbiota of the same cheese, except for SdC cheese. In Art, Cur, and Del cheeses, the different sites of the same cheese wheel were closely placed in 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 the rind and core based on the first principal component, which 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 the 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 colonization by lactobacilli (basonym *Lactobacillus*) of the related NWS 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 at all sampled times. In Del samples, instead, the satellite microbiota changed according with time; *Aeromonas* was found in samples after 1 d, *Escherichia-Shigella* and *Acinetobacter* were observed after 30 d, and no taxon exhibited homogeneous detection among samples ripened for 60 d. In line with 16S based profiling of the NWS, SdC samples reported the highest abundance of *Streptococcus* in cheeses, whereas 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*, *Leuconostoc*, and *Weissella* after 60 d. Intermediate relative abundances of *Lactobacillus* and *Streptococcus* featured the Art cheese microbiota, whereas *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 qPCR to genotype LAB subtaxa. This approach allowed us to assess the dynamic changes in LAB subpopulations within bulk milks, 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 (*L. acidophilus*, *Lc. rhamnosus*, *Ll. hilgardii*, and *Lt. curvatus*) did not provide amplification (Supplemental Figure S3, see Notes).

In the Art samples (Figure 5A), all species included in clusters A1 to A3 were lactobacilli deriving from bulk milks, among which those included in cluster A3 reported the lowest CopyN in the related NWS. An exception was in subcluster A4.2, encompassing *S. thermophilus* and *Leuconostoc* spp., taxa that equally colonized the related bulk milks and NWS; the microbial cluster A4 included various species of lactobacilli, and *P. pentosaceus* and *W. cibaria* exhibited high CopyN in NWS and very low CopyN in bulk milks. In cheeses, clusters A1 and A2 did not show clear tendencies in colonizing specific sites. The microbial cluster A3 was detected starting in d 1 samples, and its CopyN increased in cheeses profiled at 60 d. *Latilactobacillus sakei*, which was purged out from the clustering, was detected at a high CopyN in NWS, rind, and under-rind sites at 1 d, and only in the under-rind of the cheese wheel ripened for 60 d. Species of subcluster A4.1 reported unvaried CopyN from 1 d after the cheesemaking until 60 d, whereas *S. thermophilus*

