MICROBIOLOGICAL SAFETY AND QUALITY ASPECTS OF FERMENTED DAIRY PRODUCTS

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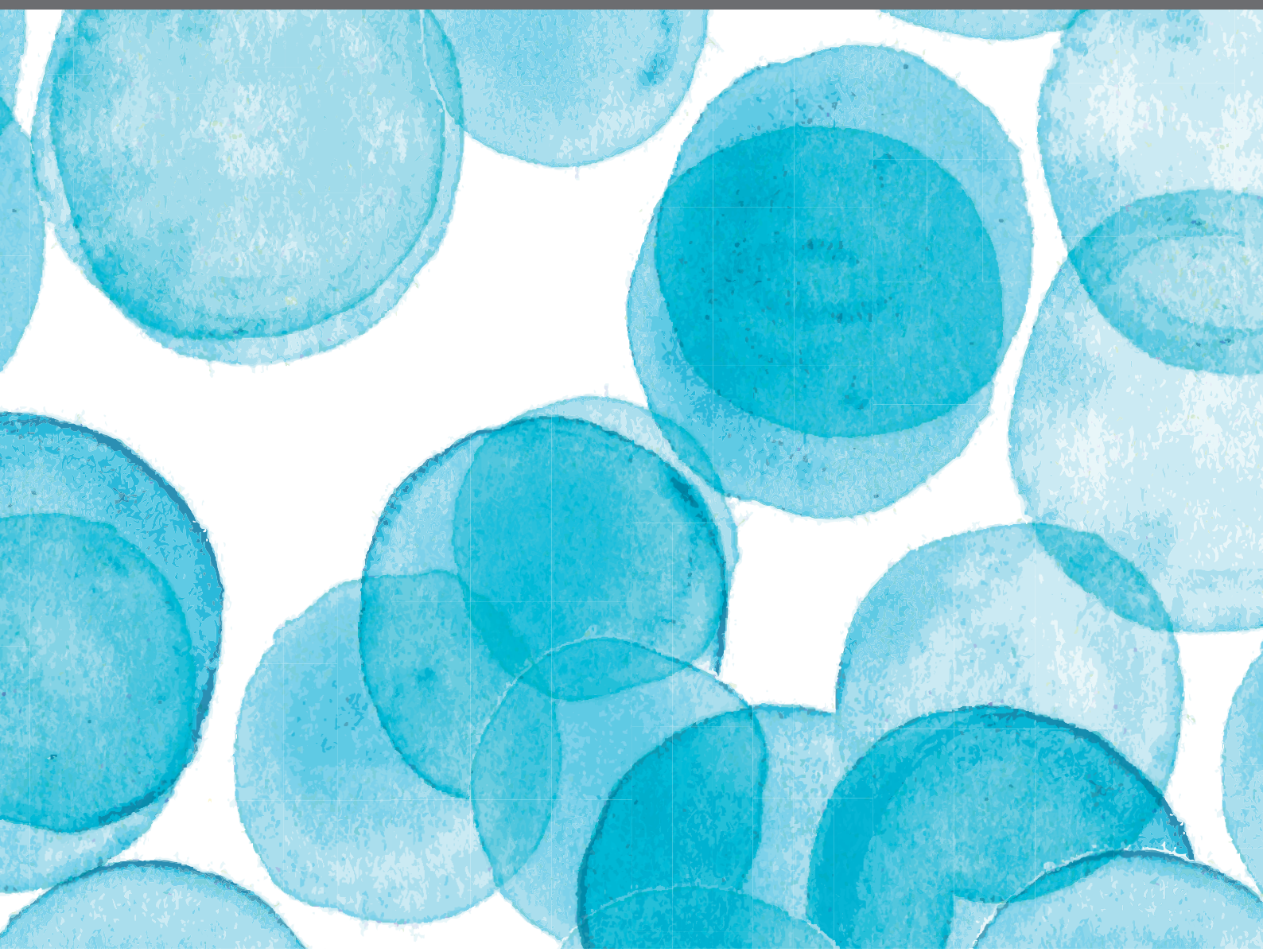

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MICROBIOLOGICAL SAFETY AND QUALITY ASPECTS OF FERMENTED DAIRY PRODUCTS

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Editorial: Microbiological Safety and Quality Aspects of Fermented Dairy Products

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Editorial on the Research Topic

Microbiological Safety and Quality Aspects of Fermented Dairy Products

Fermented dairy products include a wide range of foods that are highly appreciated worldwide. The production processes, the type of milk, practices adopted during feeding, milking, and beyond can affect the quality and safety characteristics of these products. A vast array of microorganisms can be found in milk, and microbial succession during fermentation of multiple products, as well as during cheese ripening contributes to the desired properties of these foods. In addition to sensory and safety aspects, microorganisms present in fermented dairy foods can positively affect the health of people due to their potential probiotic nature and the production of beneficial metabolites such as vitamins, antioxidants, and antimicrobial compounds.

Despite recent innovations on microbial cultivation techniques, and the widespread use of a myriad of newly developed "omic" techniques, including genomic approaches through the use of High Throughput Sequencing in combination with advanced metabolomics, there are many dairy fermented products with limited information about their microbial composition (diversity) and dynamics (succession). This is particularly true for traditional regional products, which represent a rich niche for discoveries involving microorganisms with better conditions for recovery from disturbances and more reliable and robust for industrial production. The unknown microbiota can reveal new mechanisms of interactions and functions and new bioactive molecules with beneficial effects on the sensory aspect of food and on human health.

The goal of this Research Topic was to deepen the current knowledge in the microbial safety and quality aspects of fermented dairy products, gathering studies on the different types of products, practices, and how the microbiota affected the quality and safety attributes of these much-appreciated foods. This Research Topic brings a series of 14 articles related to the **microbiological safety and quality aspects of fermented dairy products**.

Two review articles provide important information for the cheese sector. The first presents an updated view on the diversity and safety of artisanal cheeses produced in Brazil, and the second addresses safety and technological issues regarding yeast contamination in white-brined cheeses. Pineda et al. tackle product diversity according to Brazilian geographical regions, the microbiological safety, and the huge challenges faced by the Brazilian artisanal cheese sector. The authors highlight that one of the main challenges is related to risks associated with foodborne pathogens when the quality of the raw milk is unsatisfactory. Artisanal cheese regulations have

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constantly been revised and adapted, considering the small-scale production of Brazilian artisanal cheeses, thus research collaborative efforts may help the sector to move toward better quality and safety goals. It is noteworthy that the economic income of thousands of rural families depends on artisanal cheese production in the country and there is an increased appreciation for artisanal cheese consumption in recent years. Geronikou et al. summarize the current knowledge on the identification of contaminant yeasts in white-brined cheeses, their occurrence and spoilage potential, interactions with other microorganisms, as well as guidelines to prevent cheese spoilage. The proliferation of spoilage yeast depends on ripening and storage conditions at each specific dairy, product characteristics, nutrients availability, and interactions with the microbial consortium, ultimately leading to decreased sensorial properties, shorter shelf life, and impaired quality. White-brined cheeses are worldwide produced with many different process flow charts. However, even small variations might have an impact on the parameters and, thereby, on the yeast species being able to proliferate. Detailed knowledge on variations on spoilage potential at strain level is still missing for this type of cheese. Furthermore, the acquisition of scientific knowledge about yeast interactions with other microorganisms related to milk and the cheese matrix will add to an optimized production of white-brined cheese of enhanced quality.

Milk is susceptible to contamination with pathogenic microorganisms. Microbial food safety along the dairy chain is an important topic, from public health and industry perspectives. The three papers submitted that dealt with food safety addressed issues related to the foodborne pathogen *Listeria monocytogenes*. This important microorganism can adapt and survive in food and food processing facilities where it can persist for years. The paper presented by Ricci et al. evaluated the heat resistance of *L. monocytogenes* in matrices involved in the production of Mozzarella di Bufala Campana Protected Designation of Origin (PDO) cheese. The 12 tested *L. monocytogenes* strains showed heterogeneous heat resistance profiles that ranged from 7 to <1 Log_{10} CFU/mL reductions after 8 min at 60°C. *D*-values and *z*-values, calculated for the most heat resistant strain, between 60 and 70°C were affected by the food matrix, being especially increased in drained cheese curd. As cheese curd stretching is not an isothermal process, a built secondary model was used to simulate microbial inactivation, and the authors were able to estimate the lethal effect of the process to be around 4 Log_{10} reductions. The data supplied may prove to be useful for Mozzarella cheese producers in determining appropriate time and temperature (kinetic parameters of heat inactivation) for producing fresh pasta filata cheese avoiding the presence of *L. monocytogenes* and improving microbial safety, efficacy, and sustainability of the process.

The paper carried by Bechtel and Gibbons analyzed the population genetic structure of 504 *L. monocytogenes* strains isolated from food with publicly available genome assemblies. The purpose of the study was to understand if genetically distinct populations are associated with particular foods. Using comparative and population genomic analysis, they identified genes that were present at a greater frequency in the population associated with cheese. The results suggest that particular *L. monocytogenes* genotypes may be associated with the colonization and persistence in certain food environments, such as dairy and cheese, and indicate potential candidate genes involved in the specialization to particular food substrates. This type of study can be very useful for tracking certain genotypes and to better understand food-borne outbreaks. Similarly, Mafuna et al. characterized 143 *L. monocytogenes* isolated in South Africa for their strain's genetic relatedness, virulence profiles, stress tolerance, and resistance genes. Examination of genes involved in adaptation and survival showed that some sequence types are well-adapted in food processing environments due to the significant over-representation of benzalkonium chloride resistance genes, stress tolerance genes, prophage (φ) profiles, plasmids profiles, and biofilm formation associated genes. The information provided in this study is important for a better understanding of the adaptation and survival of *L. monocytogenes* in the food-processing environments and the efficacy of using biocide disinfectants in facilities. Later in this editorial, a paper conducted in Italy by Cremonesi et al. will approach the effect of chlorine usage for sanitation procedures on the raw milk microbiota.

The next eight papers deal specifically with the analyses of the cheese microbiome. The composition of the microbiota has an important impact on the quality and safety of cheese due to the growth and interaction between microorganisms during processing and ripening. Therefore, much effort has been made by the authors to create molecular tools and omic systems to investigate the microbial community composition of cheese.

Dreier et al. developed a high-throughput quantitative real-time polymerase chain reaction (HT-qPCR) approach for rapid and cost-efficient quantification of microbial species in cheese. Preliminary results from model and downgraded commercial cheeses showed that the application of microfluidic HT-qPCR to complex fermented products could be of interest for identifying the microbial origin of quality defects. This new system is a promising approach that will allow, particularly in the production of raw milk cheese, simultaneous monitoring of the quality-relevant ripening microbiota, thereby opening up new perspectives for the control and assurance of high product quality. Afshari et al., Bottari et al., and Unno et al. used integrated molecular, analytical chemistry methods, and omics approaches to valuating the complexity of the microbiota and their metabolites involved in the cheese ripening.

The paper by Afshari et al. used bacterial 16S rRNA-gene sequencing, untargeted metabolomics, and data integration analyses to characterize and differentiate commercial Cheddar cheeses. Microbiota and metabolite compositions could be used to distinguish diverse time-ripened cheeses. Individual amino acids and carboxylic acids were positively correlated with the ripening age for some brands. The results suggest that multi-omics analyses have the potential to be used for discovering biomarkers for validating cheese age and brand authenticity. The paper by Bottari et al. used culture-dependent and independent microbial counts, high throughput 16S rRNA sequencing, length heterogeneity (LH-PCR), and ultra-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UPLC/ESI-MS) analysis to evaluate whether the

composition of the bacterial community of Parmigiano Reggiano PDO cheese along with the specific peptide composition are more affected by the ripening times or by the cheese making process. The ripening time had the greatest impact on microbial dynamics and, consequently, on peptide composition. The potential use of peptides as markers of a specific microbial composition affords the possibility of taking advantage of it to protect and valorize the specificity and connection of traditional cheese to its production territory. The paper by Unno et al. integrated metagenomics using metagenomic amplicon sequencing and metabolomics with high-performance liquid chromatography (HPLC), and headspace gas chromatography mass spectrometry (HS-GC/MS) to reveal the microorganisms and components relationships in surface mold- and bacterial smear-ripened cheeses. Correlation analysis revealed that the abundance of specific bacteria was related to the formation of specific organic acids, free amino acids, and volatile compounds in these kinds of cheeses. The study will contribute to elucidate the role of non-starter cultures and possibly to select candidates for adjunct culture.

According to Johnson et al., one aspect of cheese quality that remains poorly understood is the variability of microbial subpopulations due to temporal or facility changes within production environments. In their work, the aim was to quantify the microbiome variability by measuring day-day and facility-facility changes. Microbial communities were characterized using 16S rRNA metabarcoding and by plating on selective growth media. Interestingly, facility differences were greater on food contact surfaces, whereas daily differences within each facility were mostly explained by variation in the milk and cheese. The results highlighted the complexity of the Cheddar cheese facility microbiome and demonstrated daily and facility-facility microbial variations, which might influence cheese quality.

The paper produced by Mancini et al. focused on the microbial and bacteriophages characterization from facilities that use natural whey starter (NWS) cultures for Trentingrana (Grana-like) PDO cheese production. Using culture-dependent methods and metataxonomic analysis, *Lactobacillus helveticus* was found occurring as the dominant and *Levilactobacillus brevis* as codominant NWS cultures. One hundred and twenty distinct phages were identified from 303 bacterial isolates from the NWS cultures. The authors found a possible correlation between bacterial starter biodiversity and the number of recovered lytic phages. They speculated that the presence of high biodiversity of NWS dairy bacteria biotypes is relevant to avoid phages dominance in NWS cultures, observing the recovery of a lower number of phages from the dairy plants with the higher biodiversity in *L. helveticus* biotypes. Phage predation can cause loss of NWS activity and generates a defective cheese. Also using the case of Trentingrana cheese, Cremonesi et al. evaluated the influence of chlorine products (sodium hypochlorite detergent) usage for sanitizing equipment on raw milk, NWS, and cheese microbiota and volatilome. Samples were subjected to culture-dependent and metagenomic analyses. Cheese volatilome was determined by solid-phase micro extraction-gas chromatography-mass spectrometry (SPME-GC-MS). As expected, a difference in microbial population related to chlorine usage in bulk milk, vat, and NWS samples was evidenced. The paper results support the idea that chlorine influences the milk and cheese microbiome and, consequently, cheese quality, safety, and sensory attributes. Furthermore, chlorine replacement is not associated with an increase of spoilage bacteria, staphylococci, and coliforms, but it leads to an increase in the milk microbial biodiversity. Further studies on sanitation strategies alternative to chlorine-based protocols are needed.

Cheese has been demonstrated to be an optimal carrier product to deliver viable probiotic bacteria. Pisano et al. produced probiotic Caciotta cheeses from pasteurized ewes' milk by using combinations of autochthonous microbial cultures, containing probiotic strains. It was demonstrated, using multivariate statistical analysis of ^{1}H nuclear magnetic resonance spectroscopy-based metabolomics data, significant variations in the cheese' profiles both in terms of ripening time and strains combination when compared to a control cheese produced using commercial starter cultures. The data obtained have indicated the applicative potential of autochthonous lactic acid bacteria cultures, containing probiotic *Lactobacillus* and *Kluyveromyces* strains, for the production of potential functional cheese.

In the final paper, Perkins et al. combined culture-dependent techniques with whole-genome shotgun sequencing to light up the phylogenetic relationships among *Geotrichum candidum* and *Galactomyces* spp. strains of environmental and dairy origin. *G. candidum* is a yeast culture used on mold- and smear-ripened cheeses during ripening to promote the development of sensorial properties in the cheese surface. It was proposed a new multilocus sequence typing (MLST) scheme to optimally genotype isolates, substantially improving the knowledge on this culture to allow a better selection and control of *G. candidum* strains throughout the ripening.

AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phenotypic and Genetic Characterization of the Cheese Ripening Yeast *Geotrichum candidum*

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The yeast *Geotrichum candidum* (teleomorph *Galactomyces candidus*) is inoculated onto mold- and smear-ripened cheeses and plays several roles during cheese ripening. Its ability to metabolize proteins, lipids, and organic acids enables its growth on the cheese surface and promotes the development of organoleptic properties. Recent multilocus sequence typing (MLST) and phylogenetic analyses of *G. candidum* isolates revealed substantial genetic diversity, which may explain its strain-dependant technological capabilities. Here, we aimed to shed light on the phenotypic and genetic diversity among eight *G. candidum* and three *Galactomyces* spp. strains of environmental and dairy origin. Phenotypic tests such as carbon assimilation profiles, the ability to grow at 35°C and morphological traits on agar plates allowed us to discriminate *G. candidum* from *Galactomyces* spp. The genomes of these isolates were sequenced and assembled; whole genome comparison clustered the *G. candidum* strains into three subgroups and provided a reliable reference for MLST scheme optimization. Using the whole genome sequence as a reference, we optimized an MLST scheme using six loci that were proposed in two previous MLST schemes. This new MLST scheme allowed us to identify 15 sequence types (STs) out of 41 strains and revealed three major complexes named GeoA, GeoB, and GeoC. The population structure of these 41 strains was evaluated with STRUCTURE and a NeighborNet analysis of the combined six loci, which revealed recombination events between and within the complexes. These results hint that the allele variation conferring the different STs arose from recombination events. Recombination occurred for the six housekeeping genes studied, but most likely occurred throughout the genome. These recombination events may have induced an adaptive divergence between the wild strains and the cheesemaking strains, as observed for other cheese ripening fungi. Further comparative genomic studies are needed to confirm this phenomenon in *G. candidum*. In conclusion, the draft assembly

of 11 *G. candidum*/*Galactomyces* spp. genomes allowed us to optimize a genotyping MLST scheme and, combined with the assessment of their ability to grow under different conditions, provides a reliable tool to cluster and eventually improves the selection of *G. candidum* strains.

Keywords: yeast, cheese, *Geotrichum candidum*, *Galactomyces candidus*, multilocus sequence typing, assimilation of carbon compounds, genome assembly

INTRODUCTION

The yeast species *Geotrichum candidum* (teleomorph *Galactomyces candidus*) is commonly found in water, air, soil, cereals, ripened fruits, milk, and especially on the surface of mold- and smear-ripened cheeses like Camembert, Tilsit, and Pont-L'Évêque (Marcellino et al., 2001; Boutrou and Guéguen, 2005; Thornton et al., 2010; de Hoog and Smith, 2011; Eliskases-Lechner et al., 2011; Desmasures, 2014). *G. candidum* was long considered an undesirable microorganism because its presence in cheese was uncontrolled, but is now recognized for its contribution to cheese ripening, due to its capacity to consume lactic acid and generate alkaline products (Marcellino et al., 2001; Spinnler et al., 2004; Boutrou and Guéguen, 2005). This yeast also contributes to the development of flavor in the cheese due to its proteolytic and lipolytic activities (Boutrou et al., 2006; Gente et al., 2007). Additionally, *G. candidum* is a good competitor against unwanted microorganisms such as *Mucor* spp. and has an antagonistic action against pathogens such as *Listeria monocytogenes* (Dieuleveux et al., 1998; Boutrou and Guéguen, 2005). However, these various traits, especially those contributing to the development of cheese flavors and texture, are strain dependent. *G. candidum* species display a great diversity at the morphologic and metabolic levels (Guéguen and Schmidt, 1992; Jollivet et al., 1994; Jacobsen and Poulsen, 1995; Molimard et al., 1997). Their high level of polymorphism and widely variable phenotypic traits complicate the identification and characterization of *G. candidum* strains by traditional microbiological methods (Prillinger et al., 1999; Gente et al., 2006). Many indigenous strains of *G. candidum* have been isolated from the environment or from dairy products (Marcellino et al., 2001; Gente et al., 2002b; Lavoie et al., 2012). However, the lack of genomic and functional information has limited their potential utility.

Different *G. candidum* strains can be discerned based on chromosome sizes or sequences of their housekeeping genes (Gente et al., 2002a; Alper et al., 2013; Morel et al., 2015; Jacques et al., 2017). The ability to identify distinct phylogenetic groups within the *G. candidum* species lead to the development of two independent multilocus sequence typing (MLST) schemes to genotype isolates of *G. candidum*, but those were never compared using the same set of strains [MLST2013 (Alper et al., 2013) and MLST2017 (Jacques et al., 2017)]. Moreover, only one genome of the *G. candidum* species has been partially sequenced and subjected to further gene prediction analysis (Morel et al., 2015).

The aim of this study was to combine phenotypic assays and whole genome sequencing to elucidate the phenotypic and genetic diversity of eight *G. candidum* strains that belong to distantly related groups from a previous study using MLST (Alper et al., 2013; **Table 1**). We also used the newly sequenced genomes to optimize a MLST scheme based on the two MLST schemes available in the literature (Alper et al., 2013; Jacques et al., 2017). The phenotypic and genomic results obtained in this study increase the scientific knowledge on the ripening yeast *G. candidum* and, in the near future, could be used to improve strain selection for cheese ripening processes.

MATERIALS AND METHODS

Biological Material, Culture Conditions, and Genomic DNA Extraction

Eight *G. candidum* (identified in bold in **Table 1**) and three *Galactomyces* spp. strains were used in this study (**Table 2**). The eight *G. candidum* strains were selected as representatives of each branch in the phylogenetic tree obtained with MLST2013 (Alper et al., 2013), while the *Galactomyces* spp. strains were chosen to form an outgroup of other *Galactomyces* species. Considering that the genus *Galactomyces* is not well known, the choice of three strains of different species as outgroups allowed to draw a wider portrait of this genus. All strains were grown on YEG (Yeast Extract Glucose) agar plates [10 $g{\cdot}L^{-1}$ of yeast extract (Thermo Fischer Scientific), 10 $g{\cdot}L^{-1}$ of D-glucose (EMD Chemicals) and 15 $g{\cdot}L^{-1}$ of Bacto agar (BD Diagnostics)]. The strains were inoculated directly from 15% glycerol (*v/v*) stock cultures stored at −80°C. The plates were incubated in the dark for 4 days at 25°C. Isolated colonies were re-streaked on YEG agar plates or in YEG Broth and incubated for 7–9 days at 25°C in the dark. An additional 33 *G. candidum* strains isolated from dairy environment was used to validate the newly designed MLST scheme (**Table 1**).

The mycelia on YEG Broth were harvested and grounded into fine powder in liquid nitrogen using a CryoMill apparatus (Retsch, Germany). Cryogenic grinding was performed at −196°C with an automatic pre-cooling step at a 5 Hz frequency followed by a 2 min grinding step at a 25 Hz frequency. Genomic DNA was extracted from 30 mg of grounded mycelium using Purelink RNA/DNA viral mini kit (Invitrogen) with the following modifications. Grounded mycelium was homogenized in 200 µl of a 0.9% NaCl solution, prior to the addition of the proteinase K and the lysis buffer. DNA concentration and quality were measured using a NanoDrop ND-1000 spectrophotometer

TABLE 1 | Origins and allelic profiles of the 41 *G. candidum* strains analyzed.

Strain	Geo complex	Lineage	ST	Allele						Origin[a]	References
				ALA1	*CDC19*	*SAPT4*	*GLN4*	*PGI1*	*PGM2*		
15	A	3	1	1	1	1	1	1	1	Mold-ripened cheese, Canada	Alper et al., 2011
20	A	2	2	1	2	1	1	2	2	Mold-ripened cheese, Canada	Alper et al., 2011
21	C	1	3	2	3	2	2	2	3	Clotted carrot, Japan	Guéguen and Jacquet, 1982
28	A	5	4	1	1	1	1	1	4	Mold-ripened cheese, Canada	Alper et al., 2011
34	A	2	2	1	2	1	1	2	2	Mold-ripened cheese, Spain	Alper et al., 2011
37	A	2	2	1	2	1	1	2	2	Smear cheese, France	Alper et al., 2011
38	A	2	2	1	2	1	1	2	2	Mold-ripened cheese, France	Alper et al., 2011
39	A	5	4	1	1	1	1	1	4	Smear cheese, France	Alper et al., 2011
40	**A**	**5**	**4**	**1**	**1**	**1**	**1**	**1**	**4**	**Smear cheese, France**	Dieuleveux et al., 1997
48	B	4	5	1	1	2	3	2	4	Bioreactor contaminant	Alper et al., 2011
70	**C**	**1**	**3**	**2**	**3**	**2**	**2**	**2**	**3**	**Smear cheese, Canada**	Alper et al., 2011
73	A	5	4	1	1	1	1	1	4	Smear cheese, France	Gente et al., 2002a,b, 2006
74	B	4	6	1	4	2	1	3	5	Grass, France	Gente et al., 2002a,b, 2006
75	B	4	7	1	5	2	1	4	4	Corn silage, France	Gente et al., 2002a,b, 2006
76	A	5	8	1	1	1	1	2	4	Milk, France	Gente et al., 2002b, 2006
77	**A**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**Milk, France**	Gente et al., 2002b, 2006
244	**C**	**4**	**9**	**3**	**3**	**2**	**4**	**2**	**6**	**Milk, Canada**	Lavoie et al., 2012
317	**B**	**4**	**10**	**4**	**4**	**2**	**3**	**2**	**7**	**Organic milk, Canada**	Lavoie et al., 2012
436	A	2	2	1	2	1	1	2	2	Industrial strain–company A	Alper et al., 2011
562	A	5	4	1	1	1	1	1	4	Milk, MPP 1, Canada	Lavoie et al., 2012
563	**A**	**5**	**4**	**1**	**1**	**1**	**1**	**1**	**4**	**Milk, Canada**	Lavoie et al., 2012
645	A	5	4	1	1	1	1	1	4	Smear cheese, Canada	Lavoie et al., 2012
655	A	5	4	1	1	1	1	1	4	Mold-ripened cheese, Canada	Lavoie et al., 2012
664	B	4	11	4	4	2	1	2	4	Milk, MPP 1, Canada	Lavoie et al., 2012
690	B	4	12	5	6	2	1	4	7	Milk, MPP 3, Canada	Lavoie et al., 2012
1024	A	3	13	5	1	1	1	1	1	Industrial strain–company B	Alper et al., 2011
1025	A	3	13	5	1	1	1	1	1	Industrial strain–company B	Alper et al., 2011
1026	A	4	14	1	1	1	1	2	8	Industrial strain–company B	Alper et al., 2011
1028	**A**	**2**	**2**	**1**	**2**	**1**	**1**	**2**	**2**	**Industrial strain–company A**	Alper et al., 2013
1031	A	3	13	5	1	1	1	1	1	Industrial strain–company A	Alper et al., 2013
1032	A	3	13	5	1	1	1	1	1	Industrial strain–company A	Alper et al., 2013
1033	A	3	13	5	1	1	1	1	1	Industrial strain–company A	Alper et al., 2013
1034	A	2	2	1	2	1	1	2	2	Industrial strain–company A	Alper et al., 2013
1035	C	1	15	3	4	2	2	2	3	Industrial strain–company A	Alper et al., 2013
1036	A	2	2	1	2	1	1	2	2	Industrial strain–company A	Alper et al., 2013
1037	A	3	13	5	1	1	1	1	1	Industrial strain–company A	Alper et al., 2013
1038	A	5	4	1	1	1	1	1	4	Industrial strain–company A	Alper et al., 2013
1039	A	5	4	1	1	1	1	1	4	Industrial strain–company A	Alper et al., 2013
1040	A	5	4	1	1	1	1	1	4	Industrial strain–company A	Alper et al., 2013
1041	A	3	1	1	1	1	1	1	1	Industrial strain–company A	Alper et al., 2013
1146	**A**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**Artisanal sheep cheese, Slovakia**	**CBS 11176**

[a]*MPP: Milk Production Plant. Bold: Strains sequenced in this study.*

TABLE 2 | Identification and origins of the *Galactomyces* spp. strains.

Species	Strain	Origin	References
Galactomyces geotrichum	1147	Soil extract, Puerto Rico	CBS 772.71
Galactomyces reessii	1148	Cold water retting, Indonesia	CBS 179.60
Galactomyces citri-aurantii	1150	Orange orchard soil extract, California	CBS 175.89

(Thermo Fisher Scientific Inc., Wilmington, NC, United States). The purified DNA was stored at −80°C until used.

Isolation of Arthrospores, Phenotype Assessment, Growth at 35°C and Assimilation of Carbon Compounds

The arthrospores of eight *G. candidum* and three *Galactomyces* spp. strains were isolated from the mycelium with a sterile cotton swab using 0.5% (*v/v*) Tween 80, suspended in YNB starvation broth [6.7 $g{\cdot}L^{-1}$ of Bacto Yeast Nitrogen Base without amino acids (BD Diagnostics) and 100 $g{\cdot}L^{-1}$ of D-glucose (EMD Chemicals)], and incubated for 48 h at 25°C in the dark (Kurtzman et al., 2011b). Microscopic images were taken, and spore concentration was determined using a hemocytometer and optical microscopy (BX61, Olympus). Thereafter, the solution was diluted with sterile carbon-free YNB solution to obtain a concentration of $1{\cdot}10^6$ $spores{\cdot}ml^{-1}$. In order to compare the morphology between strains, isolated colonies were re-streaked in three points on YEG agar plates and photographed after 7 days of incubation at 25°C using a standardized photography setup (Pitt and Hocking, 2009).