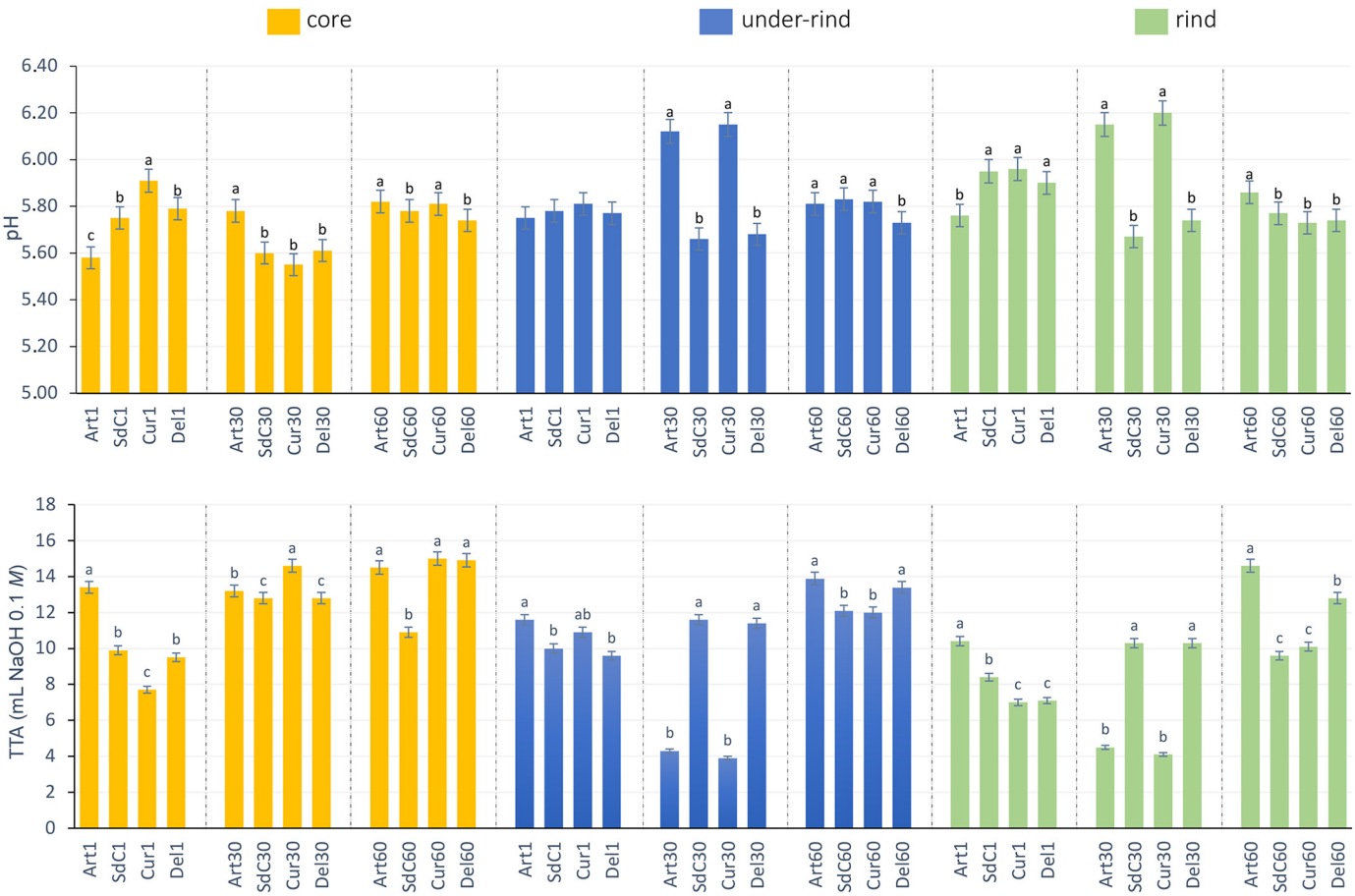

Figure 2. Values of pH and total titratable acidity (TTA) featuring 4 different Apulian Caciocavallo samples (i.e., Art, SdC, Cur, and Del) respectively profiled at different time points (i.e., 1, 30, and 60 d after cheesemaking) and 3 different sites in the cheese wheel (i.e., core, under-rind, and rind). Error bars show the SD. Within each panel, different letters above bars indicate a significant difference ($P < 0.05$).

and *Leuconostoc* spp. reported an equal detection after 1 d in all 3 sites of the cheese wheel, and their CopyN 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 NWS; however, these species were purged out from the clustering due to their controversial detection in cheeses. Species included in cluster B1 were found in NWS, but at lower CopyN in the related bulk milks, and they exhibited clear tendencies in colonizing rind and under-rind sites of the cheese wheel during 60 d of ripening. *Lactobacillus acidophilus* showed similar CopyN in both milks and NWS, but it showed controversial tendencies in cheeses during ripening. Microbial cluster B2, including 2 lactobacilli (*Ls. fermentum* and *Lp. plantarum*), along with *S. thermophilus* and *Leuconostoc* spp., featured in both bulk milks and NWS, reporting a predilection to colonize the cheese wheel 1 d after making. In fact, B2 cluster CopyN decreased after 60 d. Grouping 5 species of lactobacilli, cluster B3 displayed a high CopyN in bulk milks and a low CopyN in NWS, and these lactobacilli persisted in cheeses from 1 d to 60 d of ripening.

Two microbial clusters (C1 and C2) were found when processing Del samples (Figure 5C), whereas 4 LAB species (i.e., *P. pentosaceus*, *Ls. fermentum*, *L. acidophilus*, and *Leuconostoc* spp.) were purged out from the clustering. The C1 cluster included those species showing low CopyN in bulk milks but detected with high CopyN in NWS. All species allocated in C1 were found in cheeses from 1 d until 60 d showing the highest CopyN in the under-rind sites 1 d after the cheesemaking. 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 exceptions. In detail, *Lc. rhamnosus*, *Lc. casei*, and *Ll. hilgardii* exhibited the highest CopyN in under-rind sites at both 1 d and 60 d after cheesemaking. The opposite was true for *W. cibaria* amplicons, which were lower in the under-rind and higher in other sites than *Lc. rhamnosus*, *Lc. casei*, and *Ll. hilgardii*. The other C2 members (*Lt. curvatus*,