Their ability to grow at 35°C was evaluated by inoculating *G. candidum/Galactomyces* spp. strains on GYP agar plates [40 $g{\cdot}L^{-1}$ of D-glucose (EMD Chemicals), 5 $g{\cdot}L^{-1}$ of yeast extract (Thermo Fischer Scientific), 5 $g{\cdot}L^{-1}$ of Bacto peptone (BD Diagnostics) and 20 $g{\cdot}L^{-1}$ of Bacto agar (BD Diagnostics)] for 4 days at 35°C in sealed plastic bags (containing O_2) immersed in a water bath to ensure a constant temperature (Kurtzman et al., 2011b). Plates were inoculated in a single point with 1 µl from the starved and diluted yeast cell suspension ($1{\cdot}10^6$ $spores{\cdot}ml^{-1}$). Growth was assessed by the presence or the absence of a colony. Preliminary screening of carbon compound assimilation abilities was performed on the same 11 *G. candidum/Galactomyces* spp. strains using YT MicroPlates (BIOLOG) according to the manufacturer's protocol, with the following modifications. All wells of the microplate were filled with 100 µl of the starved and diluted yeast cell suspension ($1{\cdot}10^6$ $spores.ml^{-1}$). Plates were incubated in the dark for 10 days at 25°C in unsealed plastic bags to avoid drying out of the wells. *Yarrowia lipolytica* LMA-23 was used as a positive control, while the negative control consisted of sterile distilled water. Yeast growth was determined after 3 and 10 days by optic density (OD) measurement with a BioTek microplate reader Synergy HI and Gen5^{TM} software v2.07. A scan area measurement of the OD was done at a wavelength of 590 nm. Carbon compounds, namely adonitol (Acros Organics), D-arabitol (Acros Organics), D-(-)-amygdalin (Acros Organics), bromosuccinic acid (Sigma-Aldrich), α-ketoglutaric acid (Sigma-Aldrich), L-(-)-malic acid (Acros Organics), L-glutamic acid (MP Biomedicals), for which variations in assimilation profiles were observed between strains (data not shown) were selected for further tests. Additional carbon sources, namely lactose (Laboratoire MAT), D-glucono-1,5-lactone (Sigma-Aldrich), sodium citrate (BDH), sodium DL-lactate (Sigma-Aldrich) were also selected because their assimilation allows the formal identification of *Geotrichum* and *Galactomyces* strains at the species level and because of their relevance in the dairy environment (de Hoog and Smith, 2011; Hill and Kethireddipalli, 2013). Each carbon source was added at 0.5% concentration (w/v) in 4.9 ml of YNB culture media. For acidic or basic compounds, the pH of the solutions was adjusted between 5.2 and 5.6, with HCl (1N) or NaOH (1N). Spores of all *G. candidum* and *Galactomyces* spp. strains (**Tables 1**, **2**) were then inoculated as described for the YT MicroPlates protocol, in triplicate, using the standard method of Wickerham and Burton and incubated for 21 days at 25°C in the dark (Wickerham and Burton, 1948; Kurtzman et al., 2011b). A 15 s vortex agitation was done on each test tube at days 7 and 21. Growth evaluation was determined by eyes using the descriptors established by Kurtzman et al. (2011b).

Library Preparation, DNA Sequencing and Genome Assembly

Sequencing library construction was performed from 50 ng of total genomic DNA using the Illumina Nextera DNA library preparation kit (Illumina, San Diego, CA, United States) and Nextera index kit (Illumina), according to the manufacturer's instructions. Purified libraries were quantified using PicoGreen (Promega, Madison, WI, United States), diluted at 2 µM, multiplexed and sequenced on the Illumina HiSeq 2000 platform (101 bp paired-end reads). For strain LMA-244, the Illumina MiSeq platform (300 bp paired-end reads) was also used. FASTQ file generation and demultiplexing were performed using bcl2fastq v1.8.4 (Illumina, San Diego, CA, United States). Both single- and paired-end reads generated were quality filtered using FastQc v0.11.4 and trimmed with Trimmomatic v0.35 in order to cut adapters or indexes, to remove low quality bases (quality score <10) from the end of the reads, and to discard reads below 21 nt (Andrews, 2010; Bolger et al., 2014). Reads that remained unpaired were retained as single-end reads. Genome assembly was performed using SPAdes v3.11.1 (Bankevich et al., 2012). The genome of strain CLIB 918 as reference was used for the assembly of the eight *G. candidum* genomes (Morel et al., 2015) and *de novo* sequencing and assembly was performed for the *Galactomyces* strains. Based on the optimization of the total number of contigs, the number of contigs ≥1,000 bp and the N50 value, the k-mer option -k 21, 33, 55, 77 were used for all strains.

Scaffolds were filtered using the khmer software with a length cut-off of 1,000 bp (Crusoe et al., 2015). Genome assembly statistics and completeness were assessed using QUAST web interface and BUSCO v3 (Gurevich et al., 2013; Waterhouse et al., 2017). BUSCO measures the completeness of a genome assembly by comparing predicted genes in the assembly to a dataset of near-universal single-copy orthologs selected from OrthoDB v9 (Waterhouse et al., 2017).

Genomic Content Comparison

A measure of the similarity between all the strains was generated by comparing the k-mer (31 nt) content (presence or absence) of the whole assembled genomes (scaffolds) using Ray Surveyor software, which is a functionality of Ray v3.0.0 (Deraspe et al., 2017). Considering the set of all 31 bp k-mers of an assembled genome i, $A_i = (k_1, k_2, \ldots, k_{IA})$, and the set of all 31 bp

k-mers of an assembled genome *j*, $A_j = (k_1, k_2, \ldots, k_{lB})$, the set of all 31 bp k-mers shared between the assembled genomes *i* and *j* is defined as $K_{i,j} = (A_i \cap A_j)$. In order to have comparable values because of the varying genome sizes of the *G. candidum* and *Galactomyces* spp. strains, the normalized count of all 31 bp k-mers shared between the assembled genomes *i* and *j*, defined as $K'_{i,j} = \frac{|k_{i,j}|}{\sqrt{|k_{ii}| \times |k_{jj}|}}$, was rather used. Hence, a similarity heatmap was generated with gplots packages in the R environment using the values of the normalized set $K'_{i,j}$ of shared 31 bp k-mers for all pairwise strain combinations (Warnes et al., 2016). These normalized set of values were also transformed in Euclidean distance metrics with the distance matrix computation function in the R environment and used to generate a UPGMA dendrogram with MEGA v6 (Tamura et al., 2013; R Development Core Team, 2015).

MLST Loci Selection

Two MLST schemes were developed and published independently for *G. candidum* isolates typing. MLST2013 targets six housekeeping genes coding for an alanyl-tRNA synthetase (*ALA1*), a pyruvate kinase (*CDC19*), an acetyl-CoA acetyltransferase (*ERG10*), a glutaminyl-tRNA synthase (*GLN4*), a phosphoglucoisomerase (*PGI1*), and a phosphoglucomutase *(PGM2*) (Alper et al., 2013). MLST2017 targets five loci coding for a nucleoporin (*NUP116*), a dihydroorotate dehydrogenase (*URA1*), an oritidine-5′-phosphate decarboxylase (*URA3*), a yeast subtilisin-like protease III (*SAPT4*), and a phospholipase B (*PLB3*) (Jacques et al., 2017).

All 11 loci targeted in MLST2013 and MLST2017 were amplified for the eight isolates of *G. candidum* (identified in bold in **Table 1**), using the primers available in **Table 3** and the following parameters: initial denaturation step of 2 min at 94°C, followed by 35 cycles (denaturation 30 s at 94°C, annealing for 30 s at 56°C and extension of 1 min at 72°C) and a final extension of 3 min at 72°C. PCR products were visualized by electrophoresis on 0.8% agarose gel, stained with Gel Red, and illuminated under UV light. For all primer sets, a single band was obtained. PCR fragments were sequenced using an ABI 3730×l Data Analyzer (Thermo Fisher Scientific, Waltham, MA, United States) at the Plate-forme de séquençage et de génotypage des génomes (Centre de recherche du CHU de Québec, Université Laval, Québec, Canada).

The dendrogram obtained with Ray Surveyor software was used as a reference to compare the phylogenetics trees constructed with the MLST2013 and MLST2017 schemes. Once the optimum consensus MLST scheme was designed, all six target loci (*ALA1, CDC19, SAPT4, GLN4, PGI1,* and *PGM2*) (**Table 3**) were amplified, using the same PCR conditions, on an additional set of 33 *G. candidum* strains (**Table 1**).

MLST Data Treatment and Bioinformatics Analysis

The Staden Package was used for alignment, edition and construction of consensus sequences based on the ABI sequence chromatograms (Staden, 1996). Consensus sequences were entered in Unipro UGENE v1.31.1 software and multiple sequence alignments were performed using MUSCLE (Edgar, 2004; Okonechnikov et al., 2012). Sequences were trimmed to be in frame and encode the same number of amino acids. Evolutionary histories were inferred using the PhyML Maximum-likelihood method with bootstrap test of 1,000 replicates (Felsenstein, 1985).

For the new consensus MLST scheme (MLST2019), the number of polymorphic sites, the dN/dS ratio (dN is the number of non-synonymous substitutions per non-synonymous site and dS is the number of synonymous substitutions per synonymous site), and the average number of nucleotide differences between populations were determined using DnaSP software v6 (Rozas et al., 2017). The allelic profile of each locus was determined based on nucleotide polymorphisms. The allelic profile of each locus was then assigned a unique number in order of discovery (e.g., *Ala1-1, Ala1-2*. . .). Afterward, each unique combination of six loci numbers (1-1-1-1-1-1) was used to determine the sequence type (ST; e.g., ST1) of each strain (Jolley and Maiden, 2014).

Phylogenetic Analysis

The MLST2019 data were used to predict the population structure of *G. candidum* by using the STRUCTURE v2.3.4 software (Pritchard et al., 2000). Analyses were performed with a length of Burnin period of 80,000 and 80,000 rounds of calculation after the Burnin period. The number of assumed populations was determined by running simulations with $K = 2$ up to $K = 10$ with five iterations for each assumed population (Porras-Hurtado et al., 2013), and was best estimated when the log (probability of data) ceased to increase rapidly. The population structure was then calculated with the optimal ancestral subpopulation ($K = 5$) with default parameters, including 30 iterations of the analysis for $K = 5$. Recombination analysis was performed for the whole data set, within and between the Geo complexes using the NeighborNet method and the *phi*-test for recombination implemented in the software SplitsTree v4 (Bryant and Moulton, 2004; Huson and Bryant, 2005). Additionally, the linkage disequilibrium between and within the loci was verified with the Index of Association (I_A) and the Standardized Index of Association ($I_A{}^S$) using the LInkage ANalysis (LIAN) software v3.7 (Smith et al., 1993; Haubold and Hudson, 2000).

Data Availability

Raw sequence data for the Whole Genome Shotgun projects were deposited at GenBank under the Bioprojects accession numbers PRJNA482576 for LMA-40, PRJNA482605 for LMA-70, PRJNA482610 for LMA-77, PRJNA482613 for LMA-244, PRJNA482616 for LMA-317, PRJNA490507 for LMA-563, PRJNA482619 for LMA-1028, PRJNA490528 for LMA-1146, PRJNA486748 for LMA-1147, PRJNA486749 for LMA-1148, and PRJNA486756 for LMA-1150.

MLST partial gene sequences were deposited at GenBank under the accession numbers from MH745581 to MH745588 for *ALA1*, from MH745589 to MH745596 for *CDC19*, from MH745597 to MH745604 for *ERG10*, from MH745605 to MH745612 for *GLN4*, from MH745613 to MH745620 for *NUP116*, from MH745621 to MH745628 for *PGI1*, from

TABLE 3 | Primer sequences of 11 MLST target loci.

Locus	Primer sequence 5′ → 3′	Locus size (bp)	References
ALA1	Fwd-GCTCTTCGTGAGGTTCTTGG	424	Alper et al., 2013
	Rev-ACCTCGTAGGCATCAGTGCT		
CDC19	Fwd-CGCCAGTCAGAGAAGGAATA	355	Alper et al., 2013
	Rev-GTCGACCTGGTTCTTGACAC		
ERG10	Fwd-AACACAACATTTCCCGTGAG	652	Alper et al., 2013
	Rev-AGAGCTTAGCGTCAGCACTGA		
GLN4	Fwd-TGTTCTCAGAGGGTTTCCTG	558	Alper et al., 2013
	Rev-CCACATCTGAGGATTGTCGT		
PGI1	Fwd-ACCGCTGAGACTCTTCGCAA	472	Alper et al., 2013
	Rev-CTCCATGGAAAGCTGCTGGA		
PGM2	Fwd-GAACGGTGTCTACGGTCTTG	548	Alper et al., 2013
	Rev-TCAATGTACAGACGGATGGTC		
NUP116	For-ACCGCTACAACTGGATTTGG	425	Jacques et al., 2017
	Rev-GAGACCTGTTTGAGGGCTTG		
URA1	For-CAAGCCAATTGTGCTGAGAA	465	Jacques et al., 2017
	Rev-GGTGTCGTAGGGCAGTTGAT		
URA3	For-GCCAAAAAGACCAACCTGTG	470	Jacques et al., 2017
	Rev-CCTCATCCATACGGTTCTGC		
SAPT4	For-ATCATTAACACCCCGGCATA	501	Jacques et al., 2017
	Rev-GTGTCACCAAGCAGAGCAAA		
PLB3	For-AAGAATATCTGGGATCTTTC	393	Jacques et al., 2017
	Rev-TGAAGAAGAAGTACCAAGAA		

MH745629 to MH745636 for *PGM2*, from MH745637 to MH745644 for *PLB3*, from MH745645 to MH745685 for *SAPT4*, from MH745686 to MH745693 for *URA1*, and from MH745694 to MH745701 for *URA3*.

RESULTS

Colony Morphology

Macroscopic and microscopic observations on YEG culture media revealed three different morphotypes among the 11 *G. candidum* and *Galactomyces* spp. strains (i.e., yeast-like, intermediate or mold-like morphotypes) (**Figure 1**), as previously described (Boutrou and Guéguen, 2005). Strains LMA-77, 563, 1028, and 1148 were characterized by mold-like, white, filamentous, resistant-to-the-touch and not-greasy colonies, with a predominance of vegetative hyphae, whereas LMA-1146 and 1150 were characterized by yeast-like, cream-colored, not resistant-to-the-touch and greasy colonies, with abundant production of arthrospores. Strains LMA-40, 70, 244, 317, and 1147 showed an intermediate morphology, characterized by white-felted, slightly resistant-to-the-touch and not-greasy colonies, with abundant production of arthrospores.

Carbon Assimilation Profile

These 11 *G. candidum* and *Galactomyces* spp. strains were previously identified by rDNA sequencing (Alper et al., 2011). To confirm their identification at the species level, we performed carbon assimilation tests in YT MicroPlates and growth tests at 35°C on GYP agar (**Table 4**). Most of the carbon compounds tested showed similar assimilation profiles for all *G. candidum* and *Galactomyces* spp. strains. A positive assimilation was observed for glucose, galactose, sorbose, xylose, glycerol, and succinate. A negative assimilation was observed for inuline, sucrose, raffinose, melibiose, lactose, trehalose, maltose, methyl-glucoside, cellobiose, salicin, rhamnose, ribose, gluconate, glucosamine, and xylitol. A variable assimilation profile was observed for D-arabitol, α-ketoglutarate, malate, L-arabinose, adonitol, citrate, gluconolactone, lactate, and bromosuccinate. Among these variable assimilation profiles, α-ketoglutarate and malate could be used to distinguish *G. candidum* strains (*G. candidus*) from other *Galactomyces* species. Additionally, all *G. candidum* strains showed variable assimilation profiles for five carbon compounds (adonitol, D-arabitol, α-ketoglutaric acid, malic acid, and sodium citrate) (**Figure 2**, right panel). Apart from strain LMA-1028, all *G.* candidum strains grew positively at 35°C. On the contrary, none of the three *Galactomyces* spp. strains grew at this temperature (Data not shown).

Sequencing, Genome Assembly and Genomic Content Comparison

The statistics for the eight *G. candidum* genome assemblies (**Table 1** in bold) were highly variable, except for the assembly size, the GC content and the BUSCO completeness (**Table 5**). Genome sizes varied from 23.1 Mb (LMA-40) to 24.2 Mb (LMA-70), with an average GC content of 42%. Similarly, the statistics for the three *Galactomyces* spp. *de novo* assemblies were highly variable depending on the species, except for the BUSCO completeness. The genome length varied between 22 Mb (*Galactomyces geotrichum* LMA-1147) and 26 Mb (*Galactomyces*

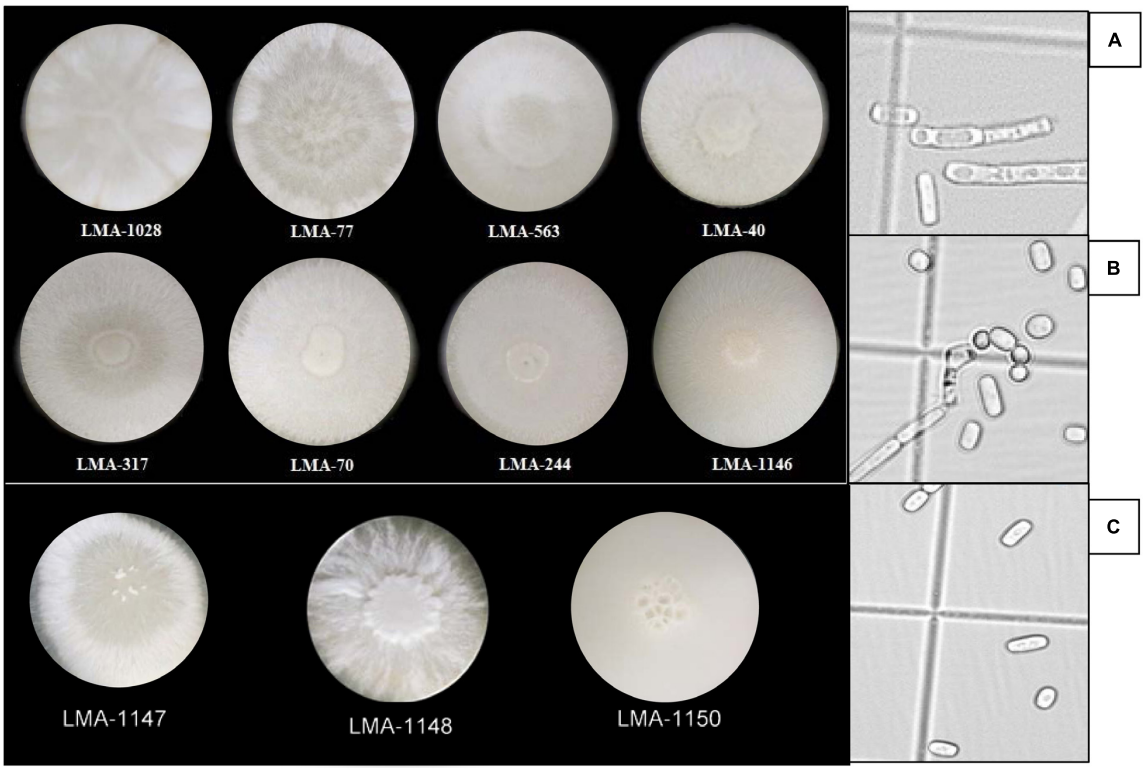

FIGURE 1 | Morphology of *Geotrichum candidum* and *Galactomyces* spp. strains on YEG culture media. (Left) Macroscopic observation of colonies. (Right) Microscopic observation of arthrospores. **(A)** Mold-like (example: LMA-1028). **(B)** Intermediate (example: LMA-244). **(C)** Yeast-like (example: LMA-1146).

citri-aurantii LMA-1150), and the GC content between 37% (*Galactomyces reessii* LMA-1148) and 40% (*G. geotrichum* LMA-1147). Although different strains and/or species were assembled, for all partial genomes, the BUSCO completeness percentage was close to 90% which suggests a good quality draft genome for all isolates sequenced.

The similarity among the partially assembled genomes of the *G. candidum* and *Galactomyces* spp. strains was assessed by comparing their k-mer content, using the software implementation Ray Surveyor (Deraspe et al., 2017). The dendrogram generated from the $K'_{i,j}$ Euclidean distances and the heatmap representation of the normalized similarity values ($K'_{i,j}$) uncovered separate groups within the strains (**Figure 2**, left and center). First, the *G. candidum* strains (LMA-40, LMA-70, LMA-244, LMA-317, LMA-563, LMA-1028, and LMA-1146) were clearly separated from the other *Galactomyces* (strains LMA-1147, LMA-1148, and LMA-1150). These two groups shared 3% or less of their k-mer content. Even though *G. candidum* strains were closely related, they could be separated into three major groups based on their k-mer content (**Figure 2**).

Multilocus Sequence Typing Schemes for *G. candidum*

We observed similarities between both previous MLST schemes, according to the clustering of the strains (**Supplementary Figures S1**–**S3**). LMA-40, -77, -563, -1028, and -1146 were grouped together whereas LMA-70 and -244 were grouped together at the opposite side of the tree. On the other hand, LMA-317 clustered differently in the MLST2017 scheme compared to MLST2013. In MLST2017, LMA-317 clustered in a third group of isolates located between the other complexes. Also, for LMA-40, -77, -563, -1028, and -1146, clustering between strains of the same clade was different in the two schemes. This observation led us to evaluate the resolution level of both schemes (**Supplementary Figures S1**, **S2**) by constructing a maximum-likelihood phylogenetic tree for each scheme. We compared both trees to the dendrogram that we constructed using the distance matrix based on whole genome content (**Figure 2** left panel). Both MLSTs showed similar clustering to the one using whole genomic content, but none had the same precision as the whole genome comparison. Following these observations, we selected six (6) target loci from both MLST schemes to develop an improved genotyping method for *G. candidum* isolates (**Supplementary Figure S3**), named MLST2019. We selected the loci *ALA1, CDC19, SAPT4, GLN4, PGI1,* and *PGM2* because they clustered the isolates the same way as the shared k-mer comparison using the whole genomic content. We then genotyped 33 additional *G. candidum* strains using MLST2019.

We applied the MLST2019 scheme to a total of 41 strains of *G. candidum* (for which 40/41 were the same as MLST2013) and observed 15 STs distributed into three complexes, namely GeoA, GeoB, and GeoC (**Figure 3**). GeoA complex, supported

TABLE 4 | Carbon assimilation profile for the *G. candidum/Galactomyces* spp. strains.

Genus	***Geotrichum candidum***								***Galactomyces* sp.**		
Strain (LMA)	77	1146	1028	40	563	317	70	244	1147	1148	1150
Geo complex	A	A	A	A	A	B	C	C			
ST	ST1	ST1	ST2	ST4	ST4	ST10	ST3	ST9			
Substrate											
D-arabitol	W	+	−	+	−	+	−	+	−	−	−
α-ketoglutarate	W	+	W	+	W	+	−	−	−	−	−
Malate	+	W	W	W	W	+	W	+	−	−	−
Glucose	+	+	+	+	+	+	+	+	+	+	+
Inuline	−	−	−	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−
Melibiose	−	−	−	−	−	−	−	−	−	−	−
Galactose	+	+	+	+	+	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−	−	−	−	−
Trehalose	−	−	−	−	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	−	−	−	−	−
Methyl-glucoside	−	−	−	−	−	−	−	−	−	−	−
Cellobiose	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−
Sorbose	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	−	−	−	−	−	−	−	−	−	−	−
Xylose	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	−	−	−	−	−	−	−	−	−	−	W
Ribose	−	−	−	−	−	−	−	−	−	−	−
Glycerol	+	+	+	+	+	+	+	+	+	+	+
Adonitol	−	−	+	+	−	W	−	−	−	−	+
Mannitol	+	+	+	+	+	+	+	+	+	−	+
Succinate	+	+	+	+	+	+	+	+	+	+	+
Citrate	−	−	−	−	W	W	W	W	W	−	+
Gluconate	−	−	−	−	−	−	−	−	−	−	−
Glucosamine	−	−	−	−	−	−	−	−	−	−	−
Xylitol	−	−	−	−	−	−	−	−	−	−	−
Gluconolactone	+	+	+	+	+	+	+	+	+	−	W
Lactate	+	+	+	+	+	+	+	+	W	+	+
Bromosuccinate	+	+	−	+	+	+	+	+	N/A	N/A	N/A
Amygdalin	W	W	W	W	W	W	W	W	N/A	N/A	N/A
Glutamate	+	+	+	+	+	+	+	+	N/A	N/A	N/A

+: Positive assimilation; −: Negative assimilation; W: Weak assimilation; N/A: Not available.

by a bootstrap value of 1, contained 31 isolates, whereas GeoB complex, supported by a bootstrap value of 0.688, contained 6 isolates and GeoC complex, supported by a bootstrap value of 1, contained 4 isolates. Most *G. candidum* strains (31/41) clustered within GeoA, which includes all the commercial starters except LMA-1035 and all the cheese isolates except LMA-70. Furthermore, the GeoA isolates had either the filamentous, intermediate or yeast-like morphotype. The GeoB complex was composed of *G. candidum* isolates with an intermediate or yeast-like morphotype and these were divided into two sub-complexes, according to their isolation source. Dairy strains LMA-317, 664, and 690 isolated from milk produced in Canada clustered separately from the non-dairy strains LMA-48, -74, and -75, isolated respectively from a bioreactor, grass and corn silage (**Table 1**). The GeoC complex was composed of strains isolated from various environments and were not clustered according to their isolation source. These strains had either a filamentous (LMA-21) or an intermediate (LMA-70, -244, and -1035) morphotype on YEG agar. LMA-1035 was a commercial ripening starter, and LMA-21, -70, and -244 were respectively isolated from rotting carrots, smear cheese and milk (**Table 1**).

The combined sequences of the six loci used in the MLST2019 scheme generated a concatenated contig of 2,859 bp with 64 polymorphic sites. The number of polymorphic sites varied from 5 for *CDC19* to 18 for *GLN4* and consisted mostly of synonymous substitutions. The number of non-synonymous substitution sites varied between 1 and 2 for most loci and up to 4 sites for the *PGI1* locus. The allelic profile for each locus was 2 for *SAPT4*, 4

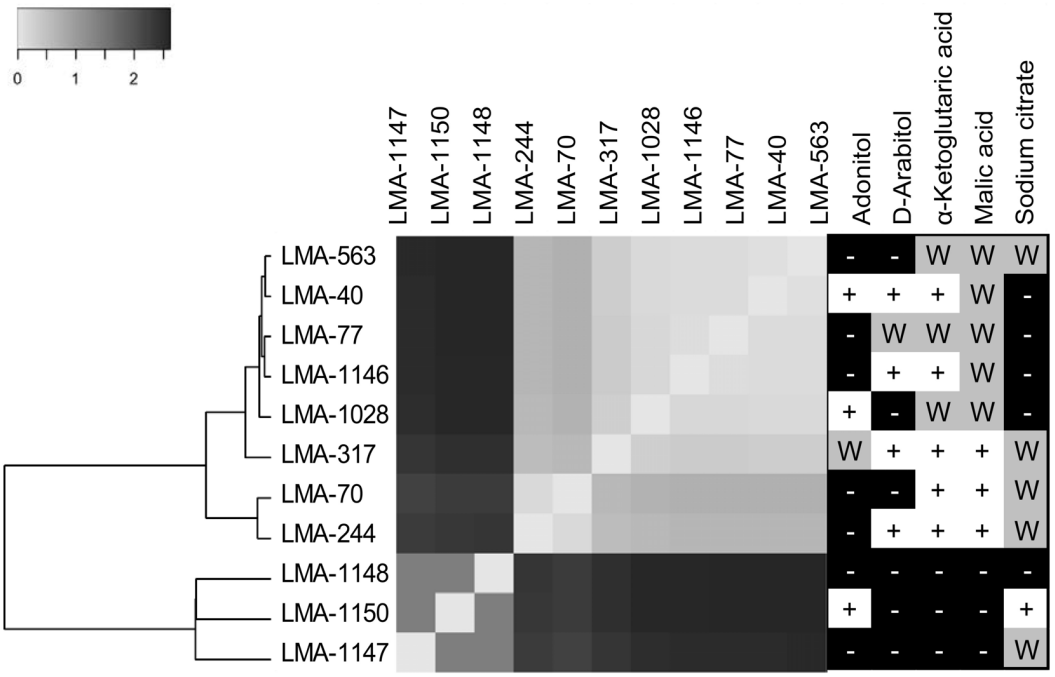

FIGURE 2 | Genomic similarity of three *Galactomyces* spp. and eight *G. candidum* strains represented by a heatmap obtained from the Euclidean distance between the normalized set of k-mers (31 bp fragments) shared between the partial genomes of *Galactomyces* spp. and *G. candidum* strains. Phenetic tree analyses were conducted in MEGA6. (Right panel) Ability of *G. candidum/Galactomyces* spp. strains to grow on carbon compounds (+ for positive assimilation, − for negative assimilation, and W for weak assimilation).

TABLE 5 | Assembly statistics for the genome of *Geotrichum candidum* and *Galactomyces* spp. strains.

LMA strain	Mean coverage	Assembly size (Mb)	Total no. of scaffolds	Scaffold N50 (bp)	Longest scaffold (bp)	GC content (%)	BUSCO Completeness (%)
40	63×	23.10	1,268	32,639	167,475	41.8	90.3
70	70×	24.17	1,450	44,909	195,322	41.4	90.2
77	58×	23.32	1,488	29,977	158,856	41.7	90.5
244	114×	23.20	836	137,000	210,021	41.5	90.3
317	19×	23.36	1,370	35,529	209,353	41.8	90.3
563	49×	23.30	1,427	31,326	124,845	41.7	89.9
1028	34×	23.42	1,332	36,124	191,607	41.7	90.5
1146	54×	23.35	1,363	32,892	152,162	41.7	90.3
1147	64×	21.96	3,121	13,090	102,582	40.4	89.8
1148	105×	22.56	2,775	13,420	64,496	36.6	89.5
1150	55×	25.98	2,668	16,289	69,389	39	90.9

for *GLN4* and *PGI1*, 5 for *ALA1*, 6 for *CDC19*, and 8 for *PGM2*. The proportion of variable sites varied between 1.27 and 3.23%. For each locus, the number of alleles was always lower than the number of polymorphic sites, which resulted in a number of variable alleles per variable sites ranging from 0.13 to 1.20. The dN/dS ratio was always close to 0 and mostly lower than 0.2, except for *PGI1* (**Table 6**).