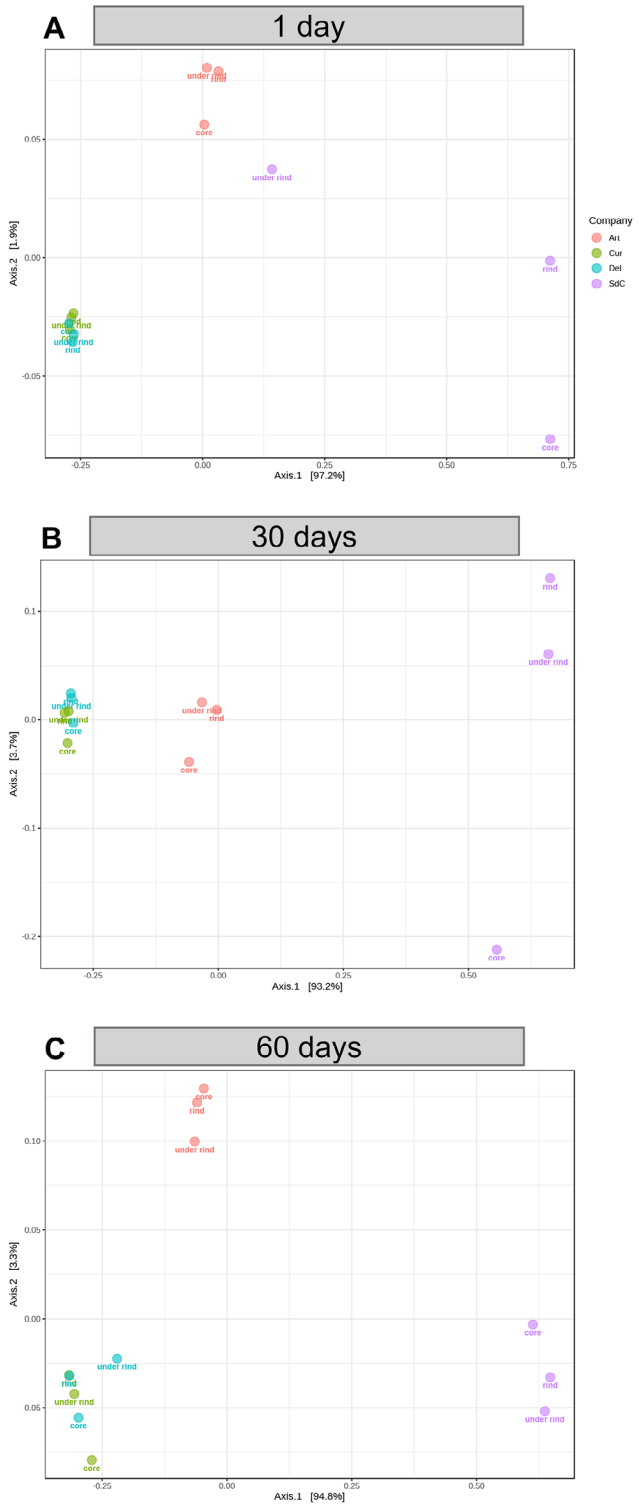

Figure 3. β-Diversity analysis (permutational ANOVA) at feature level (i.e., amplicon sequence variants) of Caciocavallo cheese microbiota found in samples delivered at different times of ripening (1, 30, and 60 d) from 4 different Apulian cheese-producing dairies (i.e., Art, SdC, Cur, and Del). Differences in microbiota divided by 3 different sites in the cheese wheel (i.e., core, under-rind, and rind) profiling were also considered. For all 3 plots (A, B, and C), significance (*P*-value) was lower than 0.001.

Ls. reuteri, *L. delbruecki*, and *S. thermophilus*) did not provide specific evidence in discriminating 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 CopyN detection in NWS, bulk milks, or both, respectively. Based on the Caciocavallo ripening time, species of the cluster D1 can be further distinguished in those exhibiting a higher CopyN 1 d after cheesemaking (i.e., *P. pentosaceus*, *Lp. pentosus*, *S. thermophilus*, *Fl. sanfranciscensis*, *W. cibaria*, and *Ls. fermentum*) than those reporting higher CopyN at 60 d (i.e., *Lt. sakei*, *Lv. brevis*, *Lc. paracasei*, *Lc. buchneri*, and *Lc. parabuchneri*).

Dynamic VOC Changes

In Caciocavallo cheeses, dynamic VOC changes were inspected by processing samples collected at different ripening times (i.e., 1, 30, and 60 d after cheesemaking). The metabolite relative concentrations were normalized, and a clustering analysis was run. Variables (i.e., VOC) were grouped in 6 different clusters (i.e., A–F; Figure 6), among which the VOC of 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, whose NWS had also provided the lowest *S. thermophilus* CopyN (Supplemental Figure S3). Cluster C and cluster D grouped most of the secondary metabolites, including alcohols (3-methyl-1-butanol, 2-ethyl-1-hexanol, ethanol, 2-heptanol, 2-butanol), aldehydes (nonanal), lactones (delta-dodecalactone), ketones (acetoin, 2-butanone), and organic acids (acetate) that derive from lactic acid, residual bioavailable lactose, and free amino acid fermentation. As an example of cluster C, ethanol was found in samples during the entire ripening time, from 1 to 60 d. The metabolites from cluster D instead mainly featured in SdC samples at 30 d and Art samples at 60 d. Cluster E, including 2 esters and 1-butanol, showed positive heats in few samples and was the most representative of SdC samples ripened for 60 d. Cluster F, instead, included the highest number (7) of different organic acids, delta-decalactone, and 1-hexanol, which, therefore similarly featured in our samples.

DISCUSSION

Caciocavallo cheese is one of the most typical Italian pasta filata cheese and, within this niche of products, has a great popularity (De Pasquale et al., 2014). As a fermented dairy food, cheese reports diverse microbiological and sensorial quality features, which are determined by multiple factors, such as the milk source, manufactur-

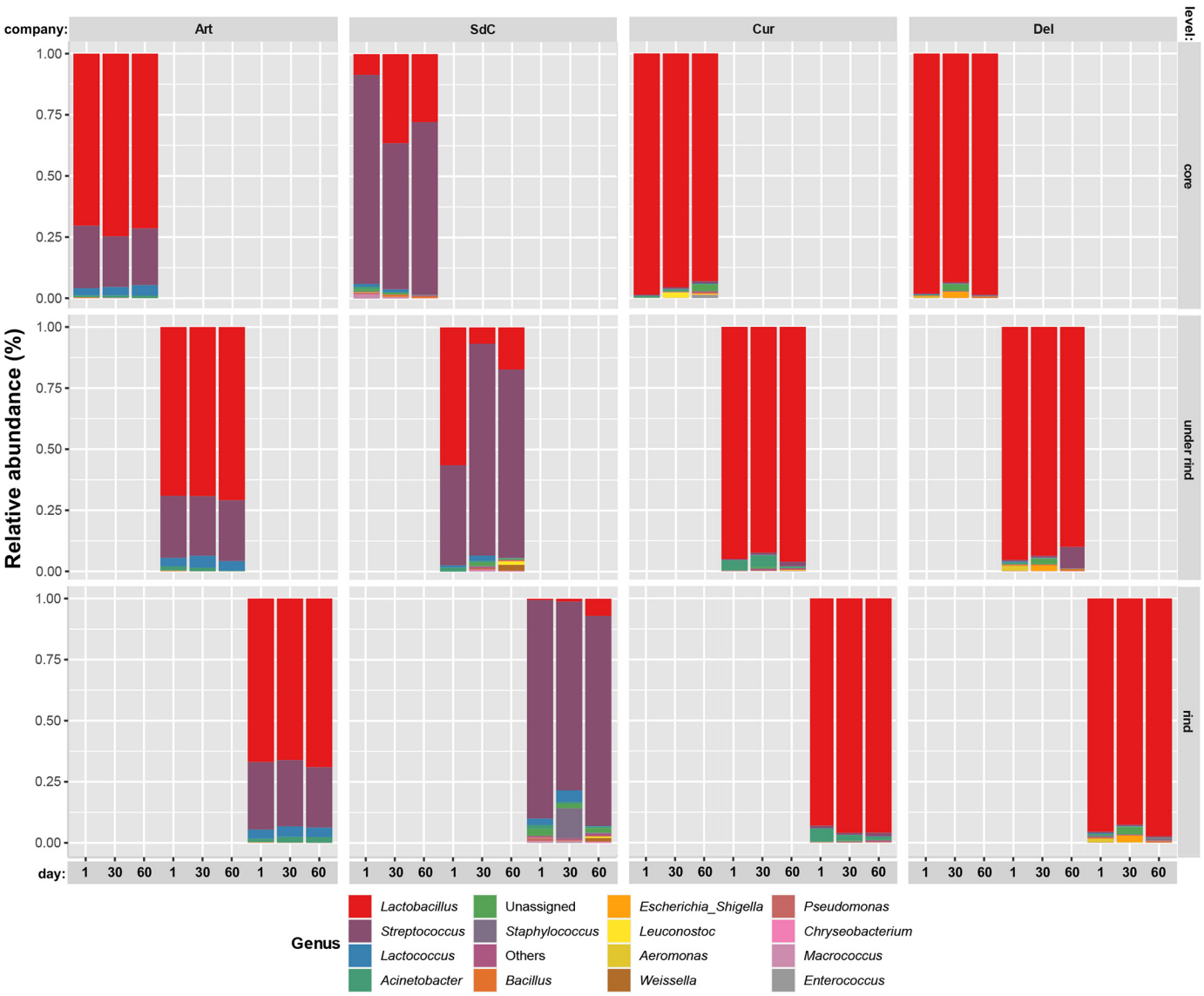

Figure 4. Metataxonomics (16S rRNA gene sequencing) of microbiota at genus level from 4 different Apulian Caciocavallo samples (Art, SdC, Cur, and Del) profiled at different time points (i.e., 1, 30, and 60 d after making) and 3 different sites in the cheese wheel (i.e., core, under-rind, and rind).

ing process, biotechnological parameters (including the use of starters), and facility-specific and environmental microbiomes (Gobbetti et al., 2018).

In an effort to elucidate the influence of microbiota from bulk milks and nonstarter cultures (NWS) on shaping the microbial communities and 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 dairies was conditioned by previous evidence discussing how local traditions influenced the cheesemaking processes leading to a different cheese microbiota development (Uzun et al., 2020). Because no differences have been found for both pH and TTA between the bulk milks specifically used to produce the analyzed Caciocavallo cheeses when grouped based on cheesemaking site, the strategy we adopted to limit the geographical location (Bonizzi et al., 2009) and seasonality (Celano et al., 2022a) allowed us 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