Evaluation of the Population Structure of *G. candidum*

Using MLST2019 ST profiles in STRUCTURE software, we statistically estimated the number of ancestral genotypes (K) of the yeast *G. candidum*. The STs were clustered in 5 subpopulations ($K = 5$), called lineages or clades (1, 2, 3, 4, and 5) (**Figure 4** and **Table 1**). When compared to MLST2019 clusters (GeoA, B, and C), lineages 2, 3, and 5 form the GeoA complex while lineage 1 corresponds to GeoC. The lineage 4 corresponds mostly to GeoB except for strains LMA-1026 and LMA-244, which come from GeoA and GeoC, respectively (**Figure 3** and **Table 1**). The subpopulations observed did not seem to cluster the strains either by their ability to assimilate carbon sources, their colonial morphology or their isolation source, although lineages 2, 3, and 5 do not contain environmental isolates (**Table 1** and **Figure 4**).

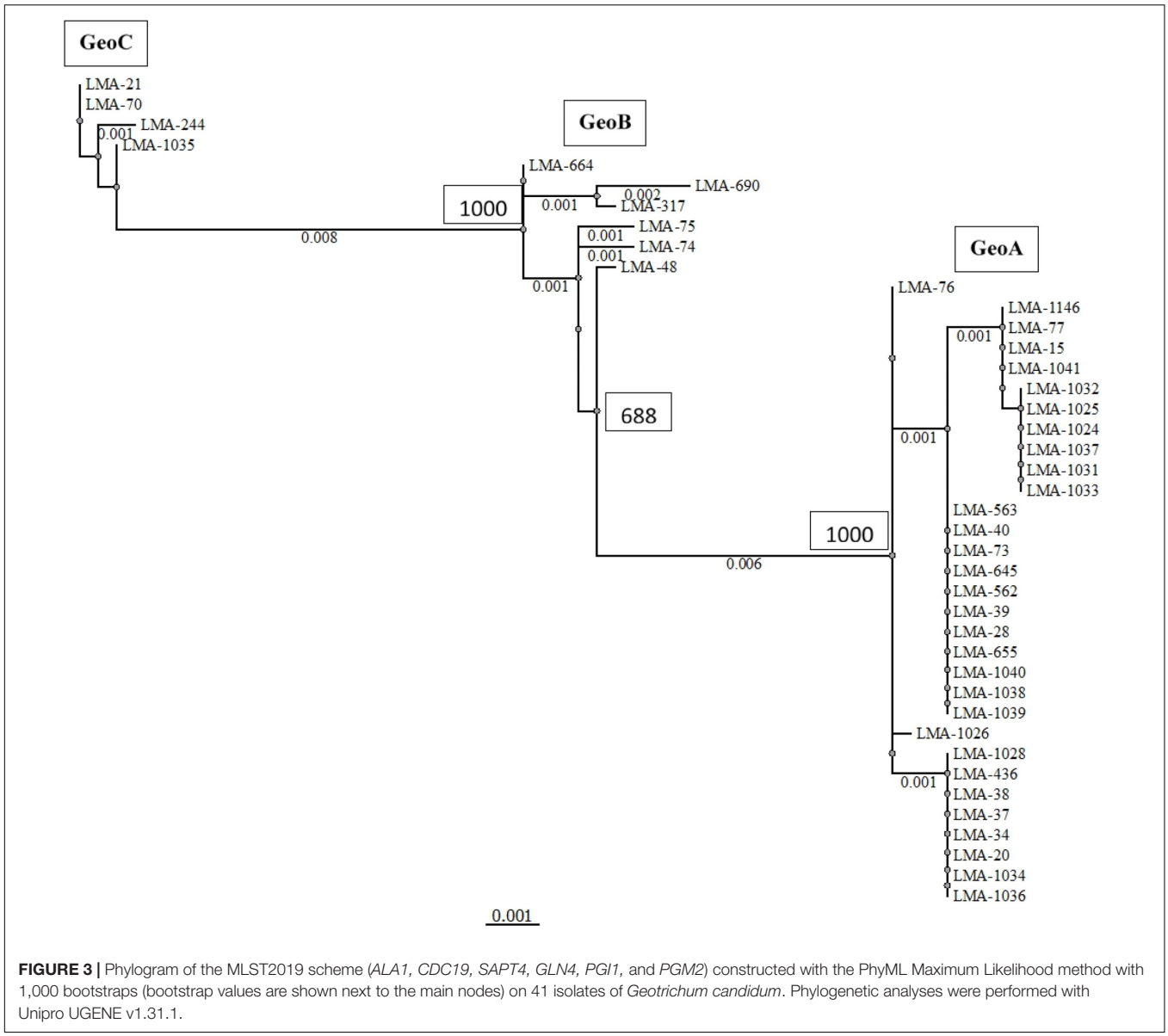

FIGURE 3 | Phylogram of the MLST2019 scheme (*ALA1, CDC19, SAPT4, GLN4, PGI1,* and *PGM2*) constructed with the PhyML Maximum Likelihood method with 1,000 bootstraps (bootstrap values are shown next to the main nodes) on 41 isolates of *Geotrichum candidum*. Phylogenetic analyses were performed with Unipro UGENE v1.31.1.

TABLE 6 | Loci characteristics targeted in the consensus MLST scheme used to genotype 41 isolates of *G. candidum*.

Locus	Locus size (bp)	Number of polymorphic sites*	Number of alleles	Proportion of variable sites (%)	Number of alleles per variable site	dN/dS
ALA1	424	8 (1)	5	1.89	0.63	0.000
CDC19	355	5 (1)	6	1.41	1.20	0.171
SAPT4	501	16 (2)	2	3.19	0.13	0.079
GLN4	558	18 (2)	4	3.23	0.23	0.028
PGI1	472	6 (4)	4	1.27	0.67	0.616
PGM2	548	11 (1)	8	2.01	0.73	0.048

**Numbers in parentheses are the number of non-synonymous substitution sites detected in the locus.*

All MLST2019 loci and Geo complexes were investigated for genetic exchanges by a NeighborNet analysis, based on nucleotide sequences. The six targeted loci (*ALA1, CDC19, SAPT4, GLN4, PGI1,* and *PGM2*) showed a tree-like subpopulation structure, which suggests that the allele diversity is due to clonal descent within a given locus rather than recombination (**Supplementary Figure S4**). The NeighborNet analysis was then performed for GeoA, GeoB, and GeoC complexes separately,

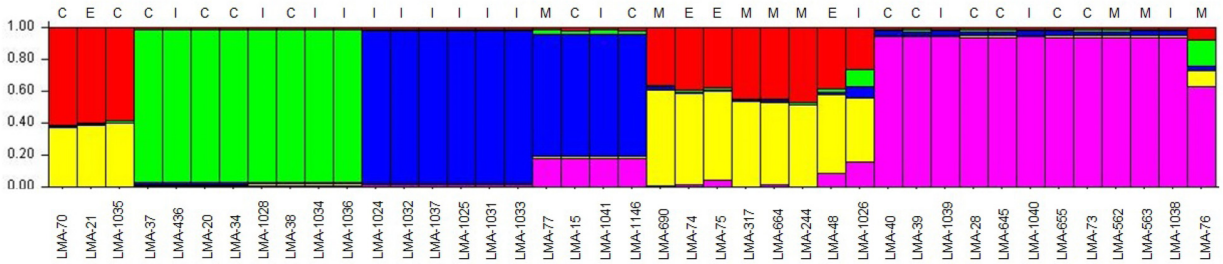

FIGURE 4 | Estimation of the population structure of 41 *G. candidum* strains based on their sequence type profile. For each of the strains, on the abscissa, the estimated contribution, on the ordinate, to its genotype from each of five hypothetical ancestral genotypes is represented by bar plot of different colors. The source of isolation of each strain is shown above the histogram: cheese strain (C), environmental strain (E), industrial strain (I), milk (M). Each color represents a lineage in the *G. candidum* population. Lineage 1 (60% red and 40% yellow), lineage 2 (green), lineage 3 (blue), lineage 4 (close to 60% yellow and 40% red), and lineage 5 (purple).

for all pairs of complexes GeoA-B, GeoB–C, and GeoA–C, and for all complexes together GeoA–B–C (**Figure 5**). Tree-like structures were observed within GeoA, and GeoC complexes, and between GeoA and GeoC. The GeoB individual complex and all other comparisons (GeoA–B, GeoB–C, and GeoA–B–C) showed parallelogram-shaped (network-like) structures, suggesting intergenic recombination (data not shown). The recombination hypothesis was verified doing a *phi*-test in SplitsTree (**Table 7**; Bruen et al., 2006). The recombination events were supported statistically within the GeoB complex ($p = 0.03$), between the GeoA and the GeoB complexes ($p \leq 0.001$) and on the whole data set ($p \leq 0.001$) while the absence of recombination was statistically supported for the GeoA ($p = 1.0$), between GeoA and GeoC ($p = 0.2$) and between the GeoB and GeoC ($p = 0.3$). Because only four strains formed the GeoC complex, not enough data was available to perform the *phi*-test.

Finally, we verified the linkage disequilibrium of all loci within and between the Geo complexes by using the value of the Index of Association (I_A) and the Standardized Index of Association ($I_A{}^S$) calculated using LIAN software. Both indexes measure the extent of linkage equilibrium within a population by quantifying the amount of intergenic recombination and by detecting association among alleles at different loci (Agapow and Burt, 2001). If the value of the indexes differs significantly from zero, it is a sign of linkage disequilibrium and of a clonal population, while a value of 0 is a sign of linkage equilibrium due to frequent recombination (Xu et al., 2014). The $I_A{}^S$ values were close to 0 for GeoB, GeoA–B, GeoB–C, and GeoA–B–C complexes, and were significantly different from 0 for GeoA (0.4, $p = 0.5$), GeoC (-0.13, $p = 1$), and GeoA–C (0.37, $p \leq 0.0001$) complexes, confirming what was observed in the NeighborNet analysis (**Table 7**).

DISCUSSION

Phenotypic Identification and Characterization

The strains used in this study were successfully associated to *G. candidum* or a *Galactomyces* species following their phenotypic characterization (de Hoog and Smith, 2011). The strains LMA-1147 (*G. geotrichum*), LMA-1148 (*G. reessii*), and LMA-1150 (*G. citri-aurantii*) had the typical carbon assimilation profiles and the ability to grow at 35°C. Based on the same criteria, all other isolates except LMA-1028 were positively identified as *G. candidum*. It was also possible to distinguish *G. candidum* strains from the *Galactomyces* species, based on their weak or positive utilization of α-ketoglutaric acid and malic acid (**Table 4**), as already observed (Kurtzman et al., 2011a). In addition, the pairwise comparison of the shared k-mer content (31-mers) confirmed the identification and phylogeny of the four species studied (**Figure 2**).

The *G. candidum* strains displayed variability in the assimilation of a few carbon compounds (**Figure 2**, right panel) which could reflect subgroups with different functional traits. In contrast with previous reports, we found that LMA-40 can use adonitol and did not detect variable assimilation of DL-lactate and D-mannitol among the *G. candidum* strains (Marcellino et al., 2001; Alper et al., 2013). In addition, using traditional microbiological tests, we observed for the first time the variable assimilation of malic acid and α-ketoglutaric acid, the weak assimilation of amygdalin, and the positive assimilation of bromosuccinic acid in *G. candidum*. Some of the carbon compounds tested in this study can be metabolized during cheese ripening by different microorganisms, including *G. candidum* strains. Lactate assimilation by *G. candidum* during the early phase of cheese ripening has been described several times and is positively associated with deacidification of the cheese surface, which is mandatory for the growth of acid-sensitive bacteria (Fox and Law, 1991; Marcellino et al., 2001; Gripon, 2002; Leclercq-Perlat et al., 2004; McSweeney, 2004; Lessard et al., 2014; Arfi et al., 2005; Boutrou and Guéguen, 2005; Castellote et al., 2015; Dugat-Bony et al., 2015). Citrate metabolism in lactic acid bacteria leads to the production of CO_2 and diacetyl, which contributes to the typical aspect and flavor of Gouda, Cottage, Quark, and Cheddar cheeses (Fox and Law, 1991; Molimard and Spinnler, 1996; McSweeney, 2004). However, citrate utilization as the sole carbon source by *G. candidum* strains was found to be weak or negative (**Figure 2**, right panel), suggesting that metabolites produced by *G. candidum* through citrate catabolism would not be major contributors to the typical flavor of surface-ripened cheese

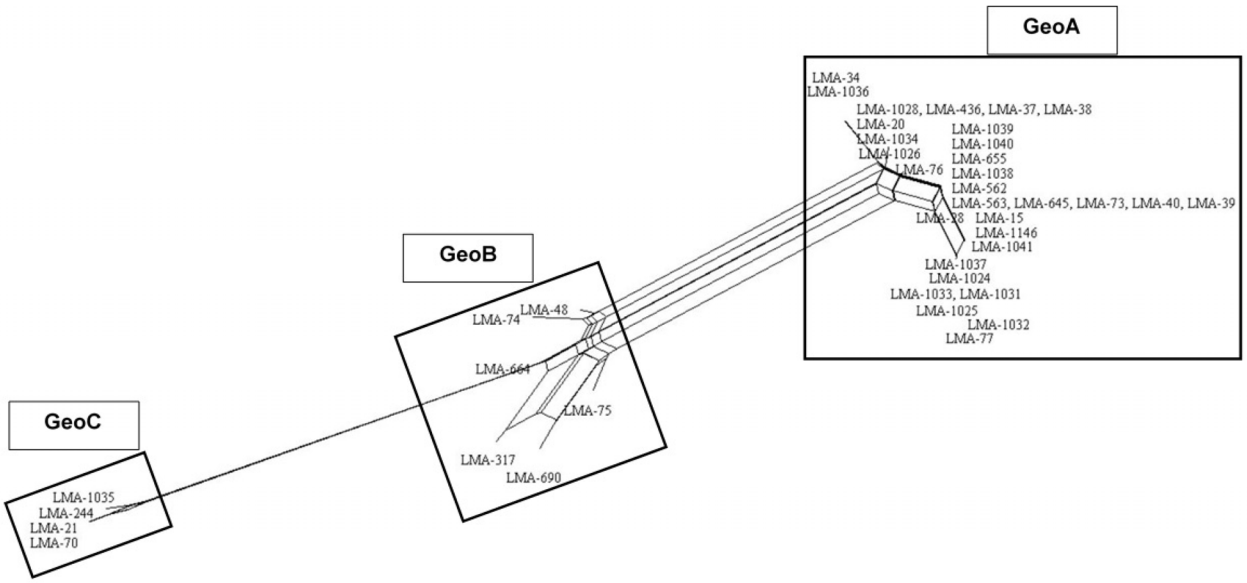

FIGURE 5 | NeighborNet analysis of the combined six loci (*ALA1, CDC19, SAPT4, GLN4, PGI1,* and *PGM2*) obtained from 41 *G. candidum* isolates. Numbering in the figure corresponds to sequence type. Parallelogram formation indicate recombination events. NeighborNet analysis was performed with SplitsTree version 4.

varieties (Marcellino et al., 2001; Leclercq-Perlat et al., 2004; Lessard et al., 2014). Glutamic acid, which can be consumed by all *G. candidum* strains tested, is produced through the transamination of L-methionine. This enzymatic reaction, in the presence of an amino-acid acceptor, usually the α-ketoglutaric acid, contributes to ammonia and aroma production by *G. candidum* strains during cheese ripening (Brennan et al., 2004; Boutrou and Guéguen, 2005; Cholet et al., 2007; Lessard et al., 2014; McSweeney, 2004). Finally, no relationship was observed between the genetic population structure and the phenotypic characteristics.

TABLE 7 | Recombination and linkage disequilibrium statistics.

Geo group	*Phi*	I_A	I_A^S	*p*-value
A	1.0	0.21	0.4	0.5
B	0.03	−0.33	−0.07	0.9
C	NA	−0.63	−0.13	1
A–B	≤0.001	0.32	0.06	0.05
B–C	0.3	−0.2	−0.04	0.8
A–C	0.2	1.9	0.37	0.0001
A–B–C	≤0.001	0.36	0.07	0.03

Genome Comparisons and MLST Analysis

Before this study, the only genome sequence of *G. candidum* available in public databases was for the strain CLIB 918, which was isolated from Pont-L'Evêque cheese (Morel et al., 2015). The draft genome assemblies for eight additional *G. candidum* strains presented in this study contained from 836 to 1,488 scaffolds (≥1,000 bp) with N50 values between 29,977 and 137,000 bp (**Table 4**). The high number of short scaffolds observed might arise from the short reads produced through the Illumina HiSeq technology and the presence of repeated regions, which are abundant in eukaryotic genomes (Paszkiewicz and Studholme, 2010; Yandell and Ence, 2012; Kuo et al., 2014). Using the *G. candidum* CLIB 918 draft genome sequence as a reference allowed major improvements in the scaffolding step for the *G. candidum* strains. The assembly size, N50 value and length of the longest scaffold increased with CLIB 918 as a reference, in comparison to assemblies without CLIB 918 (**Supplementary Table S1**). Overall, the three *Galactomyces* spp. draft genome assemblies provided similar estimations of the genome sizes and GC contents to those previously proposed using the thermal denaturation method (Smith et al., 1995). These assemblies contain close to 3,000 scaffolds (≥1,000 bp) with N50 value around 13,000 bp. The absence of an available reference genome for the scaffolding of *Galactomyces* spp. strains could explain the more fragmented assemblies, compared to those of *G. candidum* strains.

Sequencing the whole genome of eight *G. candidum* and three *Galactomyces* spp. strains allowed us to use the pairwise comparison of their shared k-mer content as the reference clustering method for optimizing current MLST schemes. Two previous MLST schemes were compared for the genotyping of a common set of eight *G. candidum* isolates (Alper et al., 2013; Jacques et al., 2017). Genotyping with MLST2013 gave rise to two groups of isolates, as previously obtained, whereas a third group was obtained with MLST2017 (Alper et al., 2013; Jacques et al., 2017). Although a few subgroups differ between MLST2013 and MLST2017, both schemes clustered LMA-70 and LMA-244 together and apart from the other *G. candidum* strains.

Considering that neither MLST2013 nor MLST2017 allowed the same sub-grouping as the shared k-mer content, a selection of six loci was used to create an optimized MLST scheme (MLST2019) that would increase the discrimination power between the isolates. The MLST2019 scheme uses the loci *ALA1*, *CDC19, SAPT4, GLN4, PGI1,* and *PGM2*, which, once concatenated, generate a sequence of 2,859 bp. The evolution rate was assessed using the dN/dS ratio for each locus (between 0.000 and 0.616) and suggests that they are not subjected to positive selection, making them suitable gene targets for MLST (Shabbir et al., 2013; Yu et al., 2015).

The evidence for genetic diversity could indicate an adaptive divergence, a population differentiation under selection, between the wild strains and the cheese ripening strains of *G. candidum*, as observed for other fungi used in the agri-food industry such as *Penicillium roqueforti* (Dumas et al., 2018), *Penicillium camemberti* (Cheeseman et al., 2014), and *Saccharomyces cerevisiae* (Legras et al., 2018). This hypothesis is supported by the observation that most of the non-dairy strains are clustered in GeoB and GeoC complexes, whereas GeoA complex contains all industrial cheesemaking strains studied here, except for LMA-1035, and other isolates from milk or cheese. When analyzing the population structure of the *G. candidum* isolates, we observed that the Geo complexes (**Figures 3**, **5**) were similar, but there was a discordance in comparison to the estimation of the ancestral subpopulations (lineages) (**Figure 4**). The five *G. candidum* lineages were clustered accordingly to MLST2019, except for linage 4 which contains all GeoB strains and includes LMA-244 and LMA-1026. According to the concatenated MLST loci and the whole genome sequences, these strains are part of GeoC and GeoA complexes, respectively (**Figures 3**, **5**). The fact that LMA-244 and -1026 share alleles with strains of the complex GeoB could hint at a speciation of environmental strains (GeoB) to form new complexes GeoA and GeoC (Dumas et al., 2018). Additionally, the calculation of I_A and ${I_A}^S$ indices, both close to 0, supports the hypothesis that the strains studied are in linkage equilibrium and that recombination did play a role in increasing the STs diversity, which was statistically demonstrated, except for GeoB–C (**Table 7**; Jolley et al., 2001; Cangi et al., 2016). In this particular case, the low number of strains (LMA-21, -70, -244, and -1035) in the GeoC complex might explain the discordance between both analysis. The isolation and genome sequencing of more strains clustering in GeoC would be needed to further characterize the species *G. candidum*. Moreover, a lower diversity was observed in the GeoA complex than in GeoB and GeoC, as represented by the fewer number of branches and their smaller size (**Figure 3**). A similar phenomenon has also been observed for domesticated *P. roqueforti* strains, for which cheese complexes had fewer and smaller branches compared to the other clusters (Dumas et al., 2018). Furthermore, GeoC isolates were distant from the GeoA/B complexes on a phylogenetic level, and isolates from the environment (LMA-21) and the dairy industries (LMA-70, -244, and -1235) were obtained for the GeoC complex. These findings suggest that the GeoC complex could represent a new pool of *G. candidum* strains with an interesting biotechnological potential for the development of cheese products with typical properties.

CONCLUSION

In conclusion, the aim of this study was to combine traditional microbiological techniques with new whole genome shotgun sequencing to illuminate the phylogenetic relationships among *G. candidum* and *Galactomyces* spp. strains of environmental and dairy origin. Thereby, we report the draft assembly of 11 *Geotrichum*/*Galactomyces* spp. strains, increasing the amount of genomic data available for *G. candidum* and other *Galactomyces* species, for which no genome sequence assemblies were available. These strains were identified based on their ability to grow under different conditions and we propose a new MLST scheme to optimally genotype *G. candidum* isolates, because it better represents the whole genomic content of this species and allows a clear visualization of its population structure. Further studies are needed to test the adaptive divergence hypothesis and future comparative genomics studies will shed light on the differences between GeoC and GeoA/B. Also, the sequencing of additional genome, especially from non-dairy niche, are likely to improve the new MLST scheme and give a better understanding of the diversity within each complex. Phenotypic and genomic tools such as whole genome sequencing and the MLST scheme substantially improve our knowledge on this ripening yeast and could ultimately allow a better selection and control of *G. candidum* strains throughout the ripening of cheeses.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the raw sequence data for the Whole Genome Shotgun projects were deposited at GenBank under the Bioprojects accession numbers PRJNA482576 for LMA-40, PRJNA482605 for LMA-70, PRJNA482610 for LMA-77, PRJNA482613 for LMA-244, PRJNA482616 for LMA-317, PRJNA490507 for LMA-563, PRJNA482619 for LMA-1028, PRJNA490528 for LMA-1146, PRJNA486748 for LMA-1147, PRJNA486749 for LMA-1148, and PRJNA486756 for LMA-1150. MLST partial gene sequences were deposited at GenBank under the accession numbers from MH745581 to MH745588 for ALA1, from MH745589 to MH745596 for CDC19, from MH745597 to MH745604 for ERG10, from MH745605 to MH745612 for GLN4, from MH745613 to MH745620 for NUP116, from MH745621 to MH745628 for PGI1, from MH745629 to MH745636 for PGM2, from MH745637 to MH745644 for PLB3, from MH745645 to MH745685 for SAPT4, from MH745686 to MH745693 for URA1, and from MH745694 to MH745701 for URA3.

AUTHOR CONTRIBUTIONS

VP, SV, M-HL, ED-B, MF, and SL were involved in planning the study and writing the manuscript. SV performed phenotypic characterization, DNA extraction, and genome assembly and

comparative genomics analysis. M-HL performed DNA libraries for HiSeq sequencing. VP performed DNA extraction for MLST schemes comparison, MLST analysis, and genome assembly and comparative genomics analysis. P-LP and JC helped with the development of the bioinformatic scripts used for genome analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00737/full#supplementary-material

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The Interrelationship Between Microbiota and Peptides During Ripening as a Driver for Parmigiano Reggiano Cheese Quality

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Cheese microbiota contribute significantly to the final characteristics of cheeses due to the growth and interaction between cheese microorganisms during processing and ripening. For raw milk cheeses, such as Parmigiano Reggiano (PR), the microbiota derive from the raw milk itself, the dairy environment, and the starter. The process of cheese making and time of ripening shape this complex ecosystem through the selection of different species and biotypes that will drive the quality of the final product by performing functions of their metabolism such as proteolysis. The diversity in the final peptide and amino acid composition of the cheese is thus mostly linked to the diversity of this microbiota. The purpose of this study was to get more insight into the factors affecting PR cheese diversity and, more specifically, to evaluate whether the composition of the bacterial community of cheeses along with the specific peptide composition are more affected by the ripening times or by the cheese making process. To this end, the microbiota and the peptide fractions of 69 cheese samples (from curd to cheese ripened 24 months) were analyzed during 6 complete PR production cycles, which were performed in six different dairies located in the PR production area. The relation among microbial dynamics, peptide evolution, and ripening times were investigated in this unique and tightly controlled production and sampling set up. The study of microbial and peptide moieties in products from different dairies – from curd to at least 12 months, the earliest time from which the cheese can be sold, and up to a maximum of 24 months of ripening – highlighted the presence of differences between samples coming from different dairies, probably due to small differences in the cheese making process. Besides these differences, however, ripening time had by far the greatest impact on microbial dynamics and, consequently, on peptide composition.

Keywords: cheese microbiota, cheese peptides, raw milk cheese, cheese ripening, Parmigiano Reggiano

INTRODUCTION

Cheese microbiota is a complex ecosystem that can originate from raw milk, acidifying starters and adjunct cultures, and adventitious microorganisms that may come from equipment and the cheese making plant environment. The cheese microbiota is then shaped by the cheese making steps and ripening, which cause a selective pressure on microorganisms. Given this framework, other authors have underlined the importance of studying cheese microbial dynamics to better understand their effects on both quality and safety (Kamimura et al., 2019). In particular, the succession of different microbial groups and their interaction during cheese making and ripening is fundamental for the development of the unique sensory characteristics of each cheese variety (Gobbetti et al., 2018; Afshari et al., 2020). In raw milk, long ripened hard cheeses, such as Parmigiano Reggiano (PR), lactic acid bacteria (LAB) play a major role both as starters (SLAB) in curd acidification and non-starter (NSLAB) during cheese-ripening (Gatti et al., 2014; Carafa et al., 2019; Martini et al., 2020). In addition to their fermentation performances, LAB have attracted remarkable interest in the last decades for their potential health benefits through the consumption of fermented foods (De Filippis et al., 2020), and many LAB species have recently been linked to the gut microbiota in a genome-wide analysis (Pasolli et al., 2020).

Parmigiano Reggiano (PR) is an internationally appreciated, protected designation of origin (PDO) Italian cheese, artisanally produced in multiple dairies in the PDO area. It is made under strict production regulation, but small differences in operational conditions in each dairy may occur. These result in different cheese microbiota compositions, metabolites and their inter-relationships that underpin specific cheese quality attributes (Gatti et al., 2014). During cheese ripening, a complex chain of events occurs that entails a set of biochemical reactions, and proteolysis is one of the most important. Proteolysis is initiated by the starter, continued by non-starters, and completed and tailored by the proteolytic enzymes released by the bacterial community. These events lead to changes in the specific peptide and amino acid composition that constantly evolves during the aging period. Sforza et al. (2012) correlated these trends in peptides' evolution to the enzymatic activities, thus allowing for the discrimination of cheeses according to their aging times. That said, the aim of this work was to get a deeper insight into the factors affecting PR diversity by evaluating whether the composition of the bacterial community and the specific peptide composition are more affected by the ripening times or by the cheese making process. To this end, the microbiota and the peptide fractions of 69 cheese samples (from curd to cheese ripened 24 months) were analyzed during 6 complete PR production cycles, which were performed in six different dairies. Bacterial dynamics were studied taking into account both total DNA extracted from cheeses and DNA from entire or lysed cells, thus permitting a better understanding of proteolysis and peptide evolution during PR cheese ripening in the context of a unique and tightly controlled production and sampling set up.

MATERIALS AND METHODS

Cheese Sampling

Cheese samples were obtained from the "Consorzio del Parmigiano-Reggiano" (Reggio Emilia, Italy). Six dairies (designated as A–F) located in the PR PDO production area were considered for this study. For each dairy, samples were taken from the acidified curd (48 h), after brining (1 month of aging) and after 6, 12, and 24 months. For dairy C, E, and F, samples were also taken at 2, 7, and 9 months. To evaluate the microbial and peptide dynamics over time, samples were taken at different ripening times from the same original wheel. Moreover, samples were taken for each dairy from different wheels with the same ripening times (**Figure 1** and **Table 1**). A total of 69 samples were collected: 48 h (6 curd samples), 1-month-old (6 cheese samples), 2-month-old (3 cheese samples), 6-month-old (27 cheese samples), 7-month-old (3 cheese samples), 9-month-old (3 cheese samples), 12-month-old (15 cheese samples) and 24-month-old (six cheese samples). For each dairy (A–F), samples were identified with the letter W followed by a number, indicating the sampled wheel, and a slash followed by a second number, indicating the stage of ripening (e.g.,: AW1/0 corresponds to dairy A, wheel 1, months of ripening 0, that is to say, the curd 48 h after cheese-making).