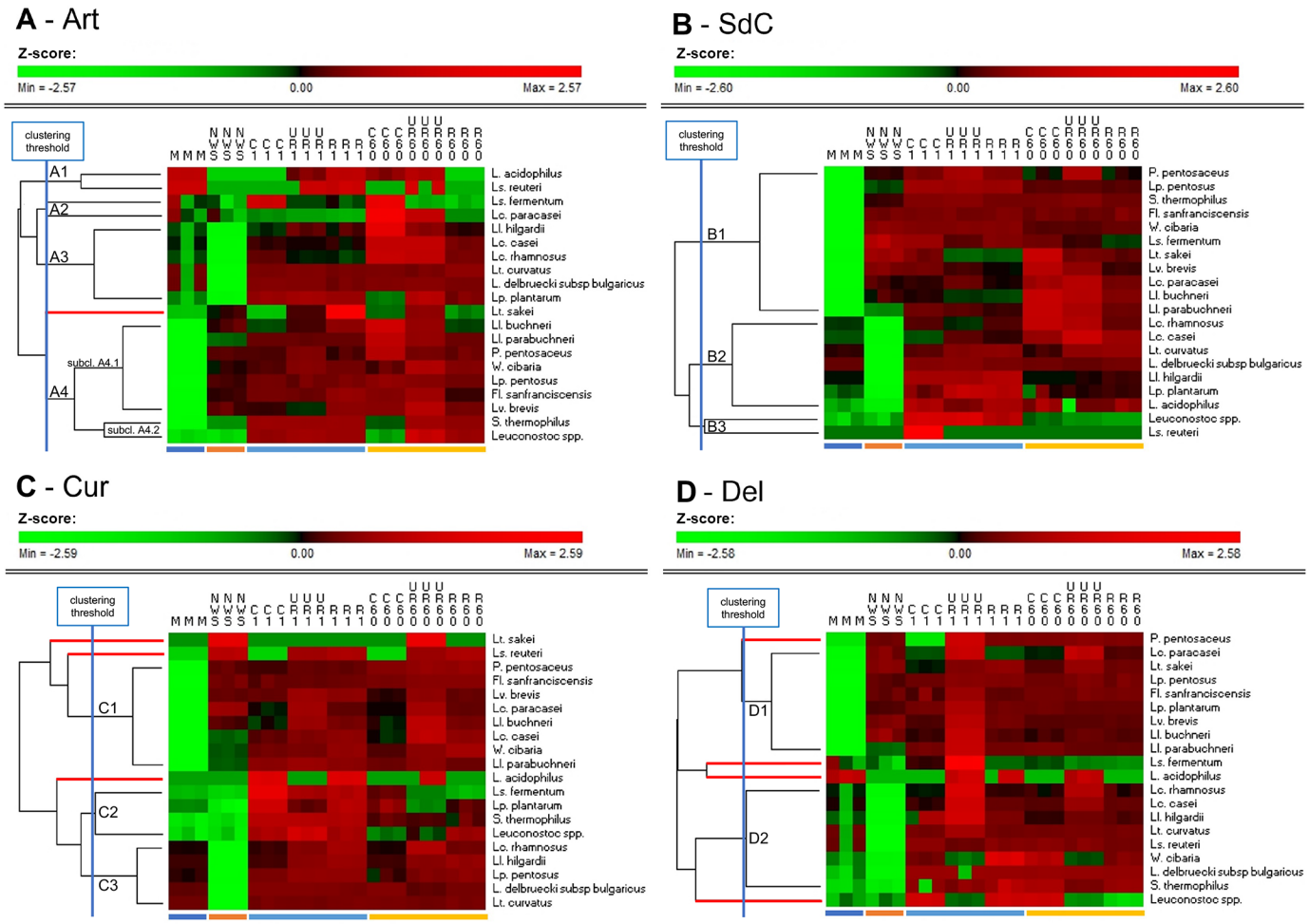

Figure 5. Based on copy number Z-scores, dynamic microbial evolution heatmap with clustering (Euclidean distance) of 20 different lactic acid bacteria (LAB; lactobacilli [n = 16], *Pediococcus pentosaceus*, *Weissella cibaria*, and *Streptococcus thermophilus*, and *Leuconostoc* spp.) found in bulk milks (M), natural whey starter (NWS), and cheeses provided by 4 different Apulian Caciocavallo cheese-producing dairy companies (Art, SdC, Cur, and Del; panels A, B, C, and D, respectively). Cheeses were screened at different time points (i.e., 1 and 60 d after cheesemaking) and samples taken from different sites in the cheese wheel (i.e., core [C]; under-rind [UR]; and rind [R]). The color bar ranges from 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. Min = minimum; Max = maximum.

most abundant cultivable bacteria found in bulk milks, especially in the Art and SdC samples. As previously explained, great attention was given to reducing inter-sample variability. However, various cofactors 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 these may have had an impact on the viable microbiota community.

The 16S rRNA targeted metagenomics data were different and allowed us 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). The operational taxonomic units detectable in both samples reporting high Shannon's values were *Acinetobacter*, *Macrococcus*, *Ralstonia*, *Lactococcus*, *Streptococcus*, *Pseudomonas*, and *Rhodococcus* genera, previously noted as the most representative colonizers of Apulian milk core microbiota (Celano et al., 2022a). In Cur and Del bulk milks, a considerable abundances of *Acinetobacter* and *Lactobacillus* were observed, respectively. This large difference might be explained by a different environmental contamination level by psychrotrophic bacteria before bulk tank storage (Hahne et al., 2019). Subsequently, both temperature and storage time can greatly influence 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