Cheeses were produced according to EU Regulation of PDO established by article 11 of regulation (EU) No. 1151/2012 (European Commission, 2012). According to this regulation, the cheese making procedure is the same for all the dairies with small variations due to operational conditions that may occur among the dairies, variations which were not considered as a relevant variable for the present study (Gatti et al., 2014). Milk from the previous day, partially skimmed by spontaneous floating, was mixed with fresh whole milk in copper vats. Calf rennet and natural whey starter were added. The curd was broken and heated at 55°C for about 50 min. Curd was then extracted and molded for 48 h, before brining. After 1 month, the wheels were extracted, washed, and ripened. Samples were obtained by coring, thus obtaining a transverse section for each wheel. Each entire section was grated and mixed before the analysis in order to have a sample representative of the whole wheel. Two separate aliquots were then prepared: one was immediately analyzed and one was kept at −20°C for the subsequent DNA and cheese water soluble extraction.

Microbial Counts

Ten grams of each grated cheese sample were suspended in 90 ml of 20 g/L trisodium citrate (pH 7.5) (Sigma–Aldrich, St. Louis, United States) and homogenized for 2 min in a blender (Seward, London, United Kingdom). Decimal dilutions of milk and homogenates were made in quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom) and spread-plated in triplicate on the appropriate medium described as follows. Cheese agar medium (CA) (Neviani et al., 2009) was used for the enumeration of cultivable NSLAB population, incubating plates at 37°C, while SLAB were counted on De Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom),

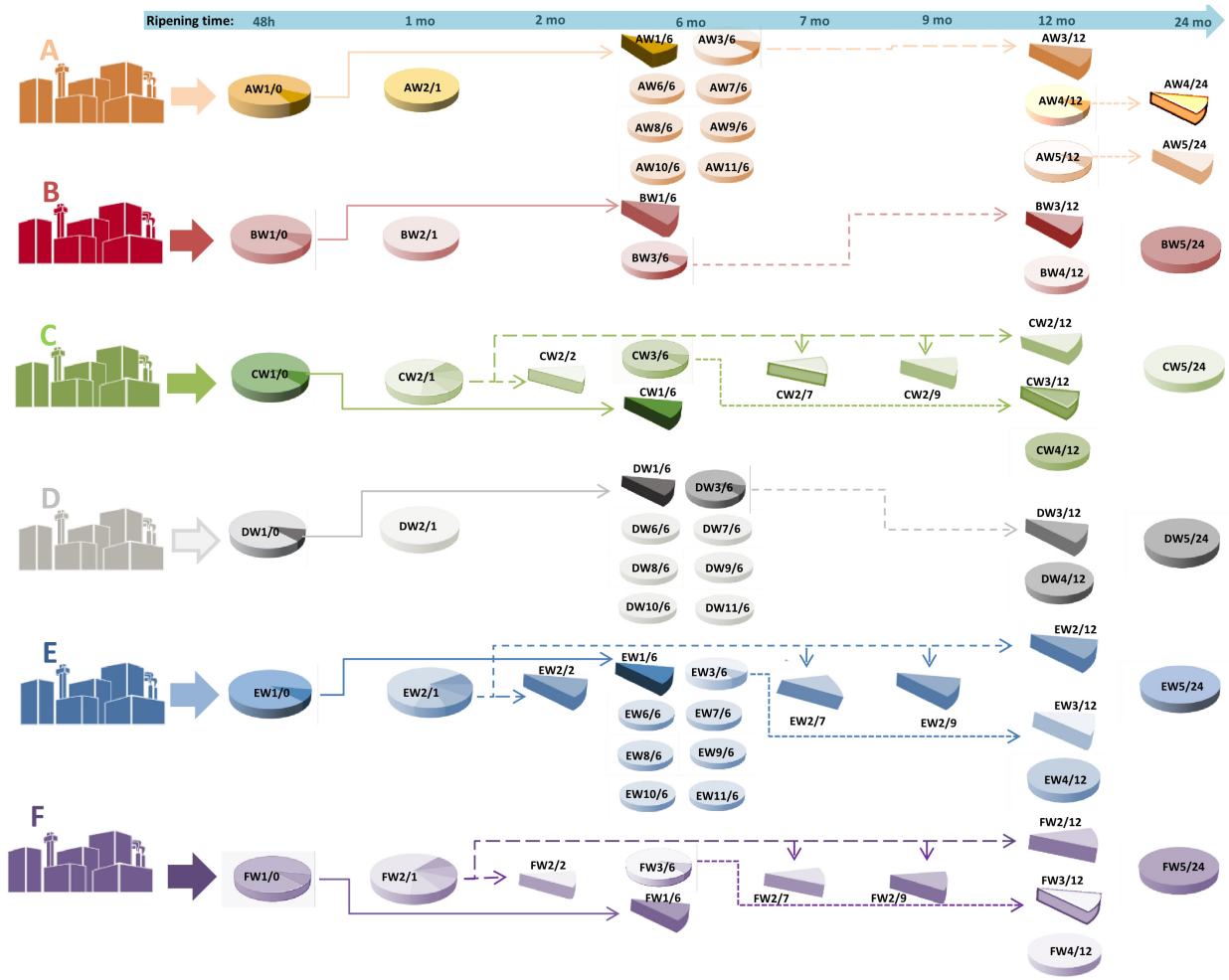

FIGURE 1 | A graphical representation of the sampling scheme. For each dairy **(A–F)**, samples were taken from the same cheese making lot (same wheel, W) at different ripening stages, and from different cheese making lots (different wheels) at the same ripening stage. As an example, AW1/0 corresponds to dairy A, wheel 1, months of ripening 0, that is to say, the curd after 48 h from cheese-making; AW1/6 is the same wheel sampled after 6 months. Samples from the same cheese making lot are connected by arrows. The time of ripening is also indicated at the top of the picture by an arrow.

TABLE 1 | Sampling scheme.

	Ripening stages							
Dairy	**48 h (acidified curd)**	**1 month (end of brining)**	**2 month**	**6 month**	**7 month**	**9 month**	**12 month**	**24 month**
A	W1/0	W2/1	–	W1/6, W3/6, W6/6, W7/6, W8/6, W9/6, W10/6, W11/6	–	–	W3/12, W4/12, W5/12	W4/24, W5/24
B	W1/0	W2/1	–	W1/6, W3/6	–	–	W3/12, W4/12	W5/24
C	W1/0	W2/1	W2/2	W1/6, W3/6,	W2/7	W2/9	W2/12, W3/12, W4/12	W5/24
D	W1/0	W2/1	–	W1/6, W3/6, W6/6, W7/6, W8/6, W9/6, W10/6, W11/6	–	–	W3/12, W4/12	W5/24
E	W1/0	W2/1	W2/2	W1/6, W3/6, W6/6, W7/6, W8/6, W9/6, W10/6, W11/7	W2/7	W2/9	W2/12, W3/12, W4/12	W5/24
F	W1/0	W2/1	W2/2	W1/6, W3/6,	W2/7	W2/9	W2/12, W3/12, W4/12	W5/24

Samples taken from the same wheel have the same W number. The number after each slash indicates the month of ripening. Dairies are written with capital letters (A–F).

incubating plates at 42°C. All the plates were incubated for 2 days under anaerobic conditions.

Culture-Independent Viable Counts

The number of viable cells was obtained by using the LIVE/DEAD® Baclight™ Bacterial Viability kit (Molecular Probes, Oregon, United States) and fluorescence microscopy (Gatti et al., 2006). The grated cheese homogenates in trisodium citrate (15 ml) were centrifuged at 10,000 rpm for 10 min at 4°C. The obtained pellets were washed twice in 15 ml 20 g/L trisodium citrate (pH 7.5) (Sigma–Aldrich, St. Louis, United States), then resuspended in 15 ml sterile water and 10-fold diluted. Subsequently, 1 ml of each sample was used for viability counts according to the manufacturer's instructions. Samples stained with LIVE/DEAD® Baclight™ were then filtered onto black polycarbonate filters (0.2 μm pore size) (Millipore Corp., Billerica, MA, United States), visualized by an epifluorescence microscope (Nikon 80i, Tokyo, Japan) and counted as described by Bottari et al. (2010). Three separate counts were performed for each sample. Results were expressed as viable cells and total cells, resulting from the sum of viable and non-viable cells (cells/mL or cells/g).

DNA Extraction

The total DNA for high throughput 16S rRNA sequencing was extracted from 69 samples using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), as described in Bertani et al. (2020). Bacterial genomic DNA for LH-PCR (length heterogeneity polymerase chain reaction) analysis was extracted using the silica column method with the General Rapid Easy Extraction System (GREES) DNA kit (InCura S.r.l., Cremona, Italy), according to the manufacturer's instructions. Two, 6, 9, 12, and 24-month-old cheeses were treated to extract DNA both from whole and lysed cells (Gatti et al., 2008; Santarelli et al., 2013).

High Throughput 16S rRNA Sequencing and Bioinformatic Data Analysis

Microbial diversity was studied through the sequencing of the amplified V3-V4 region of the 16S rRNA gene by using primers S-D-Bact-0341-b- S-17: 5′-CCTACGGGNGGCWGCAG-3′ and S-D-Bact-0785-a-A-21: 5′- GACTACHVGGGTATCTAATCC-3′ amplifying a fragment of 464 bp (Klindworth et al., 2013). Library preparation and sequencing was carried out as previously described (Berni Canani et al., 2017). The amplicons were purified using AMPure Beads XT (Beckman Coulter), quantified using a fluorimeter and combined in an equimolar pool, which was sequenced on an Illumina MiSeq platform, leading to 2x250 bp reads.

After demultiplexing, paired-end reads were joined by FLASh (Magoč and Salzberg, 2011) and a quality filtering was carried out by PRINSEQ (Schmieder and Edwards, 2011). Reads were trimmed at the first base with a Phred score <20, and those reads shorter than 300 bp were discarded. High-quality reads were analyzed by using QIIME 1.9.1 software (Caporaso et al., 2010). Briefly, OTUs (Operational Taxonomic Units) were picked at 97% similarity level using a de novo approach, and *uclust* method and taxonomic assignment was obtained by using the RDP classifier and the Greengenes database, following a previously reported pipeline (Berni Canani et al., 2017). Raw sequencing reads were uploaded to Sequence Read Archive (SRA[1]) and are stored under project accession number PRJNA649740. To avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample. Alpha-diversity analysis was carried out in QIIME on rarefied OTU tables. Evenness index was calculated as Pielou's Evenness index J', as reported in: http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.html.

The taxonomy tables were imported into the R software[2] for statistical analyses and visualization.

LH-PCR and Calculation of Diversity Indices

In order to estimate which bacterial species were still present in the cheeses at different ripening stages and which underwent lysis during ripening, LH-PCR was performed on both whole and lysed fractions of cheese samples. The primer pair 63F 5′ end labeled with 6-carboxy-fluorescein (6-FAM) and 355R was used as described by Lazzi et al. (2004). The Domain A of the variable region of the 16S rRNA gene was analyzed. Reaction, amplification, and capillary electrophoresis conditions were the same as those used by Bottari et al. (2010). The fragment sizes (base pairs) were determined using GeneMapper software version 4.0 (Applied Biosystems, Foster City, United States), a local Southern method to generate a sizing curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems, Foster City, United States) and a threshold of 150 fluorescence units. Each peak, corresponding to amplicon of specific length on the electropherogram profile, was attributed to bacterial species according to published databases (Lazzi et al., 2004; Gatti et al., 2008), and the areas under the recognized peaks were used to estimate the amount of the assigned species in the samples. Total area under all the peaks (sum of attributed and unattributed peaks) of the LH-PCR electropherograms was used for measuring the total amount of DNA arising from both intact and lysed cells (D'Incecco et al., 2016). Ecological indices throughout ripening were calculated based on LH-PCR results. Diversity indices (Shannon and Simpson) were calculated as follows: Shannon index, $H = -\Sigma piln(pi)$, and Simpson index, $D = \Sigma pi2$ where pi is the ratio between the area of each peak and the sum of all peak areas in the sample. The Simpson's index value is given as 1 – D, given that this way of presenting it means that a higher value reflects higher diversity. Richness (S = the number of species) and Evenness (E = H/Hmax; Hmax = ln S) were also calculated. For each matrix, the mean value of six samples coming from different dairies are shown and standard errors were calculated.

Cheese Water Soluble Extract

Ten grams of finely grated cheese were suspended in 45 ml of 0.1 N HCl. (L,L)-phenylalanyl-phenylalanine (Phe-Phe) was added as an internal standard (2.5 ml of a 1 mM solution).

[1]https://www.ncbi.nlm.nih.gov/sra

[2]http://www.r-project.org/

The suspension was homogenized for 1 min using an Ultra-Turrax homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) and then centrifuged at 3,400 × g for 30 min at 4°C. The solution was filtered through paper filters, and extracted 3 times with 40 ml of ethyl ether. Diethyl ether residues were removed using a Rotavapor (Buchi, New Castle, DE, United States); then, the aqueous solution was filtered through a 0.45 μm filter. 0.5 ml of 0.1% of formic acid solution were added to 1.5 ml of filtered extract, and the solution was passed through Vivaspin 2 Ultrafilter (Sartorius, Gottingem, Germany) with polyether sulfone (PES) membrane (nominal MWCO 10 kDa) previously washed and conditioned following manufacturer instructions. The filtration lasted for 45 min at 4,930 × g, using a centrifuge Hettich Universal 320R (Kirchlengern, Germany) at 23°C. Further washing steps of the ultrafilters were performed (3 times using acidified water at 0.1% formic acid). Final extracts were dried using a Rotavapor (Buchi, New Castle, DE, United States). The filtrate was dried under nitrogen, dissolved in 250 μL of 0.1% HCOOH in H_2O, and analyzed by UPLC/ESI-MS. Each sample was extracted and analyzed in triplicate.

UPLC/ESI-MS Analysis

The peptides were semi-quantified by UPLC/ESI-MS through comparison with Phe-Phe as internal standard, as described in Bottari et al. (2017) with the following modifications: no pre column was used, sample temperature: 6°C, injection volume: 2 μL, source temperature: 100°C, desolvation temperature 150°C, scan duration: 1 s.

The peptide fraction analysis yielded a TIC (total ion chromatogram) for each sample. For each signal of interest, the most intense ions were extracted, obtaining a XIC (extract ion chromatogram). The area underlying the peaks was then determined. The peptides were selected according to the signal intensity and the trend (increasing or decreasing) during ripening. Molecular masses of the most abundant peptides were obtained by analyzing the mass spectra associated with the most intense chromatographic peaks. One hundred and eighty-nine different peptides were first considered in the preliminary screening, with molecular masses ranging from about 200 to more than 7,000 Da. Peptides were then semi quantified in all the samples against the internal standard Phe-Phe according to a method previously reported (Sforza et al., 2003, 2004, 2012). Only peptides that gave a minimum chromatographic signal corresponding to 20% of the signal of the internal standard in at least one sample were considered for sequence identification. Thus, starting from the original set of 189 peptides, 34 peptides most representative of the cheese peptide profile were selected for sequence analysis.

Statistical Analysis

Statistical analyses were carried out using IBM SPSS Statistics software (version 26.0, Armonk, NY, United States). Kaiser-Meyer-Olkin measure of adequacy of sampling: 0.718. Bartlett's sphericity test sign.: 0.000. Data linearity was assessed both with Kolmogorov-Smirnov and Shapiro-Wilk tests: 38 out of 50 variables were not normally distributed. Then, bivariate correlation was performed using Spearman's coefficients, with a two-tailed significance test, and pairwise case exclusion for missing values. Significance was fixed to a $p < 0.05$. The ANOVA followed by the Tukey HSD test were performed to detect statistical differences ($p \leq 0.05$) among microbial counts and biodiversity indices as a function of ripening time.

SIMCA 16.0.1 (Sartorius Stedim Data Analytics, Göttingen, Germany) software was used to create principal component analysis (PCA) biplot to get a visual interpretation of the analyzed data.

RESULTS

Bacterial Dynamics During PR Cheese Ripening

Sixty-nine PR cheese samples (only 67 could be successfully sequenced) were analyzed by 16S rRNA gene amplicon high-throughput sequencing. A total of 3,344,483 raw reads were obtained after the sequencing step, of which 3,168,658 passed the filtering steps, with an average number of 28,034 reads/sample. The number of OTUs, the estimated sample coverage (ESC), as well as species richness (Chao1 indices) and diversity (Simpson, Shannon and Evenness indices) indicators were calculated for all samples after the rarefaction step and are reported in **Supplementary Table 1**. Good's coverage indicated that for all samples more than 99% of the bacterial diversity was described, and species richness ranged from a minimum of 24 OTUs in a 1-month-old sample to a maximum of 142 OTUs in 24-month-old samples. Diversity indices were considered according to the selected sampling times and are represented in **Supplementary Figure 1**, given that they are in good agreement with diversity indices calculated on the LH-PCR profiles.

Sequences were assigned to 21 different phyla, among which *Firmicutes* (97.4%), *Actinobacteria* (0.8%), *Proteobacteria* (0.6%), and *Bacteroidetes* (0.6%) represented between 97.9 and 100% of the bacterial population. Among *Firmicutes*, Bacilli are by far the most represented class, with values ranging between 86.5 and 99.9% of the entire microbiota. Only 23 OTUs were present in at least two samples with an abundance above 1% (**Supplementary Table 2**), and, among these, only 6 OTUs were present in at least 50% of the samples. These are the species *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lacticaseibacillus (formerly L. casei* group, Zheng et al., 2020), *Lactobacillus fermentum*, *Streptococcus thermophilus* and *Lactobacillus crispatus,* representing from a minimum of 82.5% to a maximum of 98.6% of the microbiota of the cheese samples (**Figure 2**).

The starter species, i.e., *L. helveticus* and *L. delbrueckii*, dominated through the first stages of fermentation, representing among 76.3–95.7% of the microbiota after the molding step. After the brining of the curd, these species represented between 57.4 and 94.8% of the entire bacterial population, and, in 2-month-old cheeses, these values ranged between 79.3 and 88.0%. In the subsequent sampling points, these species showed a general decrease in abundances. In the majority of samples (59 samples out of 67), the relative abundance

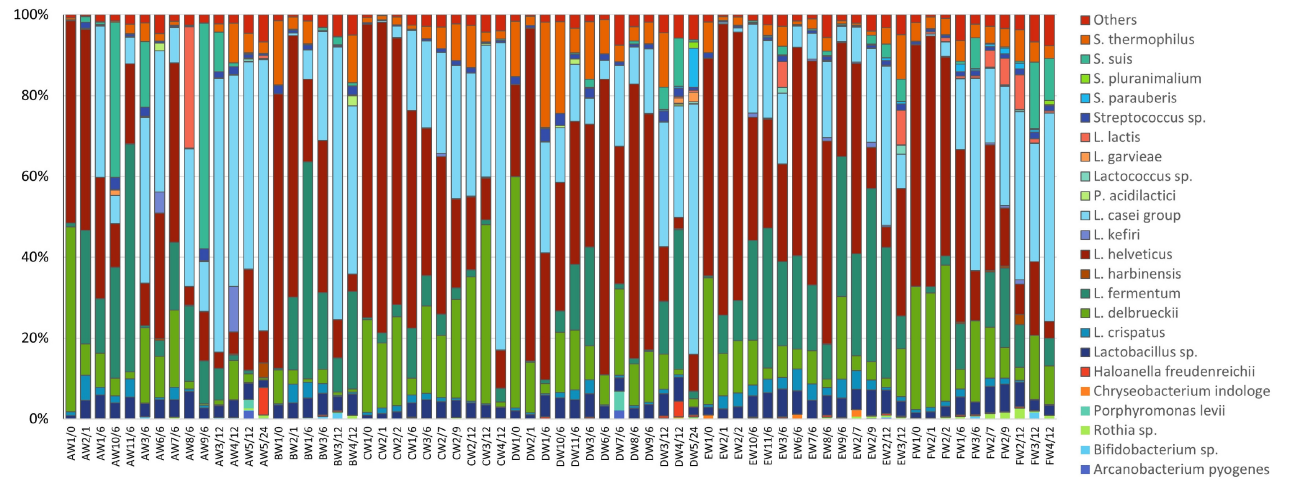

FIGURE 2 | The relative abundance of OTUs based on 16S rRNA sequencing; only bacterial species present in at least two samples with an abundance above 1% are shown. Results are reported for dairies A to F, samples are named according to the scheme reported in section "Cheese Sampling."

of *L. helveticus* was greater than that of *L. delbrueckii (*in a dairy-independent way).

For all the dairies, the species belonging to *Lacticaseibacillus* were present at low abundances in the first cheese making steps ($\leq$1% in samples between 0 and 2 month) and showed an increase from 6 months of ripening, with average values of 21.7 $\pm$ 13.9% (mean $\pm$ SD). Their relative abundance further increased after 12 months of ripening (43.2 $\pm$ 18.1%) and reached highest values in 24-month-old samples (64.5 $\pm$ 3.6%). *L. fermentum* was present in most samples and showed dynamics similar to those of *Lacticaseibacillus*, with an average relative abundance of 16.1 $\pm$ 17.7% in 6-month-old samples and 10.3 $\pm$ 11.3% in 12-month-old samples. *S. thermophilus* was also detected in the majority of samples, with values ranging from less than 0.1% up to 26%, such as in the case of *Streptococcus suis,* which ranged from less than 0.1% up to 55.7% but with high values found only in few samples from dairies A and F. Another frequent species was *L. crispatus*, which was present at low abundances (from less than 0.1% up to 6.2%), and which showed small increases in 1 month samples (until after 6–7 months of ripening) and decreases afterward. Interestingly, members of the family *Bifidobacteriaceae* were present with abundances lower than 0.1% in most samples, especially in the early cheese-making steps, reaching abundances of 1.4 and 1.6% in 12 month samples from dairies B and F, respectively.

Viable Cell Counts and Dynamics of Whole and Lysed Bacterial Cells

Bacterial profiles of 69 PR cheese samples were also described by means of culture-dependent and -independent approaches. Total, viable, and LAB counts are shown in **Figure 3**. For each ripening stage, the mean values of six samples coming from different dairies are shown. The bars represent the variability among cheeses from different dairies, having been calculated as standard error. Microbial counts that showed a general trend were SLAB (counted on MRS at 42°C) decreased since the first cheese-making steps. The number of NSLAB (counted on CA at 37°C) began, instead, to increase, starting from the curds up to 6 months of ripening stage, and decreasing thereafter. Viable cells counts were in good agreement with culture-dependent microbial counts. Species distribution revealed by LH-PCR was found to be variable, both among different dairies and within a single dairy, at different ripening times (**Figure 4**). In the early stages, and particularly for 48 h curd samples, a higher relative abundance of SLAB species, such as *L. helveticus* and *L. delbrueckii*, was observed. Longer ripened cheeses, from the sixth month of maturation, were found to contain mainly NSLAB species, such as *Lacticaseibacillus*. As far as the lysed cells fraction was concerned, LH-PCR confirmed that the species undergoing lysis are differently represented both in different dairies and during different amounts of time (**Figure 4**). From the early stages of cheese ripening, the lysis of both SLAB, such as *L. delbrueckii* and *L. helveticus*, and NSLAB, such as *Lacticaseibacillus* species, was observed. **Figure 5** presents the ecological indices during manufacture and ripening, calculated from LH-PCR results, to evaluate the microbial diversity of cheeses at different stages of ripening for the six cheese manufacturing processes. For each ripening stage, the mean value of all the samples with the same ripening times coming from different dairies are shown. The bars represent the variability among cheeses from different dairies, having been calculated as standard error. The graph includes the totality of the data relating to the presence of microbial species in function of both aging time and different dairy origin, regardless of the number of considered wheels. Among cheese ecosystems, diversity (D), Evenness (E) and Richness (S) showed changing trends, with an increase during the first two months and, then, a gradual decrease during ripening. The highest number of species in the community (S) was observed in 2-month-old

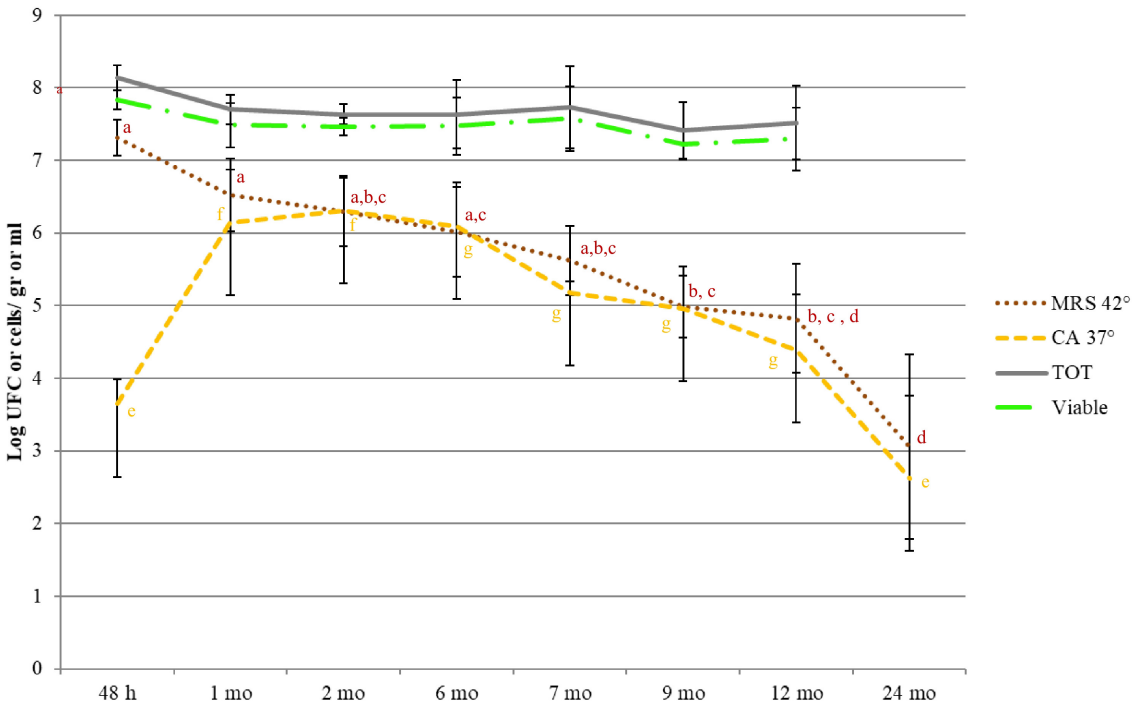

FIGURE 3 | Microbial counts (MRS 42°C: SLAB; CA 37°C: NSLAB; Total cells, Viable Cells) during cheese ripening. For each ripening stage, the means of all the samples with same aging time are shown. Bars represent differences among samples from different dairies (standard errors). Significant differences ($p < 0.05$; ANOVA followed by Tukey *post hoc* test) as a function of the ripening time are indicated by different letters. The coloring of descriptive statistics corresponds with the colors of the variables. The viable and total counts were not significantly different.

cheeses, while major differences among dairies were revealed at the beginning of cheese ripening (among 1 and 2-month-old samples) and among 7 and 9-months-old samples. Both microbial diversity and differences among dairies decreased throughout the ripening time.

Peptide Composition

Semi-quantitative data for all peptides were used as variables in a principal component analysis (PCA). The loading plot of the PCA is reported in **Figure 6**. Peptides clustered into 4 groups according to ripening stages, from curd to 24-month-old cheeses. Samples classified according to dairy of production did not cluster separately on the score plot (data not shown), indicating that the production in different dairies was not significantly responsible for peptide variability. A first cluster, corresponding to curd samples, was characterized by one peptide with a molecular weight of 2,763 Da, identified as a fraction of α_{s1} casein (CN), α_{s1}-CNf(1–23). A second cluster, corresponding to 1 and 2-month-old cheese samples, was characterized by the following peptides: α_{s1}-CNf(24–34), α_{s1}-CNf(16–20), α_{s1}-CNf(24–38), α_{s1}-CNf(24–30), α_{s1}-CNf(17–23), α_{s1}-CNf(10–14), β-CNf(47–52), β-CNf(193–209), β-CNf(1–6) and few non-identified peptides. A third cluster, corresponding to 6-month-old cheese samples was characterized by the presence of β-CNf(15–28)3P, β-CNf(12–28)4P, β-CNf(11–28)4P, β-CNf(94–107), β-CNf(195–209), β CNf(16–28)4P, β CNf(15–28)4P, β CNf(14–28)4P, β CNf(16–25)3P, β CNf(13–28)4P, and one non-identified peptide. These peptides are phospho-peptides, arising from β – casein, hydrolyzed in position 28–29 or 25–26, followed by further degradation starting from the N-terminal. The endopeptidase action is the result of endopeptidases with specificity for basic residues, given that the amino acid in position 25 and 28 is always the lysine. A final cluster, corresponding to the longer ripened cheeses (7, 9, 12, and 24 months), was characterized by the presence of NPAD (Non-Proteolytic Aminoacyl Derivatives), namely gamma- glutamyl-, lactoyl-, and pyro glutamyl-amino acids.