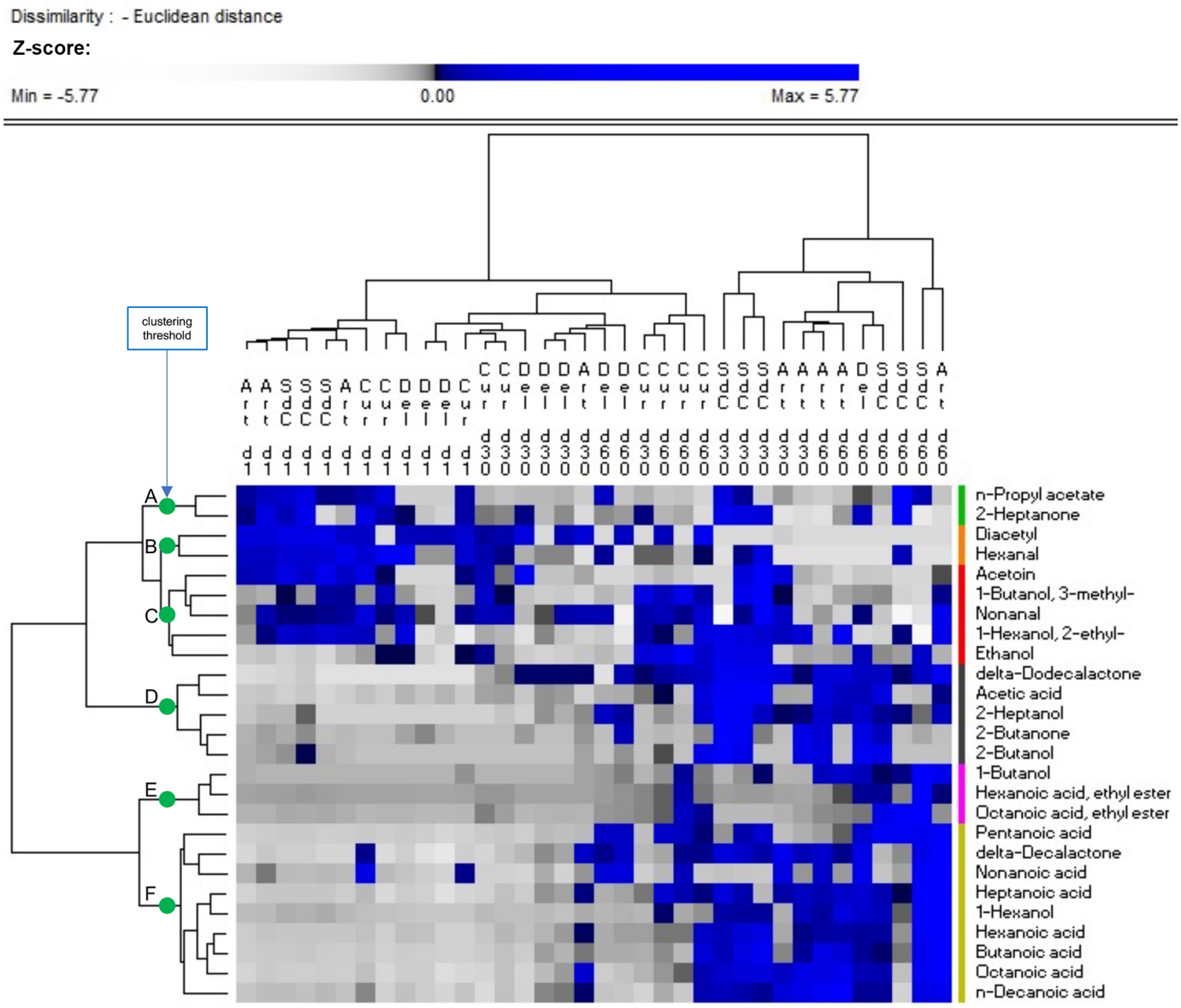

Figure 6. Based on normalized relative concentrations (Z-scores), volatile organic compound (VOC) dynamic changes were collected from Caciocavallo cheeses produced by 4 different Apulian dairy companies (Art, Cur, Del, and SdC) and screened at different time points (1, 30, and 60 d after the cheesemaking). The color bar ranges from white (lowest Z-score) to blue (highest Z-score). Min = minimum; Max = maximum.

due to their large incidence in milks and wide knowledge about their pretechnological activities during cheesemaking and ripening (Quigley et al., 2013). As observed in the related bulk milks, the highest density of viable lactobacilli, lactococci, and enterococci microbial was found in SdC NWS, whereas the lowest was found in Art. Viable staphylococci and *Enterobacteriaceae* were lower than 1 log cfu/mL in all NWS as a result of the acidification process that occurred (da Silva Duarte et al., 2020). Although based on different data, viable yeast densities were aligned with those found in bulk milks. In detail, Cur and Del NWS had the highest yeast density, whereas SdC displayed the lowest density, a result that needs to be further evaluated in light of evidence about the role of yeasts in supporting typical flavor development in cheeses (Martini et al., 2021). The 16S rRNA gene sequencing profile showed the absence of differences for Shannon's index between NWS. As also discussed by previous studies exploring difference between NWS used for Caciocavallo cheese making (Ercolini et al., 2008; Pogačić et al., 2013), the major difference we observed at the genus level involved *Lactobacillus* and

Streptococcus. In fact, although the former taxon was the most representative genus for Art, Cur, and Del NWS, *Streptococcus* was found as the major colonizer of SdC samples.