The Correlation Between Cheese Microbiota and Peptide Composition

The clustering of cheese samples according to both their microbial composition and the measured peptide fraction showed the existence of a ripening trend among the variables (**Supplementary Figure 2**). Cheese samples at 0, 1 or 2 months of ripening showed partly overlapping microbial composition and clustered with peptides n.i. PM473, β-CN f(193–209), β-CN f(1–6), β-CN f(47–52), α_{s1}-CN (10–14), α_{s1}-CN f(16–20), α_{s1}-CN f(17–23), and α_{s1}-CN f(24–34), which correspond to early peptide products from LAB proteolytic activity. At 6, 7, and 9 months of ripening, the bacterial composition of the samples was more variable, while the peptide fraction showed an evolution from early proteolysis products toward

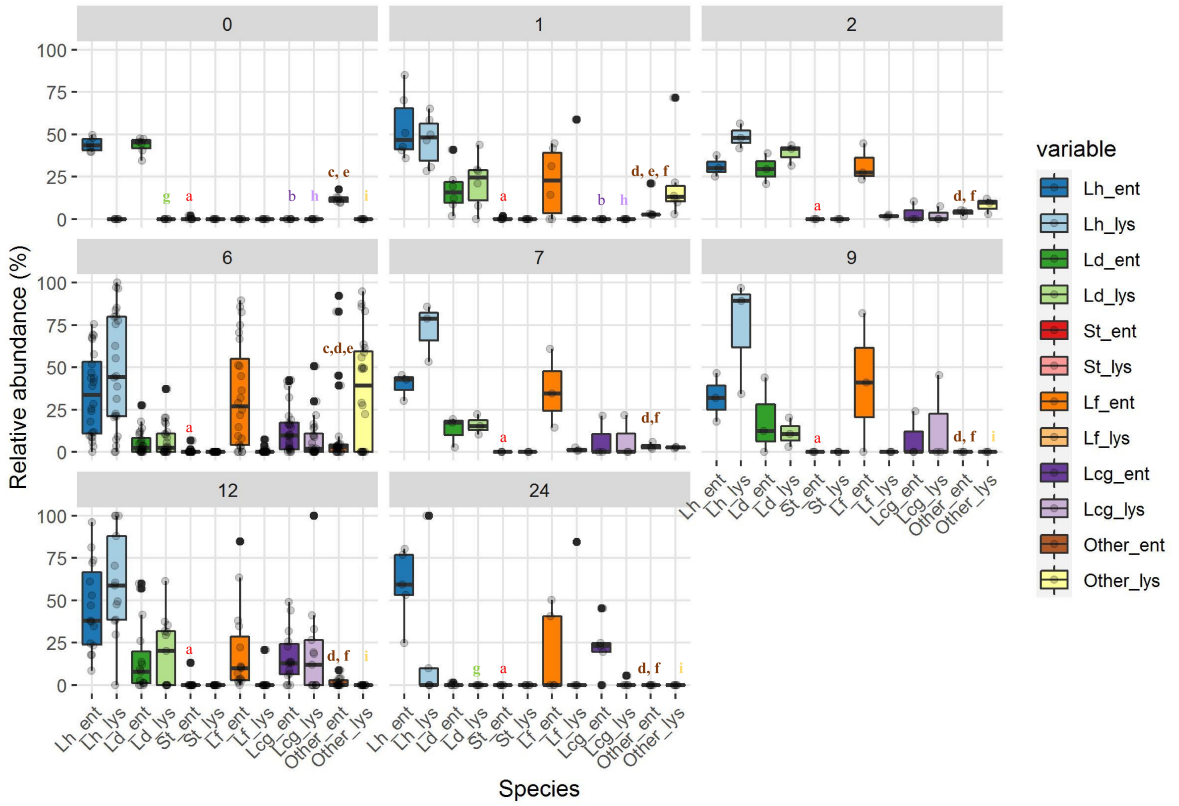

FIGURE 4 | The relative abundance of entire (indicated by _ent) and lysed (indicated by _lys) bacterial cells at different ripening stages. Ripening times are as follows: 0, curd samples, 1, 1 month samples; 2, 2 month samples; 7, 7 month samples; 6, 6 month samples; 9, 9 month samples,12, 12 month samples; 24, 24 month samples. Bacterial species are abbreviated as follows: Lh, *L. helveticus*; Ld, *L. delbrueckii*; St, *S. thermophilus*; Lf, *L. fermentum*; Lcg, *Lacticaseibacillus* (formerly *L. casei* group). Significant differences ($p < 0.05$; ANOVA followed by Tukey *post hoc* test) for each species, entire/lysed, as a function of the ripening time are indicated by different letters. The coloring of descriptive statistics corresponds with the colors of the variables. For samples that were statistically different from all the others, no letters were reported.

smaller fragments (**Supplementary Figure 2**). Longer ripened PR cheese samples (12 and 24 months) retained some variability in terms of microbial composition but were characterized by the accumulation of NPADs.

To visualize correlations existing between specific microbial taxa and proteolytic products, a heat map was built. As shown in **Figure 7**, proteolytic derivatives of caseins formed three separate clusters: a first cluster (A) is characterized by early ripening stage peptides and shows high correlation with taxa belonging to SLAB group, such as *L. helveticus* and *L. delbrueckii*, along with the less abundant species *L. crispatus*. Cluster B, characterized by proteolytic derivatives of β-caseins, showed a positive correlation with species that develop during the intermediate phase of the ripening, such as *L. fermentum* or *Pediococcus acidilactis*. The last cluster (C) was characterized by NPADs, that correlated with non-starter taxa, such as the *Lacticaseibacillus*, *Lentilactobacillus kefiri*, *Lactobacillus harbinensis,* and *Bifidobacterium* spp. The species *Lactococcus lactis* and *S. thermophilus* were also positively correlated with an increase in abundance of these compounds at later ripening stages.

LH-PCR data in cheeses at different ripening times were combined with all the identified peptides to evaluate possible correlations between the dynamics of whole and lysed bacterial cells and peptide composition from curd to 24-month-old cheeses (**Figure 8**). DNA from entire and lysed bacterial cells and peptides were grouped according to ripening time. In particular, the 4 groups, highlighted by different colors, showed a counterclockwise trend in the samples, starting from 48 h to 24 months of ripening. From this analysis, it was possible to observe that the succession of microbial species during ripening was accompanied by a marked change in peptide composition. As expected, a first cluster corresponding to the 48 h curd samples was characterized by the presence of α_{S1}-CNf(1–23) that derives from the first proteolysis stages occurring at the beginning of ripening, and the presence of entire cells of *S. thermophilus*, a minority species found in curd samples (**Figure 4**). The second cluster, corresponding to 1 and 2 month ripened cheeses, grouped together the peptides deriving from the first proteolysis stages and the entire and lysed microbial cells of *L. helveticus*, *L. delbrueckii,* and *L. fermentum*. The third cluster (6-month-old\cheeses) was characterized mostly by the presence of phospho-peptides arising from β- casein. Finally, the fourth cluster (7 to 24-month-old cheeses) was characterized by NPAD and *Lacticaseibacillus,* both entire and lysed cells.

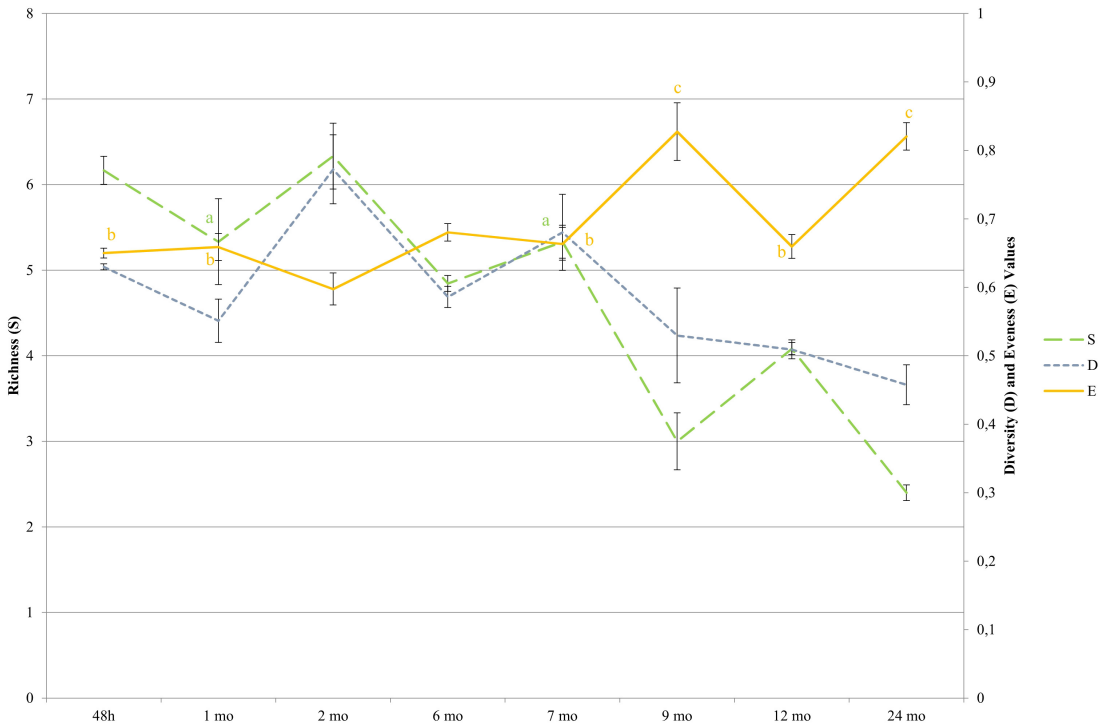

FIGURE 5 | Diversity indices during cheese ripening determined by LH-PCR. Species were detected by comparing the amplicon lengths with the LH-PCR databases. For each ripening stage, the means of all the samples with same aging times are shown. Bars represent differences among samples from different dairies (standard errors). Simpson (D = Σ pi2); the Simpson's index value is given as 1-D. pi is the relative abundance of a given LH-PCR peak; Richness (S) is equal to the number of species. Evenness (E) is the relative abundance with which each species is represented, [E = H/Hmax; were H is Shannon index (H = −Σ piln(pi)) and Hmax = lnS]. pi is the relative abundance of a given LH-PCR peak and is obtained by dividing the area of each peak with the total area of all peaks in the electropherogram profile for each sample. Significant differences ($p < 0.05$; ANOVA followed by Tukey *post hoc* test) as a function of the ripening time are indicated by different letters for each index. Simpson values were significantly different among all ripening stages, thus no letters were indicated.

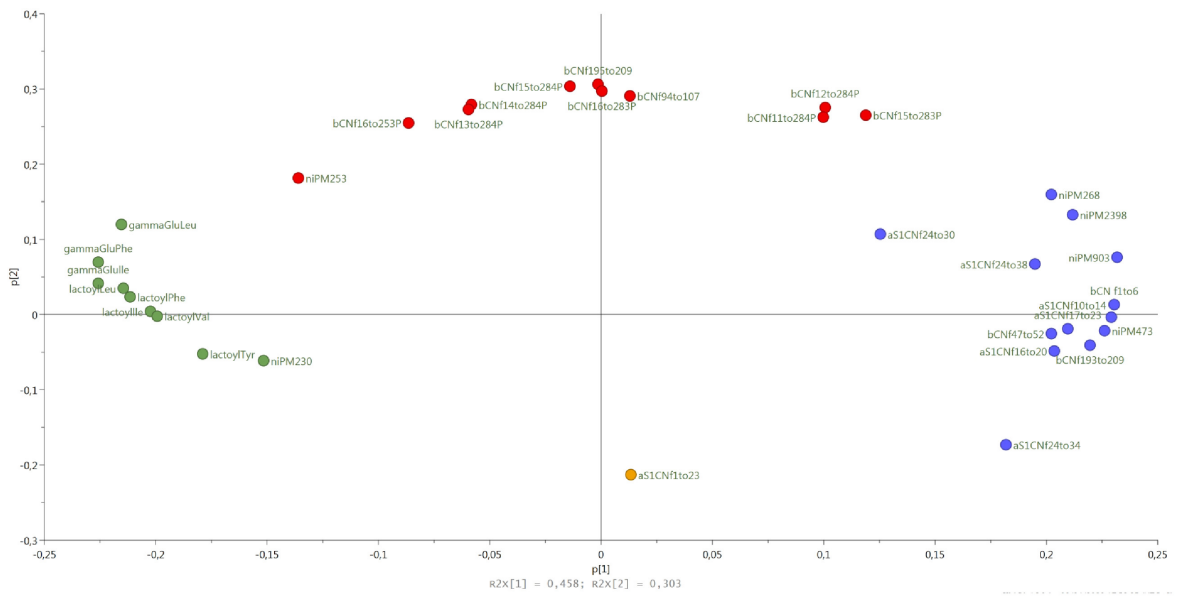

FIGURE 6 | The loading plot of the peptides semi-quantitative data colored according to the aging time: 48 h (yellow), 1–2months (blue), 6 months (red), 7–24 months (green).

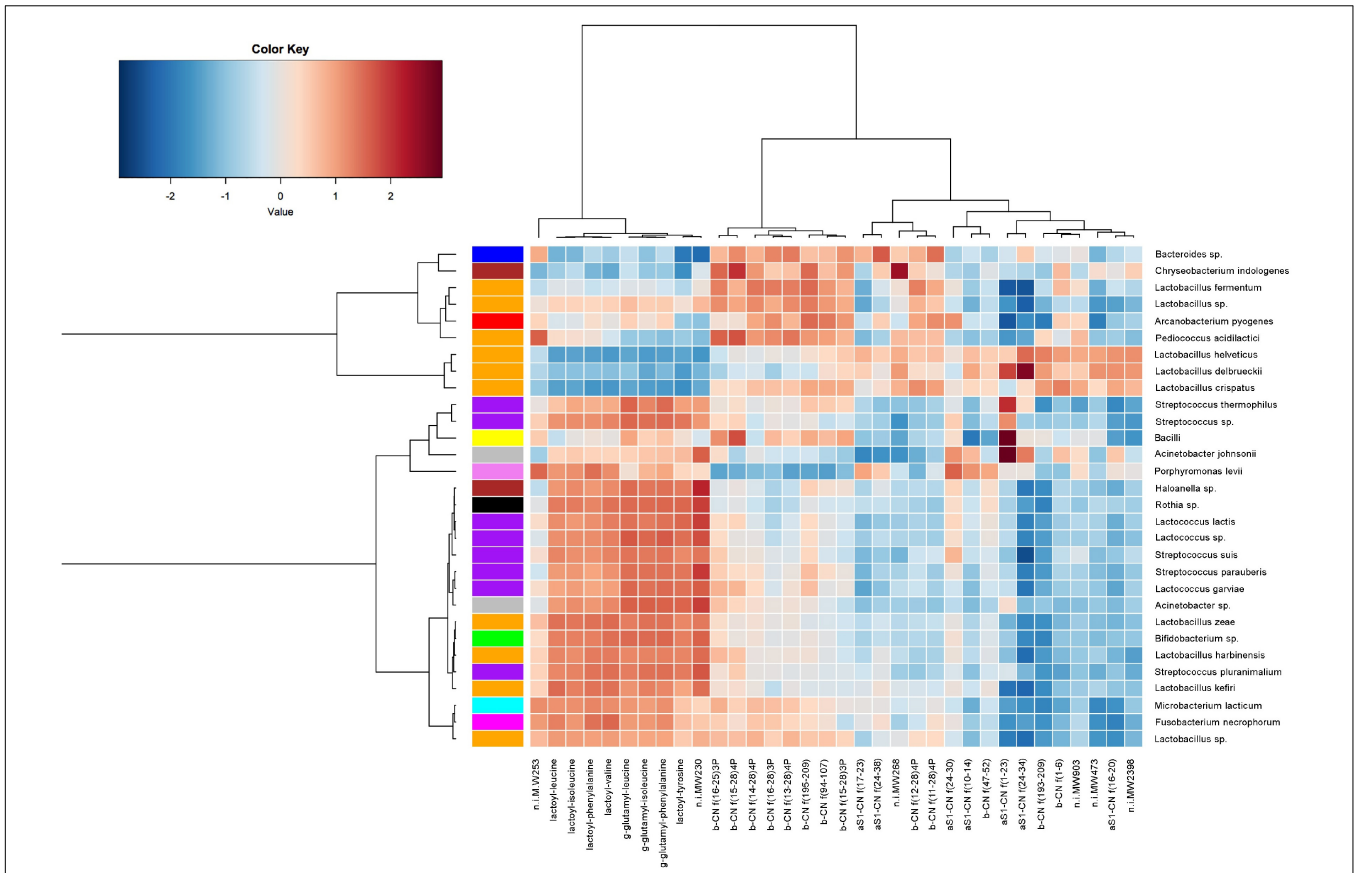

FIGURE 7 | A heat map showing Spearman's correlation between cheese microbiota and peptides abundance. Rows and columns are clustered by Euclidean distance and Ward linkage hierarchical clustering. The intensity of the colors represents the degree of association, as measured by Spearman correlations. Only taxa occurring in at least 20% of the samples were included.

DISCUSSION

The LAB viable count in the 69 PR samples analyzed in the present study showed a reduction of SLAB viability within the first month of ripening along with the NSLAB count increase. This is in good agreement with Gatti et al. (2014) who reported that in long ripened cheeses, such as PR, SLAB decrease within a few hours or a few days from the start of the cheese-making process, while NSLAB grow slowly and become the dominant microbiota in the ripened cheese. SLAB, in fact, are known to be abundantly present in the first stages of PR cheese making. They originate mainly from the natural whey starter and actively metabolize the lactose of milk (Gatti et al., 2014). Later, during ripening, when the environment becomes unfavorable due to low sugars, whey drainage, and increasing salt, NSLAB dominate thanks to their ability to use energy sources other than lactose and resistance to environmental stresses (Sgarbi et al., 2014). To get a deeper insight into the bacterial community composition and dynamics of the analyzed cheese samples, HTS and LH-PCR were used. Results obtained by amplicon sequencing showed that, despite the fact that the cheeses from different dairies exhibit a certain degree of variability, the composition of the bacterial population is characterized by the interplay of different LAB and is shaped by technological processes, as observed by other authors (Gatti et al., 2014; De Filippis et al., 2016; Gobbetti et al., 2018). Curds from all dairies were, in fact, dominated by the species *L. helveticus* and *L. delbrueckii*, which derive from the natural whey starter used for cheese making (De Filippis et al., 2014; Bertani et al., 2020). After the brining step (1-month-old samples and later), a compositional change occurs in cheese microbiota that shows a decrease in the relative abundance of the SLAB species, while NSLAB population, initially present at low abundances, find suitable conditions for development (Lazzi et al., 2014; Bottari et al., 2018). Indeed, after 6 months of ripening, cheeses from all dairies showed an increase of *Lacticaseibacillus*, which is known to dominate the microbiota of PR cheese during ripening (Gatti et al., 2014). In the 6-month-old samples, other minority species, such as *L. fermentum*, *L. crispatus*, *S. thermophilus* and *Lc. lactis,* were also present, as observed in other long-ripened, raw milk cheeses manufactured with similar technology (Alessandria et al., 2016; Levante et al., 2017).

While amplicon sequencing was performed on total DNA extracted from cheeses, LH-PCR allowed semi-quantification of DNA extracted from entire cells, to estimate which bacterial species were still present in the cheeses at different ripening

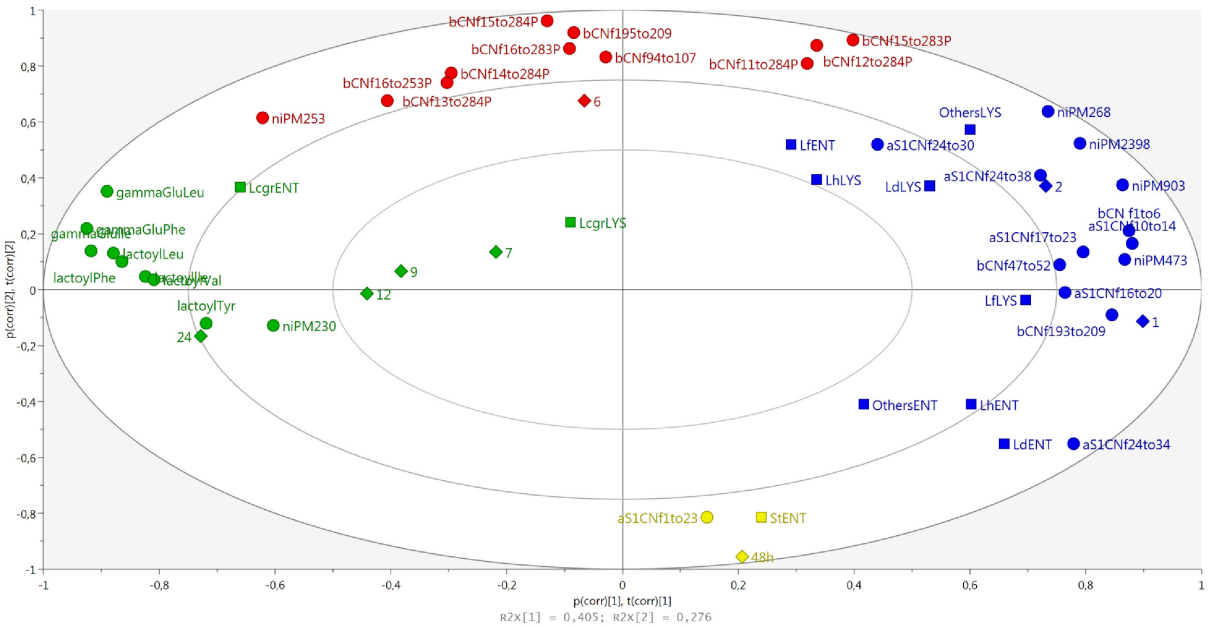

FIGURE 8 | The biplot of the distribution of microbial species and peptides according to the aging times and colored according to marked groups.

stages, and from lysed cells, permitting the estimation of which LAB underwent lysis during ripening (Gatti et al., 2008). This allowed to assess the progress of cell lysis as a function of time and the most affected microbial species. This aspect is of major importance for the evaluation of cheese ripening. In fact, after cell lysis, bacteria release intracellular enzymes important for proteolysis, thus for cheese maturation. The greater the number of growing cells, the greater the subsequent cell lysis and the number of released intracellular enzymes (D'Incecco et al., 2016; Lazzi et al., 2016). The presence of whole cells of *L. helveticus* and *L. delbrueckii* in curd samples confirmed what was previously observed, as these species come mainly from the natural whey starter and are frequently found at the beginning of ripening (Bottari et al., 2013; Gatti et al., 2014). The lysis of both SLAB and NSLAB observed since the early stages of cheese-making, confirmed that cells undergo autolysis during cheese ripening due to stressful environment conditions. The observation of an autolysis affecting SLAB first, and NSLAB at a later ripening stage, sustains the hypothesis that NSLAB can better survive to those conditions (Gatti et al., 2014). Biodiversity indices indicated that cheese curds were characterized by a high level of richness, followed by a slight decrease after brining (1 month old samples) and a subsequent increase in 2 month old samples. This is consistent with the growth trend observed by culturing, and could be correlated to the growth of NSLAB reported during the first months of ripening (Gatti et al., 2014). NSLAB in fact, arising mainly from milk, enrich microbial diversity of curd, which is mostly represented by whey SLAB.

Differences, in terms of biodiversity, among the various dairies, were small at curd stage. This is likely due to the prevalence of SLAB coming from the starter, which, although prepared differently by each dairy, generally contains mainly *L. helveticus* and *L. delbrueckii* and possibly *S. thermophilus* and *L. fermentum* (Bottari et al., 2010, 2013; De Filippis et al., 2014; Bertani et al., 2020). Major differences revealed among dairies between 1 and 2 months old samples and 7 and 9 months old samples, are consistent with the growth of NSLAB coming mainly from raw milk and their higher adaptability to specific environmental conditions, driven by cheese making parameters (Bottari et al., 2018). In the following ripening stages, biodiversity indices presented some differences according to the investigation method: while metataxonomic data showed a consistent increase of all the indicators until 9 months of ripening, LH-PCR performed on entire bacterial cells indicates that there is a decrease in richness and diversity after 2 months of ripening. This is consistent with the decrease of viable counts of both SLAB and NSLAB in longer ripened cheeses, and with the bacterial lysis occurring at higher extent after this time point. This ripening stage was previously reported as crucial for the microbial dynamics and diversity of long ripened raw milk cheeses (Santarelli et al., 2013). In the later ripening stages, both differences among dairies and the biodiversity indices showed a decrease. This is justified by microbial selection, inevitably occurring along maturation, which allows the only growth of those microbes that can use energy sources other than milk carbohydrates and tolerate decreasing a_w value (Gatti et al., 2014). This finding is in agreement with the isolation of few species from long ripened PR (Gatti et al., 2008) and is further confirmed by the E index, calculated from the LH-PCR data on entire cells in the present study, which increased over time and precisely indicates the predominance of one or a few species compared to the total.

Peptide analysis is of utmost importance in determining cheese quality and ripening. The peptide fraction of cheese is,

indeed, a direct consequence of the proteolytic events occurring during cheese ripening. Along with cheese aging, milk and bacterial proteases cleave caseins into shorter peptides, which thus accumulate in the cheese, reaching a maximum, and then decreasing due to their own degradation into shorter fragments. An exception to this phenomenon is NPADs, which are not degraded and continue to accumulate. Given the production process of a cheese, the formation of specific peptides (or peptide classes) is typical of certain stages of maturation, so they can be used as molecular markers of aging time (Sforza et al., 2012). Proteolysis also strongly influences the quality of the cheese, affecting the texture (high proteolysis correspond to soft texture), the taste (peptides are usually bitter, with the exception of NPADs which are kokumi), the flavor (amino acids are precursors of aromas), the digestibility (high proteolysis improves digestibility), allergenicity (some allergens can be degraded into non-allergenic peptides), and so on (Sousa et al., 2001; Toelstede et al., 2009; Alessandri et al., 2012). It is thus clear that analyzing the peptide fraction can provide a lot of guidance in assessing cheese quality and maturation. The differences in the microbiota (especially for the NSLAB group) of different dairies could lead to a different peptide profile due to a different kinetic and action of the proteolytic enzymes. However, analyzing samples from 48 h curd up to 24 months of aging, we found that the huge peptide evolution occurring during ripening greatly overpowered the possible small differences among cheeses from different dairies with the same aging time. The clustering of cheese samples confirmed that ripening stage is the characteristic that most affects the observed cheese peptide variability, fully confirming the data reported in Sforza et al. (2012). More specifically, four peptide groups were clustered according to the ripening stage: cluster 1 (curd, 48 h), cluster 2 (from 1 to 2 months of aging), cluster 3 (6 monthsof aging), and cluster 4 (from 7 months to 24 months of aging). Peptides typical of the earliest stages of ripening (48 h curds and 1-month-old cheeses) derive from the first proteolysis stages occurring at the beginning of ripening, when the action of chymosin on α_{s1}-CN generates the fragments available for the subsequent LAB proteolytic action. The first peptide to be formed derives from the cleavage of the peptide bond between the 23th and the 24th amino acid residue of α_{s1} casein, and constitute the N-terminal of the protein (α_{s1}-CN f1–23). This peptide is produced in the very early stages of cheese making by the action of chymosin (more thermolabile) and cathepsin D (more thermostable) (Gagnaire et al., 2011). Then, in the two months that follow, the peptide α_{s1-}CN f1–23 is broken into smaller fragments (α_{s1} CN f10–14, α_{s1}-CN f16–20, and α_{s1}-CN f17–23). As previously observed in other cheeses (e.g., Emmental) (Gagnaire et al., 2001), the N-terminal sequence of α_{s1-}casein is proteolyzed into several peptides (in addition to the aforementioned ones, also α_{s1}-CN f24–30, α_{s1}-CN f24–34, and α_{s1}-CN f24–38). On the contrary, no peptides coming from the C-terminal of the protein were detected, meaning that this part of the protein remains intact and is not attacked by proteases, neither endogenous of the milk nor microbial. In this second cluster (corresponding to the first two months of aging), β-casein peptides also appear. Differently from what observed for α_{s1} casein, in β-casein, peptides deriving both from the N-terminal and the C-terminal were identified (β CN f1–6 and β CN f 193–209). The formation of the peptide β-CN f(1–6) can be ascribed to the action of cell-envelope proteinases from thermophilic *Lactobacillus* (Gagnaire et al., 2001). The β-CN f 193–209 is generated by the cleavage of the peptide bond between Leu192-Tyr193, which can be hydrolyzed both by cathepsin D and cell-envelope proteinase of starter bacteria (*Lactobacillus* genus, mainly) (Gagnaire et al., 2001). The third cluster mainly contains phosphopeptides, and all of them derive from the region 11-28 of β-casein, in agreement with previous work (Sforza et al., 2003). These phosphopeptides share a common feature: the cleavage site in 28th position; plasmin can cleave the peptide bond at Lys28-Lys29 of β-casein (Lund and Ardö, 2004), but thermophilic starter bacteria may play an important role, as well. Finally, the fourth cluster contains NPADs, which accumulate during ripening since their chemical structure is not recognized by the proteolytic enzymes. Interestingly, the abundance of α_{S1}-CN f(1–23) – characteristic of the first stages of cheese manufacturing, within 48 h, thus derived from the proteolytic actions happening in the curd – is positively correlated with the total amount of DNA from entire cells of *L. helveticus* and *L. delbrueckii*, two species typical of PR natural whey starters (Bottari et al., 2010, 2013; De Filippis et al., 2014; Bertani et al., 2020). Indeed, SLAB develop mainly during the first days of PR aging, when lactose is still available for their metabolism (Gatti et al., 2014). Peptides found in the 6-month-old cheese samples are phospho-peptides deriving from β-CN. Their significant correlation with microbial counts on CA at 37°C is particularly interesting because CA is the medium in which NSLAB grow, meaning that degradation of β-CN to short polypeptides can mainly be attributed to NSLAB microbiota, as it has been previously hypothesized (Sforza et al., 2012). NSLAB mainly develop after SLAB, and, consistently, the amount of most β-CN peptides rises after α_{s1}-CN peptides. Peptide β-CN 193–209 (the C-terminal part of β casein), one of the most representative of this class, starts to accumulate at the beginning of cheese aging. Likewise, NPADs accumulate during aging, but they start to increase later and for up to 24 months. Thus, the trend of most of the β-CN peptides is not related with that of NPADs, while these latter are very strongly related to each other because they accumulate together during ripening, reaching their maximum amount at the end of cheese aging (Sforza et al., 2009). The accumulation of such molecules is probably due to their uniquely unconventional structure, which prevents the hydrolysis by exopeptidases. The enzymatic systems involved in their production has been reported to be likely intracellular and released after LAB death (Sforza et al., 2012; Bottesini et al., 2014). In fact, NPADs revealed a negative significant correlation with all the microbial counts with the exception of the amount of DNA from entire cells of *Lacticaseibacillus*, the dominant species in PR cheese up to 20 months of ripening (Neviani et al., 2009). This species could be responsible for the accumulation of these peptides, potentially involved in flavor formation. In addition to *Lacticaseibacillus*, other minor species from the NSLAB moiety might contribute to this accumulation, such as *Len. kefiri*, *L. harbinensis,* and *Bifidobacterium* spp. These results would sustain other findings regarding this very interesting topic (Sgarbi et al., 2013, 2014).

The aim of the research was to explore the link between the PR microbiota and the proteolysis that occurs during ripening, which is the basis for the valuable and recognizable characteristics of long-ripened cheeses. The complex organization of the sampling plan and the application of different analytical and statistical analyses enabled the conclusions and hypothesis made in previous works regarding the relation among PR microbiota's composition and dynamics and peptide evolution to be strengthened and confirmed. The highlight of this research was due to the combined approach of HTS and LH-PCR on both entire and lysed cells to evaluate their impact on proteolysis. The consequence of the bacterial presence and activity of cells and their released enzymes is not limited to the protein component of this widely appreciated cheese but also to the lipid component of the partially skimmed milk and the degradation of sugars, as well as minor components. However, the interplay of LAB and proteins has to be considered as the most relevant feature of this type of cheese. By relating the peptide evolution of cheeses of different dairies to specific microbial composition at defined times, we were able to expand on what was previously known about the most important aspects of cheese ripening.

Samples from different dairies were characterized by quite similar microbiota at curd level, due to both raw milk and natural whey starters' microbiota, but later, during ripening, the microbial composition evolved, revealing major differences among dairies between 1 and 2-month-old samples and 7 and 9-month-old samples. This differences were mainly due to the NSLAB species, which are more related to different peptide profiles given the different kinetics and activities of the proteolytic enzymes. With this observation, we highlighted once again the major role of NSLAB in ripened cheese proteolysis.

As it is widely known, PR is produced under strict regulations, although it is, nevertheless, a high quality, artisanal cheese, with uniquely appreciated features that can vary. The awareness of the association between the microbial composition (and evolution) and the proteolysis level (linked to the peptide composition), would be of practical interest for PR producers (and, more generally, for cheese producers), as the knowledge of this phenomenon allows one to monitor the cheese ripening and take corrective action in time to obtain the desired quality attributes. Moreover, the potential use of several peptides as markers of a specific microbial composition affords the possibility of taking advantage of it to protect and valorize the specificity and connection of PR cheese to its production territory.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, sra/PRJNA649740.

AUTHOR CONTRIBUTIONS

BB, MN, SS, and MG conceived the work. BB, CB, AL, and FD performed the analyses. BB, MG, SS, FD, and DE interpreted the results. EB, AL, BP, and FD performed statistical analyses. BB, EB, AL, BP, and CB drafted the original manuscript. EB, AL, and FD prepared figures and tables. BB, SS, MN, MG, BP, FD, and DE critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.581658/full#supplementary-material

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frontiers in Microbiology

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Raw Milk Microbiota Modifications as Affected by Chlorine Usage for Cleaning Procedures: The Trentingrana PDO Case

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Milk microbiota represents a key point in raw milk cheese production and contributes to the development of typical flavor and texture for each type of cheese. The aim of the present study was to evaluate the influence of chlorine products usage for cleaning and sanitizing the milking equipment on (i) raw milk microbiota; (ii) the deriving whey-starter microbiota; and (iii) Trentingrana Protected Designation of Origin (PDO) cheese microbiota and volatilome. Milk samples from three farms affiliated to a Trentingrana PDO cheese factory were collected three times per week during a 6-weeks period in which a sodium hypochlorite detergent (period C) was used and during a subsequent 6-weeks period of non-chlorine detergent usage (period NC). Samples were subjected to microbiological [Standard Plate Count; coliforms; coagulase-positive staphylococci; and lactic acid bacteria (LAB)] and metagenomic analysis (amplification of V3-V4 regions of 16S rRNA gene performed on Illumina MiSeq platform). In addition, cheese volatilome was determined by SPME-GC-MS. In the transition from period C to period NC, higher SPC and LAB counts in milk were recorded. Milk metagenomic analysis showed a peculiar distinctive microbiota composition for the three farms during the whole experimental period. Moreover, differences were highlighted comparing C and NC periods in each farm. A difference in microbial population related to chlorine usage in bulk milk and vat samples was evidenced. Moreover, chlorine utilization at farm level was found to affect the whey-starter population: the usually predominant *Lactobacillus helveticus* was significantly reduced during NC period, whereas *Lactobacillus delbrueckii* had the exact opposite trend. Alpha- and beta-diversity revealed a separation between the two treatment periods with a higher presence of *L. helveticus*, *L. delbrueckii*, and *Streptococcus thermophilus* in cheese samples after NC detergent period. Cheese volatilome analysis showed a slight decrease in lipolysis during C period in the inner

part of the cheese wheel. Although preliminary, these results suggest a profound influence on milk and cheese microbiota, as well as on raw milk cheese production and quality, due to the use of chlorine. However, further studies will be needed to better understand the complex relationship between chlorine and microbiota along all the cheese production steps.

Keywords: chlorine, whey-starter, cheese, milking equipment, biodiversity

INTERPRETATIVE SUMMARY

Chlorine compounds are commonly used in farms for sanitation purposes due to their bactericidal activity, but attention has been be paid to the effect of formation of chlorinated residues with an impact on milk. This study is aimed at evaluating the influence of chlorine products on raw milk microbiota and cheese flavor through metagenomic analysis and cheese volatilome investigation in farms associated to Trentingrana PDO cheese production. Our preliminary results support the idea that the detergents used influence raw milk microbial population and, consequently, raw milk cheese quality, safety, and sensory attributes. However, further studies will be needed to better understand the complex relationship between detergent and microbiota along all the steps of cheese production.

INTRODUCTION

Chlorine compounds are commonly used in farms, water treatment, and industrial food manufacturing processes, for sanitation purposes (McCarthy et al., 2018). These compounds, such as sodium hypochlorite (NaClO), calcium hypochlorite ($Ca(ClO)_2$) and chlorine dioxide (ClO_2), possess a high bactericidal activity against food-borne pathogens and, under proper conditions, may prevent the formation of biofilm in milking installation (Gómez-López et al., 2009; Sundberg et al., 2011).

The antimicrobial mechanism of chlorine compounds has been studied by several investigators. The most widely accepted mechanism is a damage to the protein synthesis and an increased permeability of the outer cell membrane (Gómez-López et al., 2009). Previous studies demonstrated that NaClO is effective for the milking equipment disinfection, reducing the bacteria populations by 99% on stainless steel surfaces (Greene et al., 1993; Reinemann et al., 2003). In addition, the incorporation of NaClO into cleaning products guarantees the removal of protein deposits and improves the cleaning efficiency on milking plant surfaces (Reinemann et al., 2003). For this reason, a high portion of liquid products used for milking machines cleaning and sanitation contain sodium hypochlorite (Gleeson et al., 2013). However, it has to be considered that the use of chlorine-based products, although effective and inexpensive, leads to the formation of chlorinated residues [i.e., Trichloromethane (TCM) and Chlorate], with associated health concerns that can rise to dangerous levels in milk fat products such as cream and butter, and, thus, are the subject of attention by food regulatory authorities (EFSA Panel on Contaminants in the Food Chain (CONTAM) Gleeson et al., 2013; Ryan et al., 2013; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015; McCarthy et al., 2018). In addition, the presence of chlorine can potentially influence the composition of the microbiota in milk and milk products.

Raw milk harbors one of the most diverse microbial communities detected in food matrixes (Quigley et al., 2013). The bacterial species present in raw milk depend on numerous biotic and abiotic drivers that influence and assembly the milk microbiota. Recent advances provided that, along with environment, the teat skin is one of the major source of microbial contamination of raw milk. Bacteria belonging to the families *Clostridiaceae*, *Staphylococcaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Enterococcaceae*, and *Pseudomonadaceae* may reach the milk from the teat surface (Doyle et al., 2017; Frétin et al., 2018). Other factors crucial for microbial contamination are the stage of lactation and seasonality and the dairy environment (feed, feces, humans, and air) (Zucali et al., 2011; Gobbetti et al., 2018). Moreover, the biofilms that may grow in milking equipment could be a vehicle of bacterial transfer in the dairy chain (Marchand et al., 2012). It is difficult to establish the relative importance of these sources of contamination, but each of them can influence the raw milk microbiota and, consequently, the microbial composition of bulk milk, whey starter and raw milk cheese.

In the light of the above evidence, it is important to find alternatives to the use of chlorine in dairy farms and to evaluate the potential effects of chlorine substitution, even temporary, on the milk microbiota and the presence of undesirable microorganisms. Moreover, it is of interest to deepen the relationships between the use of chlorine and the sensory characteristics of raw milk cheeses.

Trentingrana is a Protected Designation of Origin (PDO) cheese, produced in a specific alpine area of Northern Italy and its label belongs to Grana Padano PDO cheese consortium (EU, 2009). It is a hard-textured, cooked, and long-ripened (9–30 months) cheese made using raw cow milk supplemented with natural whey culture (NWC). NWC is obtained from the spontaneous fermentation (44–45°C) of the whey drained from the cheese vat at the end of cheese-making. The microbiota which develops from curd to Trentingrana ripened cheese arises only from raw milk and from NWC (Rossi et al., 2012).

The aim of this study was to determine the effect of chlorine (NaClO) use in the cleaning of milking machine on

the microbiota of raw milk, NWC and cheese in Trentingrana production, in normal conditions of use.

MATERIALS AND METHODS

Farms Characteristic and Experimental Plan

Three farms, indicated as F1, F2, and F3, respectively, located in Trentino (northeastern Italian Alps, at an altitude of about 1000 m above sea level) at a distance less than 10 km and associated to a factory producing Trentingrana PDO cheese, were involved in this study.

The experimentation was conducted over a 3 months period (from 5th December 2016 to 12th March 2017) during which the temperature variation during the day in the area where the farms and the dairy plant are located remained stable (between −2 and 9°C on the first day and between −4 and 8°C on the last day).

Two weeks before the start of the experiment each farm was preliminary visited to verify milking equipment, milking routine, washing and cooling system. In order to verify the effectiveness of cleaning procedures (with chlorine) of milking equipment and to establish a common procedure, the Lactocorder analysis was used (Bava et al., 2011).

The herds of each farm were composed by 55, 48, and 91 lactating cows (Italian Holstein-Friesian, Brown and Italian Simmental in different ratios), housed in free stall barns with cubicles, with mattress covered with sawdust; lactating cows were fed hay produced in the production area of Trentingrana and associated with concentrate. The herds were milked twice a day in DeLaval herringbone milking plants [F1 (5 + 5), F2 (4 + 4), F3 (6 + 6)]. Dairy farmers used gloves during milking. A commercial product including only detergent and emollient agents, was used for the pre-milking teat dip, and the teats were dried with disposable paper towels before the forestripping and the attachment of the milking cups. At the end of the milking a post-dipping product including lactic acid was applied. The milking equipment had automatic wash facilities ensuring uniformity and consistency of sanitizing practices.

Each auto-washer was calibrated for the cleaning product before the start date. The cleaning routine practices and products usage rates were those recommended by the producer and the water temperatures appropriate (>60°C). In the first experimental period (first 5-weeks period, named C) sodium hypochlorite (NaClO) detergent was used and in a subsequent, analogous, period (last 5-weeks period, named NC) non-chlorine liquid alkaline detergent (Perlac, Perdomini, Italy) was utilized. A 4-weeks interval was established between C and NC experimental periods in order to allow bacterial population adaptation to the new detergent.

Bulk tank milk samples were collected from three dairy farms over a 3 months period (from December 2016 to March 2017). Samples were collected in the last 3 days of each experimental week during C and NC period. One hundred-milliliter were collected from the storage tanks of each farm in sterile vials without preservative. In each sampling day, the milk obtained from the three farms was transported to a dairy factory, pooled, and, after natural creaming, used for the production of Trentingrana cheese. The whole bulk milk from the evening milking (WM), the corresponding partially skimmed milk obtained after overnight natural creaming (SM) and natural whey starter samples used for cheesemaking were collected for analysis.

Milk and whey samples intended for microbiological analysis were cooled at 4°C and analyzed within 8 h, while the samples for microbiota analysis were frozen and, then, transferred to the laboratory. Three cheese wheels for each experimental period were subjected to metagenomic and volatilomic analysis after 12 months of ripening.

A total of 45 milk samples (15 for each farm) were analyzed both for C and NC period along with 15 WM and 15 SM and 15 natural whey starters.

Milk and Natural Whey Culture Samples Microbiological Analysis

Milk samples were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology, Barcelona, Spain) and analyzed for standard plate count, coliforms and coagulase-positive staphylococci according to the procedures of the International Organization for Standardization (ISO, 1999, 2006, 2013). Lactic acid bacteria (LAB) were counted on de Man Rogosa and Sharpe (MRS) agar (Biolife Italiana, Milano, Italy) after anaerobic incubation (Anaerocult A, Merck, Darmstadt, Germany) at 30°C for 72 h and in M17 Agar (Biolife Italiana) incubated at 30°C in aerobic condition for 48 h. Heterofermentative LAB were determined by the most probable number (MPN) method using MRS broth with Durham tubes (MRS + C). Inoculated tubes were incubated at 30°C for 72 h and later at 37°C for 48 h and examined daily for gas production (Morandi et al., 2019). Positive tubes were checked microscopically to exclude the presence of yeasts and Enterobacteriaceae.

The MPN results were evaluated according to ISO 7218 (ISO, 2013).

Whey cultures were serially diluted in reconstituted (10% w/v) skimmed milk (Sacco System, Cadorago, Italy) and inoculated in Plate Count Agar (Biolife Italiana) under anaerobic conditions (Anaerocult A) at 44°C for 72 h.

The analyses were carried out in triplicate, and microbiological data were transformed by logarithm base 10 (log) and expressed in the descriptive statistic as mean and standard deviation (Std). A GLM analysis was performed with experimental period and month of analyses as fixed effects.

DNA Extraction and Purification

Milk and whey samples were thawed at room temperature. Five ml of milk were centrifuged at 500 × *g* for 10 min at 4°C; the supernatant was discarded, and the pellet was resuspended with 1 ml of saline solution (NaCl 0.9%) and centrifuged at 500 × *g* for 5 min at 4°C. The supernatant was discarded and the bacterial DNA was extracted from the samples as described previously (Cremonesi et al., 2006, 2018), by using a method based on the combination of a chaotropic agent (i.e., guanidinium

thiocyanate) with silica particles, to obtain bacterial cell lysis and nuclease inactivation. For Trentingrana PDO cheese (three samples from each treatment), 45 mL of 2% (w/v) K_2HPO_4 buffer solution (Sigma-Aldrich, Milan, Italy) were added to 5 g of cheese; the sample was, then, mixed for 90 s in a Stomacher machine (PBI, Milan, Italy). The DNA was extracted starting from 800 μL of the homogenized sample following the protocol described in Cremonesi et al. (2006) with some modifications. Briefly, 400 μL of lysis buffer (3 mol/L guanidine thiocyanate, 20 mmol/L EDTA, 10 mmol/L Tris–HCl, pH 6.8, 40 mg/mL Triton X-100, 10 mg/mL dithiothreitol) and 300 μL of binding solution (40 mg/mL silica from Sigma Aldrich, directly suspended in the lysis buffer) were added to the sample and vortexed for 30 s to obtain an emulsified solution. Then, the sample was incubated for 5 min at room temperature. After this step, the protocol was the same described in Cremonesi et al. (2006) with centrifugations at 550 × *g*. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The isolated DNA was stored at −20°C until use.

16S rRNA Gene Library Construction and Sequencing

Bacterial DNA was amplified using the primers described in literature (Caporaso et al., 2011) which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 μl volumes per sample. A total of 12.5 μl of Phusion High-Fidelity Master Mix 2× (Thermo Fisher Scientific, Waltham, MA, United States) and 0.2 μl of each primer (100 μM) were added to 2 μl of genomic DNA (5 ng/μl). Blank controls (i.e., no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystems 2700 thermal cycler (Thermo Fisher Scientific, Monza, Italy). Samples were denatured at 98°C for 30 s, followed by 25 cycles with a denaturing step at 98°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, plus a final extension at 72°C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman Coulter, Brea, CA, United States) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, United States). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., Wilmington, MA, United States), pooled in equimolar proportion and sequenced in one MiSeq (Illumina, San Diego, CA, United States) run with 2 × 250-base paired-end reads.

Microbiota Profiling

The reads obtained were analyzed by merging pairs using Pandaseq (Masella et al., 2012) and by discarding low quality reads (i.e., >25% bases with a phred Q-score <3). For computational reasons, a subset of 50,000 random reads per sample was extracted. Filtered reads were processed using the QIIME pipeline (v 1.8.0) (Kuczynski et al., 2011), clustered into Operational Taxonomic Units (OTUs) at 97% identity level and taxonomically assigned via RDP classifier (Wang et al., 2007) against the Greengenes database (release 13_8)[1]. Singletons (i.e., OTUs supported by only 1 read across all samples) were discarded as likely chimeras. OTU table was rarefied to the least-sequenced sample (i.e., 30,087 sequences). Alpha-diversity evaluations were performed using Chao1, Shannon index and observed species metrics and rarefaction curves were employed to determine whether most of the bacterial diversity had been captured. Statistical evaluation of differences in microbial alpha-diversity was performed by a non-parametric Monte Carlo-based test using 9,999 random permutations.

For beta-diversity, principal coordinates analysis (PCoA) was performed using weighted and unweighted UniFrac distances. Adonis function, which performs a partitioning of distance matrices among sources of variation using a permutation test with pseudo-F ratios, of the R package vegan (Oksanen et al., 2013) was employed to determine statistical separation of the microbiota profiles. Where appropriate, an analysis of intra- vs. inter- sample distances (i.e., comparison of distances among samples from the same experimental class vs. distances from those of the other class) were performed.

Differences in abundances of bacterial taxa among experimental groups were analyzed by non-parametric Mann–Whitney *U*-test. Unless otherwise reported, a *p*-value of 0.05 was used to assess significance. Statistical elaborations were performed using MATLAB software (R2008b, Natick, MA, United States).

Lactobacillus and *Streptococcus* Species Analysis

Characterization of *Lactobacillus* and *Streptococcus* spp. was performed by re-aligning all reads classified by QIIME within these genera to a custom reference database, which included a total of 149 *Lactobacillus* and 61 *Streptococcus* species with a genome finishing grade of "Complete," "Chromosome," or "Scaffolds" in NIH-NCBI database[2], for a total of 518 and 6,610 strains, respectively. *Lactobacillus* and *Streptococcus* spp. reads were clustered at 100% and re-classified through nucleotide BLAST (legacy BLAST, v 2.26, Altschul et al., 1990), using a *e*-value cutoff of 1e-10 and de-activating the dust-filter. Only reads matching for at least of 80% of their length were retained and, for each read, the best match (i.e., that or those with the higher bit-score) was selected. If a read had multiple classifications on different species, the classification was reset to genus level. Species-level characterization of *Streptococcus* spp. was performed on cheese samples only, whereas, for *Lactobacillus* spp., both whey starter and cheese samples were considered.

Volatilome Analysis

Cheese samples for the volatilome analysis were obtained from a half slice of the wheel 2.5 cm thick in two different position, 5 cm undercrust ("peripheral") and 5 cm from the core ("inner"), by means of a cylindrical tester of 10 mm diameter, and immediately introduced in the HS-vial for the analysis.

[1] ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz
[2] ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/

Volatilome produced by enzymatic activity during cheese ripening was determined by Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS) on the two portions (inner and peripheral) of the cheese. The extraction, separation, identification and semi-quantitation of the volatile compounds was conducted by means of a Combi-Pal automated sampler (CTC Analytics AG, Zwingen, Switzerland) coupled to an Agilent 6890 gas chromatograph with an Agilent 5975 mass spectrometric detector (Agilent Technologies, Santa Clara, CA, United States) and a polar column (Zebron ZB-WAX plus, 60 m × 0.25 mm × 10.25 μm, Phenomenex, Torrance, CA, United States). Extraction and separation conditions were described elsewhere (Battelli et al., 2019). Data were expressed as arbitrary units, as $\log_{10}$ of the peak area of the corresponding selected ion.

Statistical analysis was performed with the software package MINITAB ver. 15.1.20 (Minitab Inc., State College, PA, United States). Data were analyzed by ANOVA using the Tukey multiple comparisons method. A p-value ≤ 0.05 was considered significant.

Data Availability

Raw reads for both milk and cheese samples are available in NCBI Short Read Archive (SRA)[3] under accession number PRJNA616456.

RESULTS

Microbiological Quality of Milk and Whey Samples

The first evidence was that not using chlorine in cleaning milking equipment did not lead to any significant increase in coliforms and staphylococci content in milk within the 3-month trial period (**Table 1**). Bacterial counts tended to be lower in NC period (1.97 ± 1.02 vs. 1.60 ± 0.68 log CFU/mL for coliforms and 2.17 ± 0.48 vs. 2.07 ± 0.57 log CFU/mL for *Staphylococcus aureus*). Moreover, even if no significant differences were observed, higher levels of SPC and LAB in bulk milk were recorded comparing period C to period NC (4.06 ± 0.16 vs. 4.10 ± 0.20 log CFU/mL and). An increase in total bacteria count, although not significant, was observed also in whey-starter (**Table 2** – 8.11 ± 0.43 log CFU/mL vs. 8.66 ± 0.45 log CFU/mL in period C and NC, respectively). A slight increase in heterofermentative content was observed moving from C to NC period.

Milk and Whey Microbiota Revealed Differences Between Sodium Hypochlorite and Non-chlorine Detergent Period

The microbiota structure of milk and whey-starter samples (n = 96) was characterized by a total of 4,789,548 high quality reads, with a mean of 49,891 ± 1,067 reads per sample. Microbial profiles were evaluated: (i) for the three different farms (i.e., F1, F2, F3) and; comparing the experimental period (C, NC) (ii) per farm; (iii) on bulk (whole milk on arrival at the dairy) and vat (partially skimmed milk) milk; (iv) on whey-starter.

Rarefaction curves evaluation suggested that the depth of coverage was enough to describe the biological diversity within the samples. Farms showed a different microbial diversity, with F1 showing the highest and F2 the lowest (p-value = 0.003 with chao1, Shannon and observed species metrics, **Figure 1A**). Major differences in the principal constituents of the microbial community were revealed (p = 0.001 in all pair-wise comparisons for both unweighted and weighted UniFrac distances) (**Figure 1B**), even separating along the time points (C, interval, NC), with the only exception of F2-F3 profiles during interval period (**Supplementary Figure S1**). Independently from the experimental period a distinctive microbiota composition for the three farms was highlighted, with F1 microbiota mainly constituted by Firmicutes (average relative abundance: 60.2%), whereas F2 showed a somehow higher abundance of Proteobacteria (24.5%), and F3 was dominated by Bacteroidetes (37.2%) and Proteobacteria (35.7%) (**Supplementary Figure S2A**). This difference was evident also at genus level, with F1 mainly characterized by unclassified members of *Ruminococcaceae* and *Lachnospiraceae* families, F2 by unclassified members of *Ruminococcaceae*, *Lactobacillus* spp., and *Acinetobacter* spp., and F3 by *Chryseobacterium* spp., *Enhydrobacter* spp. and *Acinetobacter* spp. (summing up to about 70% of average rel. ab.) (**Figures 1C–E** and **Supplementary Figure S2B**).

We, then, decided to exclude the "interval" period for further evaluations, since the microbiota was still in a transitional stage and we preferred focusing on the main differences after any possible microbiota evolution in the shift from C to NC detergent. The microbial composition in the two experimental periods (C and NC) for each farm was significantly different ($p < 0.05$, weighted UniFrac for all farms) (**Figures 2A–C**). In the shift from C to NC period, F1 microbial composition showed a significant increase of *Chryseobacterium* and a significant reduction of *Oscillospira*; F2, on the other hand, was characterized by a significant increase in *Oscillospira* and *Clostridium*, and by a tendency toward an increase of *Lactobacillus* and toward a reduction of *Lactococcus*; finally, F3 showed a significant reduction of *Acinetobacter* and a trend toward the increase of *Streptococcus* (**Figures 2D–F**).

Difference between C and NC periods was evidenced also in bulk and vat milk microbiota during Trentingrana cheese-making procedure. Analysis of intra- vs. inter- period sample distances (i.e., distances among samples from the same experimental period vs. distances from those of the other period) revealed a significant separation for bulk (p = 0.002, unweighted UniFrac) and vat milk (p = 0.001 for both weighted and unweighted UniFrac) (**Figures 3A,B**). In NC period, bulk and vat milk samples both showed an increase of *Enhydrobacter* and a reduction of *Acinetobacter*; bulk milk also showed an increase of *Chryseobacterium* and a reduction of *Lactobacillus*, whereas vat milk showed a decrease of *Chryseobacterium* and *Macrococcus* (**Figures 3C,D**). Microbial composition of bulk

[3]http://www.ncbi.nlm.nih.gov/sra

TABLE 1 | Microbiological quality of bulk milk samples (n = 18 for each farm and experimental period).

Log CFU/mL	FARM 1				FARM 2				FARM 3				
	Experimental period				Experimental period				Experimental period				
	C		NC		C		NC		C		NC		*p*
	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	
SPC	4.03	0.10	4.00	0.00	4.11	0.24	4.03	0.13	4.05	0.07	4.25	0.26	NS
Coliforms	1.90	0.36	1.30	0.34	2.35	1.63	1.42	0.69	1.66	0.55	2.09	0.69	NS
S. aureus	2.54	0.32	2.33	0.49	1.65	0.23	1.54	0.42	2.31	0.32	2.33	0.38	NS
LAB in MRS	2.33	0.17	2.85	0.39	2.95	1.11	2.69	0.42	2.65	0.34	3.02	0.47	NS
LAB in M17	3.42	0.56	3.30	0.25	3.45	0.99	3.38	0.55	3.95	0.37	4.20	0.76	NS
Heterofermentative LAB	1.43	0.79	1.82	1.08	1.65	0.56	2.05	0.56	1.55	0.88	1.61	0.91	NS

Data are reported as mean and standard deviation (Std); NS stands for not significant (P > 0.05).

TABLE 2 | Partially skimmed milk by natural creaming and natural whey starter microbial counts in chlorine (C) and non-chlorine (NC) experimental periods (data are expressed as Log10 CFU/mL).

	Experimental period				
	C		NC		*p*
	Mean	Std	Mean	Std	
Naturally creamed milk					
SPC	1.00	0.00	1.07	0.13	NS
Coliforms	1.52	0.50	1.50	0.64	NS
LAB in MRS	2.60	0.48	2.57	0.55	NS
LAB in M17	3.13	0.45	3.45	0.73	NS
Heterofermentative LAB	2.02	0.86	1.90	1.06	NS
Whey starter					
Anaerobic bacteria	8.11	0.43	8.66	0.45	NS
Heterofermentative LAB	1.82	0.4	2.08	0.86	NS

Data are reported as mean and standard deviation (Std); NS stands for not significant (p > 0.05).

and vat milk resulted significantly different (p = 0.001 and p = 0.025 for unweighted and weighted UniFrac, respectively, **Supplementary Figure S3A**); the natural creaming favored the development of a more diverse microbial profile, as evident from that distances among bulk milk samples were significantly lower (p = 0.007, unweighted UniFrac) than those among vat samples (**Supplementary Figure S3B**) and by that bulk-vat paired distances did not result different from those between unpaired samples (i.e., distance between each bulk sample and all vat except from its paired one) (**Supplementary Figure S3C,D**).

Finally, whey-starter composition was quite entirely composed by *Lactobacillus* (98.8% of relative abundance), as expected; however, microbial profiles in C and NC periods remained distinct (p = 0.006, weighted UniFrac). At species-level, *Lactobacillus helveticus* was predominant during period C (62.9% of relative abundance) and significantly reduced during period NC (31.8%, p = 0.008), whereas *L. delbrueckii* had an opposite trend (28.1 and 58.6% for C and NC periods, respectively; p = 0.004) (**Figure 4**).

Cheese Results

With regard to cheese analysis, the sequencing was characterized by a total of 379,433 high quality reads, with a mean of 63,239 ± 14,054. Despite a small number of samples analyzed, both alpha- and beta-diversity analysis revealed a trend toward a separation between the two treatments (C and NC cheese samples), as it was evidenced by the significant difference in weighted UniFrac distances of cheese samples characterized by the same detergent vs. those of the other detergent (**Figures 5A–C**).

Trentingrana DOP cheese microbiota for NC treatment samples was dominated by members of the Firmicutes phylum, which accounted for about 96% of the relative abundance, while samples with C treatment revealed a composition made by a lower presence of Firmicutes (86.3% on average) and by Actinobacteria (3.7%), Proteobacteria (4.0%) and Bacteroidetes (4.4%). The overall bacterial composition of Trentingrana DOP cheese showed two main genera: *Lactobacillus* spp. (64.7 and 62.0% for C and NC, respectively) and *Streptococcus* spp. (7.1 and 28.9% for C and NC, respectively), together representing >90% of the total abundance in NC samples. Moreover, NC samples had lower presence of *Faecalibacterium* spp., *Propionibacterium* spp., and *Escherichia* spp. (**Figure 5D**).