Due to the low differentiation between species supported by 16S rDNA gene sequencing in our samples, we also carried out qPCR to genotype LAB subtaxa in NWS and evaluate their dynamic changes during ripening. Overall, the qPCR analysis showed how NWS harbored various LAB species, such as *S. thermophilus, Lc. paracasei*, *Lt. sakei*, *Ll. buchneri*, *Ll. parabuchneri*, *Fl. sanfranciscensis*, *Lv. brevis*, and *P. pentosaceus*. These results are in line with the study conducted by Ercolini et al. (2008) that investigated the microbiota of NWS used for the Caciocavallo Silano PDO cheese production. Due to the detection of some and specific LAB species discriminating NWS, authors concluded that microbiota diversity was independent from the geographical origin. Various starter lactic acid bacteria (**SLAB**; e.g., *L. acidophilus*, *Lc. casei*, *Lc. paracasei*, *Lc. rhamnosus*, *Lt. curvatus*, *Lp. pentosus*, *Ls. reuteri*, *Ls. fermentum*, *Ll. buchneri, Lt. sakei*, and *Lv. brevis* species) can be indicated as the most frequent NWS colonizers because 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 of the 4 dairy plants, Caciocavallo cheeses were collected at different time points (1, 30, and 60 d after the cheesemaking), and the profiling also considered differences driven by different sites of the cheese block (core, under-rind, and rind of the Caciocavallo cheese). The pH and TTA values in the 3 fractions were similar within the same product. The partial deacidification observed in Art and Cur cheeses during the first 30 d of ripening compared with their counterparts produced at Del and SdC may be due to an accumulating microbial density from milk storage or during the manufacturing process and from microbial interactions (Calasso et al., 2016). At the end of ripening, all cheeses showed similar values for all sampled fractions. Comparing Caciocavallo from the same ripening time, samples across the 4 facilities showed relatively higher microbiota β-diversity ($P < 0.001$) and, as the principal component analysis 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 Cur and Del dairy plants compared with Art and SdC samples. Lactic acid-producing bacteria belonging to the genera *Lactobacillus*, *Streptococcus*, and *Lactococcus* were found in Caciocavallo samples. These genera play knowing important roles in flavor formation and nutrient composition by using carbohydrates, especially lactose, proteins, fats, and peptides, to synthesize short peptides, free amino acids, short-chain fatty acids, 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). The Cur and Del samples, which showed low richness in the related bulk milks, exhibited *Lactobacillus* as dominant taxon. In turn, the low richness of the dairy bulk milk might have facilitated the colonization by lactobacilli (basonym *Lactobacillus*) in the related NWS that were used to acidify the curd. Under the conditions of the dairy facilities in this study, we can speculate that the capacity of curd contamination by *Lactobacillus* residing in milk, and especially in NWS, was markedly higher than that highlighted for *Streptococcus*. As previously shown, *Streptococcus* strains were usually detected during the manufacture of traditional Caciocavallo type cheese (Calasso et al., 2016). In line with 16S-based profiling of the NWS, the SdC samples reported the highest abundance of *Streptococcus*, with ripening time and spatial variations. *Streptococcus* genus was also found in Caciocavallo cheese produced at the Art facility, although it was not detected in the related NWS. As previously shown, the colonization may be due to the commercial starter cultures that are used to produce other varieties of cheeses in the dairy plant and are 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 *S. thermophilus* synergistically interacts with *L. delbrueckii* ssp. *bulgaricus* (Calasso et al., 2016), with both species originating from milks we assessed by qPCR analysis.

Lactococcus spp., which is traditionally used in the crafting of mesophilic lactic starter cultures for Caciocavallo Pugliese cheese production (Calasso et al., 2016), consistently appeared in both Art and 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) that is implicated in the desirable development of a buttery aroma in cheeses (Curioni and Bosset, 2002). In accordance with this observation, the 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 being assessed in cheeses until 30 d of ripening. In this respect, it should be also considered that the concentration of the precursor citrate in the related milk, as previously demonstrated, can vary according to

different factors, including the animal feeding (Dunshea et al., 2019). Moreover, Oliveira et al. (2012) also suggested that differences in acetoin concentration in dairy products can be attributed to interactions between *S. thermophilus* and other SLAB, influencing the expression of α-acetolactate synthase by *S. thermophilus*. In line with this, several alcohols (1-butanol, 2-butanol, and 2-heptanol), ketones (2-butanone and delta-dodecalactone), esters (hexanoic acid ethyl ester and octanoic acid ethyl ester), and volatile free fatty acids (**VFFA**; acetic acid) were present at the highest levels in Art and SdC cheese samples, where *Streptococcus* and *Lactococcus* were found at high frequencies. This result confirms how the microbial differences specifically observed in these samples (compared with Cur and Del) led to specific modifications in cheese biochemistry, consistent with previous studies reporting the relationship that can occur between autolysis of both *Streptococcus* and *Lactococcus* primary starters and the VOC concentration during ripening (Collins et al., 2003; De Pasquale et al., 2014).