Since *Streptococcus* and *Lactobacillus* genera constituted more than 90 and 70% of the total bacterial population for NC and C samples, respectively, a focus on these genera has been performed. Among *Lactobacillus*, *L. helveticus*, and *L. delbrueckii* were the main species in NC samples while *L. helveticus* and unclassified *Lactobacillus* were the predominant in C samples. *Streptococcus* population was dominated by *S. thermophilus* in both the experimental conditions (C and NC samples) (**Supplementary Figure S4**).

Volatilome

Supplementary Table S1 reports the relative abundance of the 19 main volatile metabolites detected in Trentingrana cheese samples, including six acids (acetic, propanoic, butanoic, 3-methylbutanoic, pentanoic, and hexanoic acid), three alcohols (ethanol, butan-1-ol, and pentan-2-ol), two aldehydes (2-methylbutanal and 3-methylbutanal), three esters (ethyl acetate,

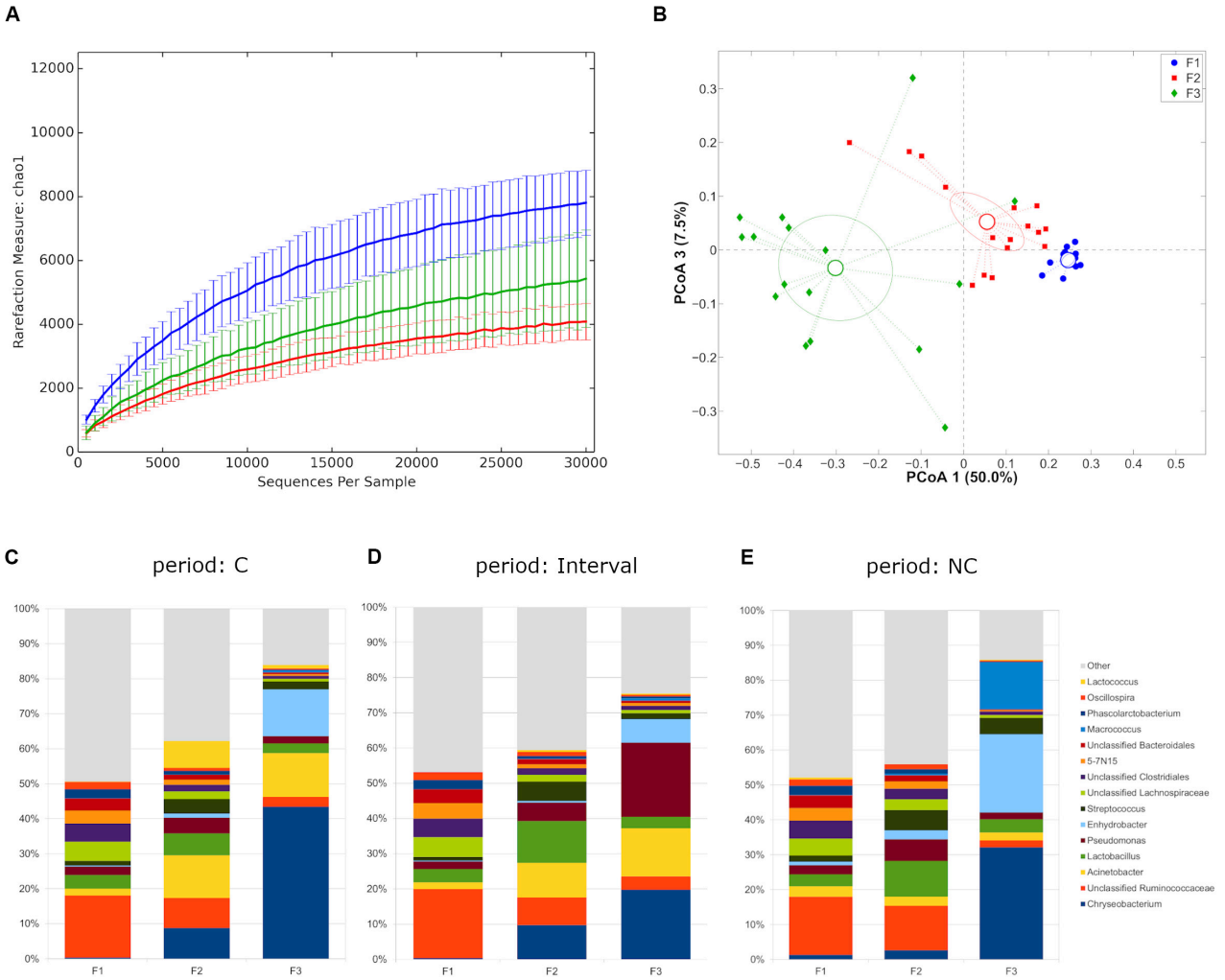

FIGURE 1 | (A) Alpha-diversity rarefaction curves (Chao1 metric) for milk samples from different farms. Data points were averaged on all time points for each farm. For each curve, average and standard deviation are shown; **(B)** Principal Coordinates Analysis from weighted UniFrac distances among samples. Each point represents a sample; data points were colored according to the farm they were collected from. Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals. The first and third principal coordinates are represented; **(C–E)** Barplots of average microbial relative abundance at genus level for the three farms tested divided on the three experimental periods [i.e., chlorine (C), interval, non-chlorine (NC)].

ethyl butyrate, and ethyl hexanoate), and five ketones (acetone, butan-2-one, pentan-2-one, heptan-2-one, and 3-hydroxybutan-2-one).

As expected, there was a strong difference between peripheral and inner portions of the cheeses, due to the heat load in the cheese core during molding that affects the ripening process (Pellegrino et al., 1997a,b). As a result, most of the carbonyl compounds and alcohols were more abundant in the inner samplings (p range: 0.002–0.037), while acids deriving from lipolysis and esters were more abundant in the peripheral ones (p range: <0.001–0.003).

Regarding C and NC periods, volatilome analysis showed an almost similar profile, with exception of butan-1-ol, propanoic and hexanoic acid that resulted to be significantly influenced by the two treatments applied (**Table 3**). Cheeses obtained in the C period were characterized by a higher level of propanoic acid, both in the inner and peripheral portions (p = 0.002 and 0.050, respectively). Moreover, only in the inner part, during C period butan-1-ol level was higher (p = 0.06) and hexanoic acid level was lower (p = 0.001) with respect to the corresponding NC period.

DISCUSSION

The purpose of this study was to investigate the influence of the use of chlorine-based products in the milking cleaning routine at farm level on bulk tank milk microbiota, and their impact on the dairy processing capacity. Furthermore, information supporting the importance of the farm environment as a unique environment was also provided.

In our study, the three farms were located in a narrow area (about 10 km from one another), had similar management

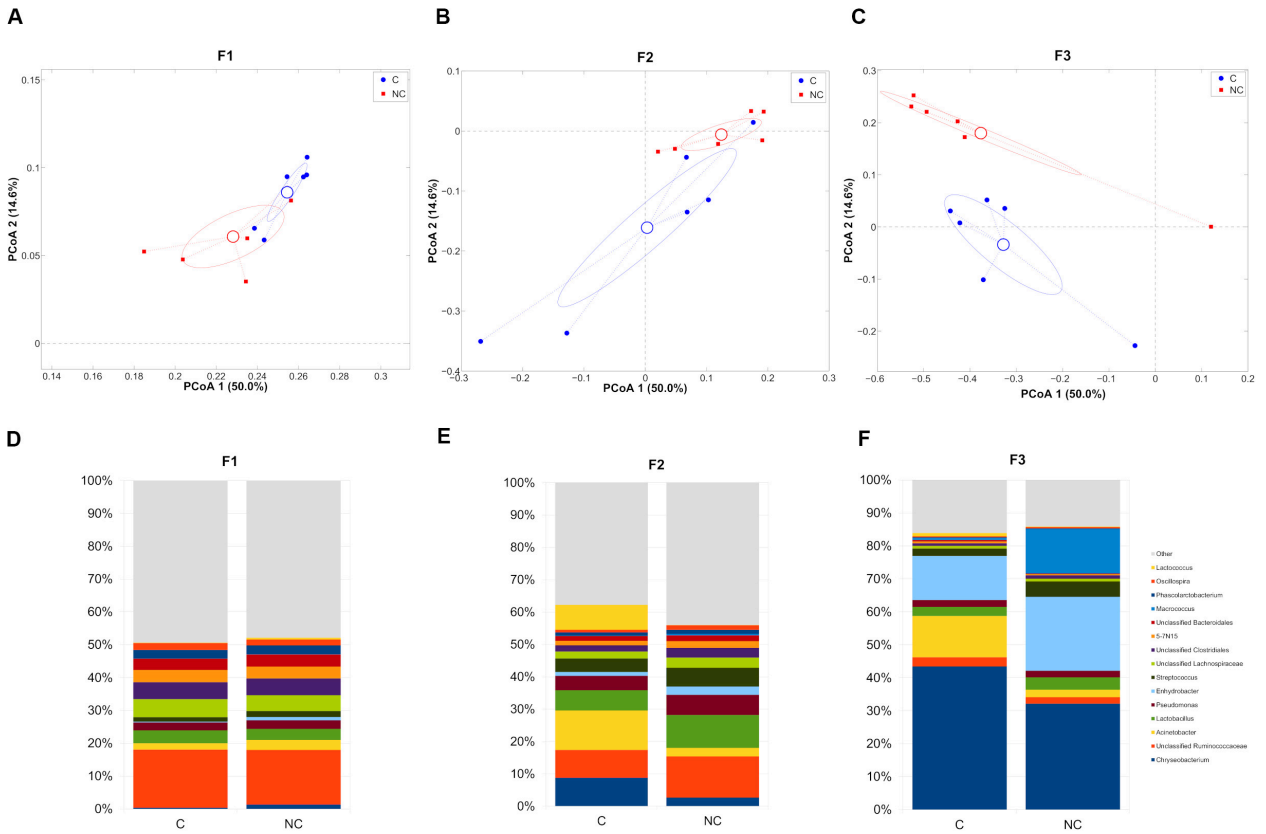

FIGURE 2 | (A–C) Principal Coordinates Analysis from weighted UniFrac distances among samples divided on the farm they were collected from. Each point represents a sample; data points were colored according to the experimental period (only C and NC periods). Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals. The first and third principal coordinates are represented. **(D–F)** Barplots of average microbial relative abundances at genus level for chlorine and non-chlorine experimental periods divided on the three farms tested.

system (herd entity and composition, herd housing and health, hygiene, udder preparation, milking equipment, cleaning and disinfecting procedures) but, despite this, they were characterized by a distinctive microbiota.

In fact, while cultural analysis revealed only slightly not significant differences between the microbial counts of bulk milks, alpha- and beta-diversities of milk microbiota samples provided a clear separation between samples from the three farms, demonstrating that the communities of milk microbiota were highly diverse. This observation clearly supports what different authors affirmed in previous studies, that multiple key factors characterizing the single farm and the exposition to niche-specific microbes during winter indoor housing, significantly influence the microbial community composition of bulk milk (Verdier-Metz et al., 2009; Mallet et al., 2012; Doyle et al., 2017; Li et al., 2018). Consistent with previous studies, the major bacterial phyla detected in the milk samples were Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Joishy et al., 2019); nevertheless, the relative abundance of useful cheese-making and spoilage micro-organisms differed from one farm to the other, with important implications from a dairy perspective. In F1 and F2 microbial taxonomic composition was dominated by Firmicutes (rel. ab. respectively 60.2% and 47.3%), while farm F3 showed a microbial composition dominated by Bacteriodetes (37.2%) and Proteobacteria (35.7%). NGS analysis revealed that farms F1 and F2 were richer in LAB (*Lactobacillus* and *Lactococcus* genera) while *Chryseobacterium*, and *Acinetobacter*, dairy spoilage associated genera (Vithanage et al., 2016), dominated in F3 milk samples.

The non-chlorine cleaning did not cause any increase in coliforms and staphylococci count, confirming that hot water usage, along with the proper use of non-chlorine products, assure the hygienic quality of milk (Gleeson et al., 2013). Our results are consistent with those reported by Pandey et al. (2014), which did not find significant differences in microbial counts comparing 200 ppm chlorine solution and 50 ppm iodophore solution usage for cleaning milking equipment.

Chlorine is known to alter both Gram-negative and Gram-positive bacterial membrane permeability providing cellular degeneration (Muhandiramlage et al., 2020). It is reasonable to hypothesize that its use in milking equipment cleaning and sanitation can influence the biodiversity of raw milk-associated microbiota.

Although the three bulk milk samples still contained a reproducible distinctive microbiota, bacterial population (beta-diversity) was different within each farm according to the

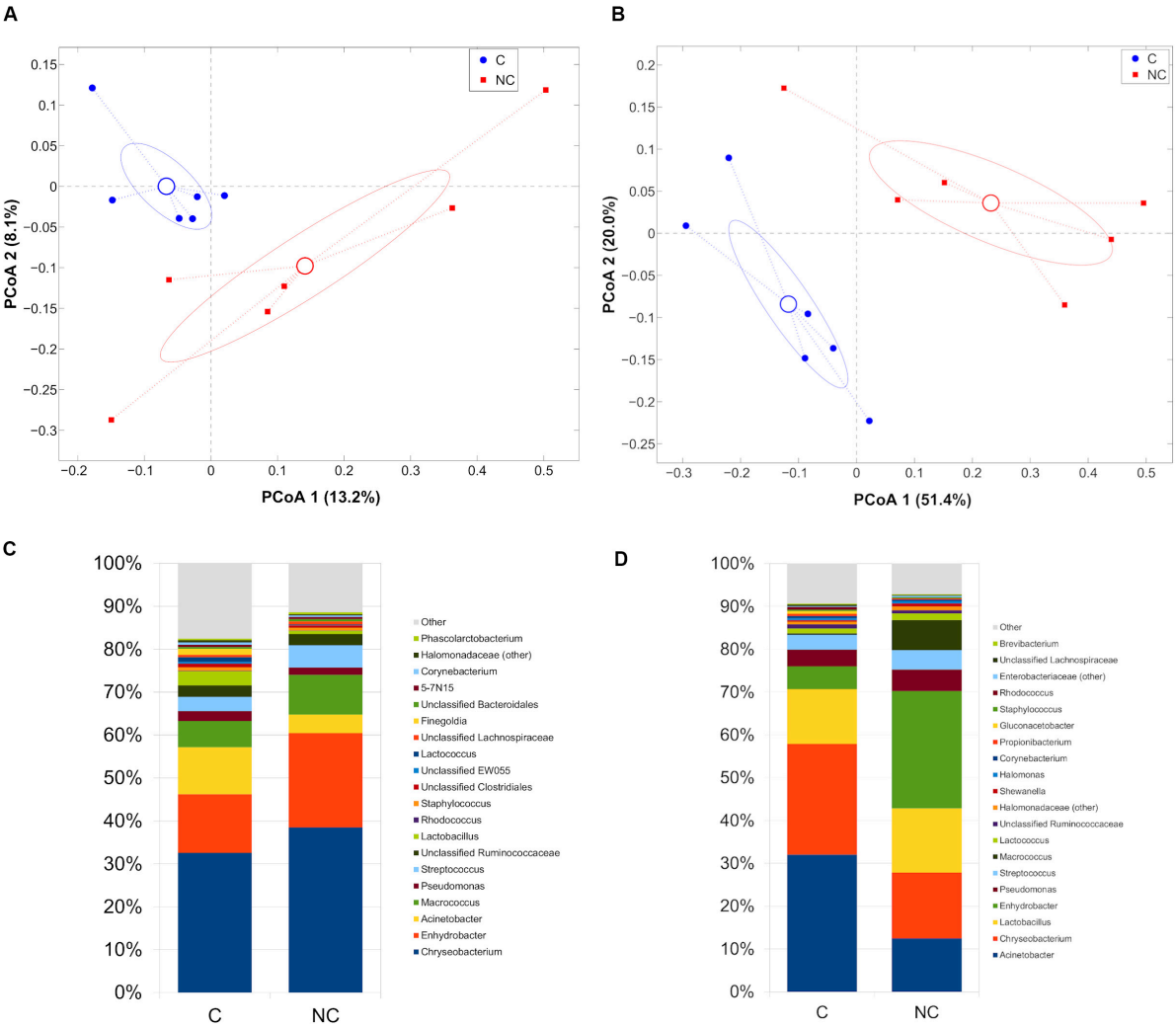

FIGURE 3 | Principal Coordinates Analysis from **(A)** unweighted UniFrac distances among bulk milk samples and **(B)** weighted UniFrac distances among vat milk samples. Each point represents a sample; data points were colored according to the experimental period (only C and NC periods). Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals. Barplots of average microbial relative abundances at genus level for chlorine and non-chlorine experimental periods for **(C)** bulk milk and **(D)** vat milk samples.

chlorine use in cleaning milking equipment. Metagenomic analysis highlighted that the influence of chlorine use on microbial population composition is still detectable for several days after the interruption of its use. Comparing C and NC periods, relative abundances analysis revealed that farm F2 and F3 had a significant decrease in *Chryseobacterium* and *Acinetobacter* genera, while in F1 milk the main reduction was observed regarding *Oscillospira* genus. Differently, *Chryseobacterium* and *Acinetobacter* increased in F1 milk moving from C to NC period, whereas in F2 the largest increase was in *Oscillospira*, *Clostridium*, *Lactobacillus*, and *Ruminococcaceae* families and in F3 a noticeable increase with regard to *Streptococcus*, *Macrococcus*, and *Enhydrobacter* was observed.

The different impact of chlorine products usage on the composition of raw milk microbial population is of great interest for dairy related processes. *Chryseobacterium* and *Acinetobacter* are Gram-negative bacteria associated to dairy spoilage, while *Oscillospira* is a gut-related genus belonging to *Ruminococcaceae* family (Joishy et al., 2019). *Chryseobacterium*, *Acinetobacter*, and *Clostridium* can cause a negative effect on the product quality, differently by *Lactobacillus*, *Lactococcus*, and *Streptococcus* which are the main actors of the cheesemaking process of raw milk cheeses. *Macrococcus* and *Enhydrobacter* have been previously reported as core bacterial genera in naturally fermented dairy products, although their role in the definition of quality attributes is still not clear (Mallet et al., 2012; Quigley et al., 2013; Zhong et al., 2016; Joishy et al., 2019).

Non-chlorine cleaning protocols have been set up and validated to reduce chlorine residues in milk and in derived dairy products (Gleeson et al., 2013). Different studies demonstrated that chlorine is highly effective in reducing the bacterial population in milk (Pandey et al., 2014), but no

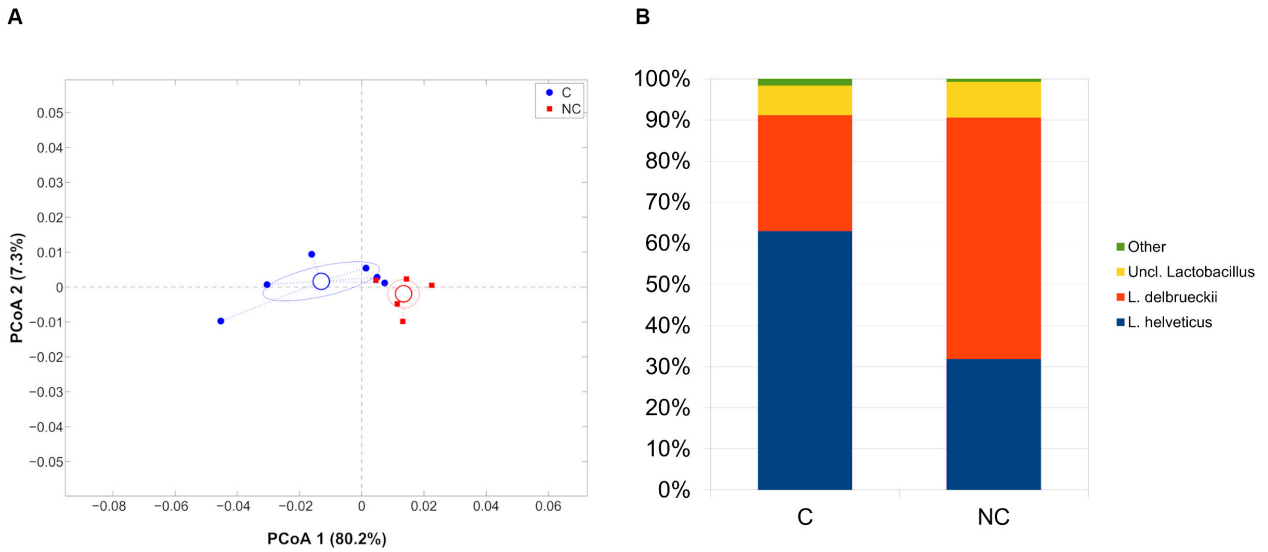

FIGURE 4 | (A) Principal Coordinates Analysis from weighted UniFrac distances among whey starter samples. Each point represents a sample; data points were colored according to the experimental period (only C and NC periods). Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals. The first and second components of the variance are shown. **(B)** Barplots of average microbial relative abundances of *Lactobacillus* species for chlorine and non-chlorine experimental periods.

TABLE 3 | Volatile organic compound (VOC) significantly affected by chlorine and non-chlorine treatment in Trentingrana cheese samples after 12 months of ripening at.

Compounds	Odor description*	Sampling	Chlorine	No-chlorine	Pooled SD	*p*-value
Butan-1-ol	fruity	inner	5.13^{B}	4.49^{A}	0.32	0.006
		peripheral	4.00	3.87	1.18	0.856
Propanoic acid	sour	inner	5.07^{B}	4.28^{A}	0.32	0.002
		peripheral	4.94^{B}	4.35^{A}	0.45	0.050
Hexanoic acid	rancid	inner	6.21^{A}	6.44^{B}	0.09	0.001
		peripheral	6.85	6.89	0.08	0.384

**Odor description reported derived from Curioni and Bosset (2002). Statistically significant differences in rows ($p \leq 0.05$) are indicated by different superscripts. Data expressed as log10 of arbitrary units (AU) of the peak area of the characteristic ion.*

information is available on its influence on the diversity and microbiota of raw milk.

Bacterial populations in raw milk consisting of the union of the three bulk milks arriving at dairy processing plant was analyzed before and after natural creaming. Natural creaming of milk in a large flat vat for about 12 h occurring at environmental temperature is known to largely affect the bacterial population of milk (Franciosi et al., 2012). As expected, high-throughput DNA sequencing analysis evidenced a significant microbial diversity in whole milk compared to partially skimmed milk. In addition, the impact of chlorine products use at farm level on milk microbiome composition after maturation was highlighted by the metagenomic data: significant changes in the core microbiota were observed with a reduction of *Acinetobacter*, *Chryseobacterium*, and *Macrococcus* genera and an increase with regard to *Enhydrobacter*.

As expected, whey-starter bacterial composition consisted mainly of *Lactobacillus* genus (Rossetti et al., 2008; Gatti et al., 2014; Morandi et al., 2019; Bertani et al., 2020). At a species-level, *L. helveticus* was predominant during period C (62.9% rel. ab.) and significantly reduced during period NC (31.8%), whereas *L. delbrueckii* had the exact opposite trend (28.1% period C; 58.6% period NC). This result is noteworthy, since we have already reported a worrying reduction of presence of several *Lactobacillus* species, foremost *L. delbrueckii*, in whey starter for Trentigrana production (Morandi et al., 2019). The loss of microbial biodiversity has been associated to a depletion of raw milk cheese sensory attributes by many authors (Broadbent et al., 2011; De Filippis et al., 2014).

Microbial diversity in cheese produced with milk collected from the three farms during C and NC experimental periods was found to be diverse, indicating the impact on the indigenous milk bacteria and in shaping cheese bacterial composition and consequently cheese quality traits.

Alpha- and beta-diversity analyses revealed significant differences among C and NC cheeses. The main differences found in the abundance of microbial groups concerned the Firmicutes taxa, which increased from 86.3 to 96% moving from C to NC period. The observed increase in the Firmicutes content is due to an increment in LAB abundances, in particular

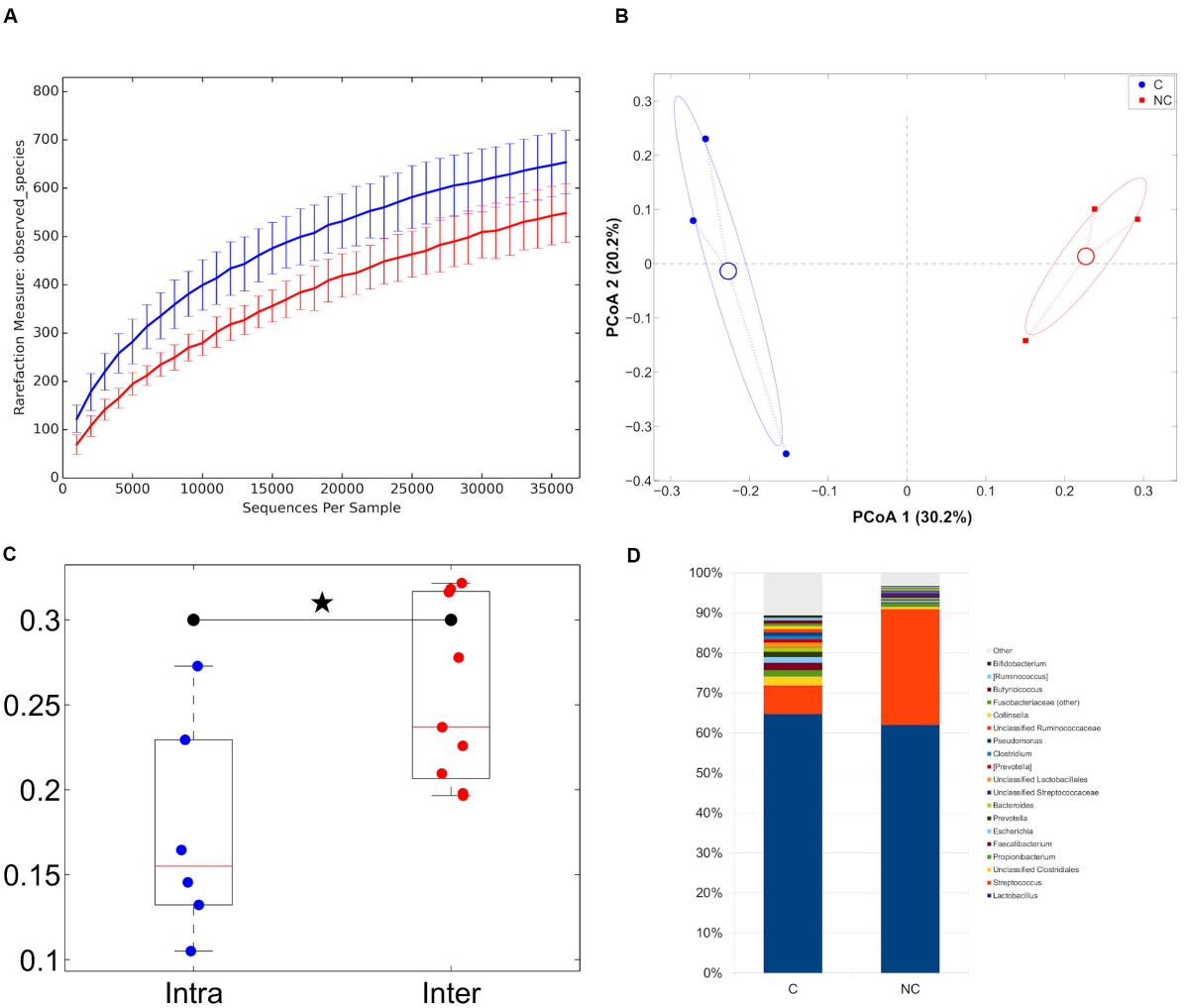

FIGURE 5 | (A) Alpha-diversity rarefaction curves (observed_species metric) for cheese samples representative of the experimental periods (C and NC). For each curve, average and standard deviation are shown; **(B)** Principal Coordinates Analysis from unweighted UniFrac distances among cheese samples. Each point represents a sample; data points were colored according to the experimental period. Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals. The first and second principal coordinates are represented; **(C)** Boxplots of intra- and inter-sample distances for chlorine and non-chlorine experimental periods; **(D)** Barplots of average microbial relative abundances at genus level for chlorine and non-chlorine experimental periods for cheese samples.

Lactobacillus and *Streptococcus* genera, that represent the key actors of the Trentingrana cheese making process for their acidifying activity in milk and their proteolytic activity in cheese, along with aroma compounds production (Morandi et al., 2019). Among *Lactobacillus* genus, as a consequence of previously underlined diversified whey starter microbial composition, a higher presence of *L. delbrueckii* was observed when chlorine products were not used at farm level. This result is of particular importance since LAB biodiversity has been associated to a higher sensory quality in cheese by different studies (Pogačić et al., 2016; Morandi et al., 2019).

This is consistent with data on the volatilome analysis of Trentingrana experimental wheels as it showed a slightly less intense lipolysis during C period in the inner part of the wheel cheese. In particular, hexanoic acid, one of the most important flavor compounds of Grana Padano cheese (Curioni and Bosset, 2002), showed significantly higher levels in NC samples. This compound derives from the lipolysis of milk triglycerides and, depending on its concentration and perception threshold, can contribute positively or negatively to the cheese aroma, being part of its typicity or resulting as a rancidity defect (Collins et al., 2003). Propanoic acid, responsible for sour notes, typically originated from the fermentation of the lactic acid produced by LAB during the cheese-making, showed a significantly higher level in the C period than in the NC period, confirming a higher development of Actinobacteria, in particular of *Propionibacterium*. Finally, in C samples a high level of butan-1-ol was detected. This compound has been identified in Grana Padano cheese and has been described as having floral, fragrant, fruity, sweet notes (Gómez-Torres et al., 2015) but, due to its

high sensory threshold, slightly contributes to cheese aroma (Qian and Burbank, 2007).