A set of additional satellite microbial genera flanked *Lactobacillus* and *Streptococcus* in the structure of the cheese microbiota, with variations depending on the dairy plant, sampling time, and (in some samples) spatial distribution. In SdC cheese, the mesophilic *Leuconostoc* genus was detected in subdominant microbiota and frequently in combination with other mesophilic LAB, e.g., *Lactococcus* (Irlinger et al., 2015). *Weissella* and *Acinetobacter*, previously associated with a variety of artisanal and surface-ripened cheeses (O'Sullivan et al., 2015), were also detected. *Acinetobacter*, a representative genus from our different dairy bulk milks (Figure 1), is a contaminant adventitious microorganism that may be persistent through the entire production chain, from the farm to the final product (Alessandria et al., 2016), and has been suggested to cause spoilage (Arslan et al., 2011; Stellato et al., 2015; de Paula et al., 2021). Herein, with exception of Art samples, *Acinetobacter* showed the capacity to consistently contaminate all cheeses, persisting over time during ripening (Figure 4). Psychrotrophic bacteria, including *Aeromonas* and *Pseudomonas*, detected in this work as satellite microbial genera in Del bulk milks and during ripening of the cheeses, have been previously isolated from a variety of cheeses, as well as raw milk (Montel et al., 2014; O'Sullivan et al., 2015), because they are particularly adapted to low-temperature milk storage conditions and therefore may have gained access to the cheese via the milk. As widely demonstrated, additional species can originate from bulk milks, such as *Lc. rhamnosus*, *Lc. casei*, and *Lp. plantarum* (Mucchetti and Neviani, 2022). The same species were here detected until 60 d of cheese ripening by qPCR, confirming evidence on how these can be potentially implicated to the flavor and aroma development (Masoud et al., 2012). According to previous studies on Caciocavallo cheese, the levels of some secondary metabolites (i.e., alcohols and acetic acid), deriving from lactic acid and residual bioavailable lactose fermentation by heterofermentative SLAB or lactic acid oxidization by nonstarter LAB, significantly increased during ripening time (De Pasquale et al., 2014; Gobbetti et al., 2015; Coelho et al., 2022). As found in other pasta filata cheeses, most VFFA (butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, and n-decanoic acid), which contribute to the cheese flavor but also serve as precursors for the synthesis of other compounds (methylketones, alcohols, and esters), were similarly abundant in all our samples (McSweeney and Sousa, 2000; De Pasquale et al., 2014).

Although previous data described differences in the spatial distribution of microbial communities between the rind and core of several cheeses (O'Sullivan et al., 2015), in our study, an exception was found for SdC Caciocavallo samples, in which no significant differences in the microbial populations present in the respective cores, under-rinds, and rinds were detected, being comparable throughout ripening. These findings suggest the existence of a core microbiota 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

Milk's microbial biodiversity is higher than its derivatives, influenced by native microbiota in the NWS during cheese production and ripening. Despite standardized production protocols, distinct microbiota clustering in samples showed how cheese-specific features are shaped by milk and NWS differences, and unique conditions of each production site. This comprehensive view revealed that the resulting microbiota contribute to cheese-specific sensory characteristics during ripening. Major volatile organic compounds were similar across samples, but

minor, crucial compounds varied. Spatial variations in microorganism abundance and taxonomy within cheese blocks reflected multiple factors, including milk colonizers and NWS, along with biotic and abiotic variables in the dairy environment, which are unpredictable and not standardizable. Further metatranscriptomics studies are needed to explore the impact of dominant and subdominant raw milk microbiota on cheese properties and to examine taxon activity in different parts of the same cheese block.

NOTES

The study was funded by the NATCASEI Project: "Management of breeding systems and environmental factors for the production and enhancement of natural starters in the cheesemaking processes," funded by the Italian Ministry of agricultural, food and forestry policies, milk fund research projects, D.M. N. 27443-25 of September 25, 2018. The authors are very grateful to the cheesemaking dairies participating in the present research. Author contributions are as follows: conceptualization (G. Ce., M. C., and M. D. A.); methodology (M. V., N. S., and G. Ce.); software (F. M. C.); formal analysis (N. S., G. Co., and G. Ce.); investigation (M. V., G. Co., F. M. C., N. S., and G. Ce.); resources (M. D. A.); data curation (M. V., F. M. C., and G. C.); writing, original draft preparation (M. V.); writing, review, and editing (M. C.); visualization (M. V.); supervision (G. Ce. and M. D. A.); project administration (G. Ce. and M. C.); funding acquisition (M. D. A.). All authors have read and agreed to the present manuscript version. Supplemental material for this article is available at https://doi.org/10.6084/m9.figshare.26013520.v4. 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.

Nonstandard abbreviations used: C = core; CopyN = copy number; LAB = lactic acid bacteria; M = bulk milk; Max = maximum; Min = minimum; NWS = natural whey starter; qPCR = real-time PCR/quantitative PCR; R = rind; SLAB = starter lactic acid bacteria; TMA = total mesophilic aerobes; TTA = total titratable acidity; UR = under-rind; VFFA = volatile free fatty acid.

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