CONCLUSION

Raw milk microbial population is critically related to milk processability, spoilage and safety characteristics, but also plays a primary role in the deriving raw milk cheese quality, safety and sensory attributes.

To better understand the major drivers affecting the composition of milk microbiota at farm level, different studies have been conducted through both culture-based and high-throughput DNA sequencing technologies. In our study, we provided some evidences deepening the relationship between the use of chlorine products in cleaning and sanitizing procedure at farm level, microbial community of raw milk and its impact on whey starter and cheese microbiome.

Our preliminary results indicate that chlorine replacement is not associated with an increase of spoilage bacteria, staphylococci and coliforms, but it leads to an increase the milk microbiota biodiversity and, consequently, it can improve raw milk production performances and the overall cheese quality.

Further studies on cleaning and sanitation strategies alternative to chlorine-based protocols are needed, in order to define both their periodicity and regularity of application.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MB, AG, MM, and LZ conceived and planned the experiments. AG collected the milk, whey and cheese samples. PC, NC, and GB carried out the experiments. BC, MB, MM, EP, and LZ contributed to the experiments and the interpretation of the results. CC, MS, and AT performed the statistical analyses. PC, SM, MS, and MB wrote the manuscript in consultation with BC and GB. All authors discussed the results and critically revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.564749/full#supplementary-material

Supplementary Figure 1 | Principal Coordinates Analysis from weighted UniFrac distances among samples divided on the experimental period (**A:** chlorine, **B:** interval, **C:** non-chlorine). Each point represents a sample; data points were colored according to the farm they were collected from. Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals. The first and third principal coordinates are represented.

Supplementary Figure 2 | Barplots of bacterial relative abundances at **(A)** phylum and **(B)** genus level. Each bar represents a sample; samples are sorted according to farm and experimental period. Colored lines below each plot indicate the experimental period (blue: chlorine; green: interval; red: non-chlorine). Only the nine most abundant phyla and the 24 most abundant genera were shown. All remaining taxa were summed up into the "Other" category.

Supplementary Figure 3 | (A) Principal Coordinates Analysis from unweighted UniFrac distances among bulk and vat milk samples. Each point represents a sample; data points were colored according to their source, independently from the experimental period. Centroids represent the average coordinate for the data points in each category and ellipses indicate the 95% confidence intervals; **(B)** Boxplots of paired unweighted UniFrac distances. For each experimental period, distances between paired bulk and vat milk samples are represented. The black star indicates a statistically significant difference (Mann–Whitney *U*-test, $p < 0.05$); **(C,D)** Boxplots of paired bulk-vat milk intra- and inter-sample distances for chlorine and non-chlorine experimental periods.

Supplementary Figure 4 | Barplots of bacterial relative abundances for *Lactobacillus* and *Streptococcus* species-level characterization. All other bacteria are grouped into "Other" category. **(A)** Barplots of single samples (chlorine: left-most samples; non-chlorine: right-most samples) and **(B)** average relative abundances for experimental period.

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REVIEW
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Occurrence of Yeasts in White-Brined Cheeses: Methodologies for Identification, Spoilage Potential and Good Manufacturing Practices

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Yeasts are generally recognized as contaminants in the production of white-brined cheeses, such as Feta and Feta-type cheeses. The most predominant yeasts species are *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Rhodotorula mucilaginosa*, and *Trichosporon* spp. Although their spoilage potential varies at both species and strain levels, yeasts will, in case of excessive growth, present a microbiological hazard, effecting cheese quality. To evaluate the hazard and trace routes of contamination, the exact taxonomic classification of yeasts is required. Today, identification of dairy yeasts is mainly based on DNA sequencing, various genotyping techniques, and, to some extent, advanced phenotypic identification technologies. Even though these technologies are state of the art at the scientific level, they are only hardly implemented at the industrial level. Quality defects, caused by yeasts in white-brined cheese, are mainly linked to enzymatic activities and metabolism of fermentable carbohydrates, leading to production of metabolites (CO_2, fatty acids, volatile compounds, amino acids, sulfur compounds, etc.) and resulting in off-flavors, texture softening, discoloration, and swelling of cheese packages. The proliferation of spoilage yeast depends on maturation and storage conditions at each specific dairy, product characteristics, nutrients availability, and interactions with the co-existing microorganisms. To prevent and control yeast contamination, different strategies based on the principles of HACCP and Good Manufacturing Practice (GMP) have been introduced in white-brined cheese production. These strategies include milk pasteurization, refrigeration, hygienic sanitation, air filtration, as well as aseptic and modified atmosphere packaging. Though a lot of research has been dedicated to yeasts in dairy products, the role of yeast contaminants, specifically in white-brined cheeses, is still insufficiently understood. This review aims to summarize the current knowledge on the identification of contaminant yeasts in white-brined cheeses, their occurrence and spoilage potential related to different varieties of white-brined cheeses, their interactions with other microorganisms, as well as guidelines used by dairies to prevent cheese contamination.

Keywords: white-brined cheese, spoilage yeasts, yeast identification, off-flavors, microbial interactions, GMP

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INTRODUCTION

Cheese making, particularly of white-brined cheeses, is one of the oldest dairy technologies, originated from the Mediterranean region and the Middle East more than 8000 years ago. Most recent scientific literature has been dedicated to white-brined cheeses produced in the Balkan Peninsula region, Turkey, Northern Africa, European countries, and some parts of Asia and Latin America (Hayaloglu, 2017). Today, many varieties of white-brined cheeses, with typical representatives being Feta and Feta-type cheeses, are produced and consumed worldwide. Only in Greece, production of Feta exceeds 110,000 tons per year (Hellenic Statistical Authority, 2018). White-brined cheeses are made from goat, sheep or cow milk, or a mixture of milks, and characterized by a creamy smooth texture and a mild salty and/or acidic taste. Traditionally, Feta-type cheeses were solely made from raw milk, but, nowadays, industrial dairies pasteurize the milk and use commercial starter cultures. Manufacture of the white-brined cheeses includes milk pasteurization, addition of the starter cultures and rennet, followed by milk coagulation and curd formation. Afterwards, curd is drained in molds, cut into pieces, salted (dry or in brine) and ripened in brine for typically several months (Hayaloglu, 2017).

Yeasts are widely spread in dairy production and frequently found in raw milk, brine, air, production surfaces, cheese vat and cloth, curd cutting knife, etc. (Sharaf et al., 2014; Banjara et al., 2015). In some types of cheeses, such as blue-veined and smear-ripened cheese, yeasts can be used as adjunct cultures, affecting the ripening process, formation of aroma compounds, and interaction with starter cultures (Ferreira and Viljoen, 2003; Kesenkaş and Akbulut, 2008; Gori et al., 2013; Ryssel et al., 2015). In white-brined cheeses, yeasts are not included as starter cultures and frequently referred as contaminants, though, sometimes, they are a part of secondary microflora (Kesenkaş and Akbulut, 2008). The most frequent yeast species in white-brined cheeses are *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Trichosporon cutaneum*, *Yarrowia lipolytica*, and *Candida* spp. (Golić et al., 2013; Karasu-Yalcin et al., 2017). It should be noted, that *G. candidum* was formerly recognized as the anamorph form of *Galactomyces geotrichum* (De Hoog et al., 1998) and, as such, both species names have been used more or less randomly in dairy literature. Based on later studies, the species *Galactomyces candidus* was accepted as the teleomorph form of *G. candidum*, and *G. geotrichum* was accepted as a separate species (De Hoog and Smith, 2004, 2011), which might cause some confusion referring especially to older literature. As most dairy isolates seem to belong to *G. candidum*, as characterized by Perkins et al. (2020), in the current review the species name *G. candidum* is generally used unless *G. geotrichum* is explicitly mentioned in the original literature.

Yeast spoilage activities might lead to alteration of the organoleptic properties, decreased shelf life, and impaired quality of the dairy products (Salustiano et al., 2003; Radha and Nath, 2014). Yeast propagation in dairy products, particularly in white-brined cheeses, depends on numerous factors, such as the composition of milk, nutrients availability, interactions with co-existing microorganisms, production, and storage conditions (Soliman and Aly, 2011; Buehler et al., 2017; Laèanin et al., 2017). Furthermore, it is well known that yeasts differ in their biochemical functions and metabolic activities and, consequently, in their spoilage behavior (Akabanda et al., 2013; Haastrup et al., 2018; Bayili et al., 2019). In this context, accurate taxonomic identification of yeast species and yeast genotyping to the strain level are essential to evaluate the spoilage potential of any yeast contaminants. However, despite the importance of yeasts as spoilage microorganisms in white-brined cheeses, limited knowledge exists on the spoilage potential of the different yeast species and the specific traits, leading to their quality defects. The aim of the present review is, therefore, to give an overview on state-of-the-art technologies for identification and detection of spoilage yeasts, the diversity of yeast species able to cause quality defects in white-brined cheeses, extrinsic and intrinsic factors influencing their spoilage potential, the role of bio-protective cultures and other microbial interactions, as well as the routes of contamination and good manufacturing practices.

IDENTIFICATION AND DETECTION OF YEASTS IN DAIRY PRODUCTS

Conventional and Molecular-Biological Methodologies for Species Identification

Traditional methods of yeast identification in dairy products are based on macro- and micro-morphological observations and physiological characteristics, such as growth requirements, assimilation, and fermentation of carbohydrates and nitrogen (Garnier et al., 2017a). Phenotypic indicators, however, are highly heterogeneous and uncertain, as different yeast species might exhibit close morphological and physiological features. Traditional culturing techniques are commonly combined with molecular approaches to identify yeast species, associated with different types of dairy products and production environments. Currently, sequencing of the D1/D2 region of the 26S rRNA gene and the internal transcribed spacer (ITS) domains (ITS1 and ITS2) divided by the conserved 5.8S rRNA gene, are the most frequently used molecular methods of yeast identification (Lopandic et al., 2006; Gori et al., 2013; Buehler et al., 2017; Garnier et al., 2017b; Haastrup et al., 2018; Tokak et al., 2019; Merchán et al., 2020). Both approaches employ extensive databases to ensure discrimination between closely related yeast species. For instance, Garnier et al. (2017b) performed 26S rRNA gene sequencing to classify spoilage yeasts to species level, e.g., *Candida inconspicua*, *Candida intermedia*, *Candida parapsilosis*, *D. hansenii*, *G. candidum*, *K. lactis*, *K. marxianus*, *Meyerozyma guilliermondii*, *Pichia fermentans*, and *Y. lipolytica*, from French dairy products (cream, fresh cheese, smear cheese, etc.) and factory air. Application of ITS sequencing in another extensive study (346 fungal dairy isolates), revealed that species within the genera *Debaryomyces*, *Candida*, and *Kluyveromyces* were dominating in raw and pasteurized milk cheeses (Buehler et al., 2017).

Recently, an enhanced focus has been directed toward specific phenotypic identification techniques, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and Fourier transform infrared spectroscopy (FTIR), enabling rapid and cost-effective taxonomic identification of dairy-associated yeasts. MALDI-TOF MS generates protein-based spectral profiles (fingerprints) acquired by desorption of specific peptide/protein biomarkers released from the cell surface by acidic treatment (Pinto et al., 2011; Chalupová et al., 2014). This method was successfully applied for the identification of contaminating yeast species in yogurts and cheeses (Kačániová et al., 2018; Halil Kılıç, 2019). FTIR is based on the detection of functional biochemical groups directly from intact cells, producing metabolic spectral "fingerprints" unique for yeast species (Wenning et al., 2002; Büchl et al., 2008; Patel, 2019). Identification of a given yeast isolate from a fingerprint-like spectrum requires a comprehensive reference database. Previously, especially the MALDI-TOF MS databases comprised predominantly clinical isolates, which presented a notable limitation for typing of the dairy-related yeasts (Dongowski et al., 2000; Larpin-Laborde et al., 2011; Moothoo-Padayachie et al., 2013). It is acknowledged, however, that the databases are being gradually updated to cover a broader range of food-related yeast species. The drawback of FTIR is the high sensitivity to growth conditions of yeasts and the sample preparation procedure, which, together with insufficient database size, might lead to poor accuracy of yeast identification (Colabella et al., 2017).

Molecular-Biological Methodologies for Identification at the Strain Level

Strain typing is essential to trace the yeasts "hot spots" in dairy production in order to prevent contamination and extend the dairy shelf life. Various genotyping or DNA fingerprinting techniques are currently applied for the identification of dairy yeasts at species and strain levels. For screening purposes, yeast genotyping and cluster analysis of the DNA fingerprints are often introduced prior DNA sequencing. As a standard strain-typing approach, pulsed-field gel electrophoresis (PFGE) is used to evaluate intraspecies diversity of chromosome arrangements or chromosome-length polymorphism (Miller, 2013; Lopez-Canovas et al., 2019). The DNA separation by PFGE relies on the ability of intact yeast chromosomes to reorient and migrate in a new direction in agarose gel in response to an alternating electric field. High discrimination power, robustness, and reproducibility of PFGE were verified for *D. hansenii* strains collected from the production of Danish surface−ripened cheeses (Petersen and Jespersen, 2004), the dairy strains of *K. marxianus* (Fasoli et al., 2015; Naumova et al., 2017), and *S. cerevisiae* (Hage and Houseley, 2013).

Other genotyping techniques, allowing to differentiate closely related yeast species up to the strain level, are based on the PCR amplification with the use of primers, targeting repeated DNA sequences along the chromosome (Lopandic et al., 2006; Gori et al., 2013). Among them, the 5′-anchored primer $(GTG)_5$ repetitive-PCR fingerprinting was applied, e.g., for characterization of yeast communities in soft cheese from Spain (Merchán et al., 2020), Danish surface-ripened cheeses (Gori et al., 2013), and spontaneously fermented milk products from West Africa (Akabanda et al., 2013; Bayili et al., 2019). Randomly amplified polymorphic DNA (RAPD), employing a single primer M13 for random amplification of complementary genome sequences, was used for taxonomic classification of yeasts in Fiore Sardo cheese (hard cheese from raw sheep's milk) (Fadda et al., 2004), fresh and sour curd cheeses (Lopandic et al., 2006), variety of Italian and Greek cheeses (Mozzarella, Caprino, Feta, etc.) (Andrighetto et al., 2000), and for differentiation of *D. hansenii* strains isolated from raw milk cheeses (Padilla et al., 2014).

Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis is another common yeast typing method, which involves PCR amplification of the 5.8S-ITS rDNA regions, followed by digestion with two or more restriction enzymes, typically *Cfo*I, *Hae*III, *Hpa*II or *Hin*fI. The RFLP of the ITS regions was efficiently applied for typing of *D. hansenii* strains, isolated from the surface-ripened cow's milk cheeses of the Danbo type (Petersen et al., 2002) and the Gubbeen Farmhouse cheese (Mounier et al., 2006), and for characterization of yeast microbiota, composed of *D. hansenii*, *K. marxianus*, *K. lactis*, *M. guilliermondii*, *Y. lipolytica*, *Trichosporon coremiiforme*, *Trichosporon domesticum*, and *Candida* spp., in goat and ewe's milk cheeses (Padilla et al., 2014).

Multilocus sequence typing (MLST) is originally based on the analysis of polymorphic sites in a number of conserved housekeeping genes, serving as unique genetic markers (alleles) within a species (Muñoz et al., 2014). Advances in the whole genome sequencing allowed to optimize MLST schemes for specific species and increase discriminative power by scattering multiple loci within a genome. According to Tittarelli et al. (2018), MLST based on five housekeeping genes (*IPP1*, *TFC1*, *GPH1*, *GSY2*, and *SGA1*) provided sufficient polymorphic sites for classification and selection of *K. marxianus* strains in Italian cheeses. Lavoie et al. (2012) performed MLST using six loci (*ADE2*, *HIS3*, *LEU2*, *LYS2*, *NMT1*, and *TRP1*) to study the occurrence of *Issatchenkia orientalis* isolates from raw milk cheese. In a recent study, Perkins et al. (2020) applied the whole genome sequence approach to elucidate genetic diversity and evolutionary pathways of *G. candidum* isolated from smear-ripened cheeses and other sources. The authors developed a new MLST scheme based on six targeted loci (*ALA1*, *CDC19*, *SAPT4*, *GLN4*, *PGI1*, and *PGM2*) and identified 15 sequence types (STs) out of 41 strains, conferring that the allele variation arose from recombination events. The authors suggested that recombination events induced an adaptive divergence between the wild strains and the cheese-making strains of *G. candidum*.

Novel Culture-Independent "Omics" Technologies

Recent advances in next-generation DNA sequencing (NGS) and bioinformatics tools have been adopted by the dairy sector to achieve deeper insights into diversity, succession, and

interactions within microbial communities in dairy products. The NGS technologies allow high throughput sequencing of total microbial DNA or RNA without any prior culturing. Generally, the workflow includes DNA library preparation by amplification of a fragment of interest, using primers with indexing adapters specific to each platform, as previously described in many excellent reviews [recently reviewed by Kumar et al. (2019)]. In dairy studies, the DNA amplicons typically target 16S rRNA gene for lactic acid bacteria and 26S rRNA or ITS genes for yeasts. In earlier studies, the 454 pyrosequencing technology (Life Sciences, Roche) was employed, e.g., to investigate microbial succession during ripening of semi-hard Danbo cheeses (Ryssel et al., 2015) and co-occurrence of potential yeast spoilers and LAB in different types of cheese and production environment (Stellato et al., 2015). Currently, the rapidly evolving NGS Illumina technology established cost-effective and accurate DNA sequencing platforms (e.g., MiSeq and NextSeq 550), generating reads of more than 300 bp, i.e., compatible to the fragments recommended for yeast identifications by ITS1-5.8S-ITS2 rDNA (Schoch et al., 2012). Especially for scientific purposes, NGS Illumina technology is being a routinely used method to examine ecology and dynamics of microbiotas in diverse fermented milk products (Jatmiko et al., 2019; Sessou et al., 2019). For example, Ceugniez et al. (2017) described the evolution of yeasts during ripening of the Tomme d'Orchies type cheeses (France), in which prevalent species, such as *Y. lipolytica*, *G. geotrichum*, *Kluyveromyces* spp., and *Debaryomyces* spp., were detected by ITS2 rRNA gene sequencing (Illumina). NexSeq sequencing targeting ITS2, together with culture-dependent 26S rRNA gene sequencing, were applied to get insight into halotolerant yeast population in the brine of semi-hard Danbo cheese (Haastrup et al., 2018). A good correlation between culture-dependent and -independent techniques was observed for the predominant microorganisms (e.g., *Debaryomyces* spp., *Candida* spp., and *Yamadazyma* spp.). For less abundant yeast species (e.g., *Trichosporon* spp.), a lower correlation was observed due to the fact that both viable and dead cells could be detected by the NGS sequencing, differences in the DNA amplicons, isolation medium, etc. (Haastrup et al., 2018). Bertuzzi et al. (2018) applied whole-metagenome shotgun sequencing to screen the microbial population on the smear-ripened cheeses and analyzed the association of metagenomic clusters with the variation of pH, color, and flavor development. Using correlation analysis, it was possible to associate individual microorganisms with volatile compounds in the cheese surface. Among them, specifically, *D. hansenii* correlated with the production of alcohols and carboxylic acids, while *G. candidum* correlated with sulfur compounds (Bertuzzi et al., 2018).

In 2014, Oxford Nanopore Technologies (ONT) released a 3rd generation portative low-cost platform for DNA and RNA sequencing. Nanopore sequencers (MinION, GridION) measure the ionic current fluctuations, when single-stranded nucleic acids pass through protein-based nanopores. Compared to Illumina sequencing, ONT generates ultra-long DNA reads and eliminates amplification bias as no amplification step is needed for library construction (Amarasinghe et al., 2020). Nanopore long-read technology (MinION) has already been tested for *de novo* sequencing and assembling of the 21 strains of *S. cerevisiae* (Istace et al., 2017) and for identification of *S. cerevisiae*, *Rhodotorula graminis*, and *Malassezia* spp. (D'Andreano et al., 2020). Recent studies of microbiota from fresh and ripened cheeses demonstrated that the contiguity of microbial genome assemblies using shotgun MinION was much higher than the Illumina-only assemblies, allowing complete mapping of genes (e.g., transposable elements), which are generally missed using a short-read sequencing strategy (Ianni et al., 2020).

SPOILAGE YEAST SPECIES IN WHITE-BRINED CHEESES

Major Yeast Genera Occurring in White-Brined Cheeses

Depending on variety of white-brined cheeses, yeasts might comprise a part of the microflora, e.g., in a smear-forming surface layer, which may positively influence cheese flavor and texture without leading to quality defects (Bintsis et al., 2000). Occurrence of yeast species in white-brined cheeses within the genera *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Torulaspora*, *Trichosporon*, and *Yarrowia* has frequently been reported (Rantsiou et al., 2008; Golić et al., 2013; Cardoso et al., 2015; Šuranská et al., 2016; Karasu-Yalcin et al., 2017), however, the detailed information on yeast taxonomy, properties, and spoilage potential is often missing. Species *D. hansenii* is most frequently isolated from the cheese environment, most likely, due to its halophilic nature and affiliation to cheese brine (Haastrup et al., 2018). A reason for lacking an overview of spoilage yeasts in white-brined cheeses lies in an extensive number of artisanal products, which are traditionally produced by the herders with different production technologies around the world. Yeast contamination, specifically in artisanal products, is difficult to control. Diversity of the spoilage consortia and yeast propagation will typically vary between the dairies due to the differences in raw materials, usage of pasteurization, rennetting temperature (30–38°C) and time (40–180 min), brining conditions and salt concentration (7–16% NaCl), ripening temperature and storage period, the standards of hygiene during cheese making, etc. (Hayaloglu, 2017). The major varieties of white-brined cheeses and the associated potential spoilage yeasts are presented in **Table 1**.

Traditional White-Brined Cheeses From Greece

One of the oldest and most popular types of white-brined cheeses is Feta, a traditional Greek cheese, classified as Protected Designation of Origin (PDO) (European Commission, 2002). Feta is a soft white cheese ripened in brine, with a rancid and slightly acid flavor, and a firm and smooth texture (Litopoulou-Tzanetaki and Tzanetakis, 2011). Traditionally, it is manufactured from non-pasteurized ovine milk or a mixture of ovine and caprine milk (up to 30%) (**Table 1**). At present, Feta is commercially made with pasteurized milk, using starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii*

TABLE 1 | Occurrence of spoilage yeasts in white-brined cheeses.

Product	Cheese Category	Raw material (milk)	Yeast species	Country	References
Feta	Soft	Ovine or a mixture of ovine with caprine	*Candida sphaerica* *Candida zeylanoides* *Debaryomyces hansenii* *Kluyveromyces lactis* *Lachancea thermotolerans* *Pichia fermentans* *Pichia membranifaciens* *Tetrapisispora blattae* *Saccharomyces cerevisiae*	Greece	Vivier et al., 1994; Rantsiou et al., 2008; Litopoulou-Tzanetaki and Tzanetakis, 2011
Halloumi	Semi-hard	Ovine, caprine or mixture of them (optionally cow)	*Candida* spp. *Debaryomyces hansenii* *Pichia membranifaciens*	Greece, Cyprus	Kaminarides et al., 2009; Mehyar et al., 2018; Kamilari et al., 2020
White cheese	Soft	Cow	*Candida aaseri* *Candida boidinii* *Candida guilliermondii* *Candida intermedia* *Candida sake* *Candida zeylanoides* *Debaryomyces hansenii* *Geotrichum candidum* *Kluyveromyces lactis* *Kluyveromyces marxianus* *Naumovozyma dairenesis* *Pichia membranifaciens* *Torulaspora delbrueckii* *Yarrowia lipolytica*	Denmark	Westall and Filtenborg, 1998
White-pickled	Soft	Ovine, caprine or bovine	*Candida zeylanoides* *Cryptococcus albidus* *Debaryomyces hansenii* *Filobasidium globisporum* *Galactomyces geotrichum* *Hanseniaspora uvarum* *Kluyveromyces lactis* *Torulaspora delbrueckii* *Torulaspora quercuum* *Trichosporon gracile* *Trichosporon ovoides* *Yarrowia lipolytica*	Serbia	Golić et al., 2013; Šuranská et al., 2016
White-brined	Unknown	Cow	*Candida* spp. *Rhodotorula* spp. *Saccharomyces* spp.	Bulgaria	Chipilev et al., 2016
Akawi	Semi-hard	Ovine, caprine or mixture of them	*Candida guilliermondii* *Debaryomyces hansenii*	Lebanon, Syria	Pachlová et al., 2016
Beyaz peynir (Turkish White cheese)	Semi-hard	Ovine, caprine, cow or mixture of them	*Candida* spp. *Candida zeylanoides* *Debaryomyces hansenii* *Kluyveromyces lactis* *Starmera amethionina* *Torulaspora delbrueckii*	Turkey	Öztürk and Şahin, 2000; Togay et al., 2020
Mihaliç	Hard	Ovine or caprine	*Candida bertae* *Candida catenulata* *Candida cylindracea* *Candida famata* *Candida inconspicua* *Candida krusei* *Candida paludigena* *Candida robusta* *Candida tropicalis* *Candida zeylanoides* *Clavispora lusitaniae* *Geotrichum candidum* *Kodamaea ohmeri* *Trichosporon asahii*	Turkey	Solak and Akin, 2013; Karasu-Yalcin et al., 2017; Togay et al., 2020

(Continued)

TABLE 1 | Continued

Product	Cheese Category	Raw material (milk)	Yeast species	Country	References
Jiben-Al-Arab	Soft	Ovine	*Candida albicans* *Candida krusei* *Candida parapsilosis* *Candida tropicalis* *Geotrichum candidum* *Rhodotorulla* spp.	Iran	Khalil et al., 2018
Domiati	Soft	Bovine	*Candida albicans* *Candida krusei* *Debaryomyces hansenii*	Egypt	Sharaf et al., 2014; Hameed, 2016
Serro Minas	Semi-hard	Bovine	*Candida atlantica* *Candida catenulata* *Candida intermedia* *Candida parapsilosis* *Candida phangngensis* *Candida silvae* *Candida tropicalis* *Debaryomyces hansenii* *Geotrichum candidum* *Kluyveromyces lactis* *Kluyveromyces marxianus* *Kodamaea ohmeri* *Rhodotorula mucilaginosa* *Saccharomyces cerevisiae* *Torulaspora delbrueckii* *Trichosporon* spp. *Trichosporon montevideense*	Brazil	Cardoso et al., 2015

subsp. *bulgaricus* (Manolopoulou et al., 2003; Rantsiou et al., 2008). After coagulation and drainage, the curd is dry-salted for 4 – 5 days and, then, laid into the brine (8% NaCl) for ripening. After dry-salting, cheese remains on the cheese-table for approximately 15 days (warm ripening at 16–18°C). The rest of ripening period lasts for at least 60 days in the barrels at a cold storage temperature (Rantsiou et al., 2008; Litopoulou-Tzanetaki and Tzanetakis, 2011). In the early 1990s, yeast species of *Tetrapisispora blattae* (former name *Kluyveromyces blattae*), *Candida sphaerica* (anamorph of *K. lactis*), and *Lachancea thermotolerans* (former name *Kluyveromyces thermotolerans*) were isolated from brine of Feta cheese (Vivier et al., 1994). Manolopoulou et al. (2003) analyzed Feta cheese from three dairies in the Peloponnese Region (Southern Greece), showing that the total yeast counts in curd varied between the dairies (10^2–10^3 CFU/g) and generally increased at the dry room ripening period (2.6×10^3–4.6×10^4 CFU/g). Rantsiou et al. (2008) used culture-dependent and culture-independent techniques to characterize the microbiota of Feta cheeses produced by four different manufactures in Greece. The yeast counts at the most production sites were of 10^3 CFU/g, presented by the dominant yeast species of *K. lactis* (79–83% of the total yeast population). The less abundant species were *P. fermentans*, *Pichia membranifaciens*, and *Candida zeylanoides*, occasionally isolated from single producers (16–20% of the total yeast population). The authors concluded that reduced diversity of yeast species in Feta was due to the adaptation to the particular environment of brine. According to Litopoulou-Tzanetaki and Tzanetakis (2011), yeasts and halotolerant microbes are the predominant microorganisms in fresh Feta cheese produced with non-pasteurized ewe's milk. The total yeast counts, primarily dominated by *S. cerevisiae* and *D. hansenii*, were highest after the fourth day of ripening (7.2×10^5 CFU/g) and, afterward, decreased during the ripening period (to 5.9×10^4 CFU/g after 60 days of ripening).

Halloumi is a traditional white-brined cheese, produced mainly in Cyprus and Greece as a PDO product in accordance with the EU Regulation No 1151/2012 (European Commission, 2015) (**Table 1**). It is commonly made from pasteurized ovine or caprine milk (or a mixture of them), though cow milk can be added as well (Kamilari et al., 2020). Cheese blocks can be wrapped in dry leaves of *Mentha viridis*, giving the characteristic minty flavor to the cheese. Contrast to Feta, Halloumi's blocks are heated after curd formation in whey at 90–95°C for at least 30 min. This step ensures the prevention of contamination, and it is essential for the characteristic flavor and elastic texture of Halloumi cheese (Kaminarides et al., 2009). There are two kinds of Halloumi cheese, the fresh and the mature (Kaminarides et al., 2009; European Commission, 2015; Kamilari et al., 2020). The latter type is immersed into the brine (14–16% w/v NaCl) for at least 40 days at 25°C before storage (Kaminarides et al., 2009; Kamleh et al., 2012). Yeasts *Candida* spp., *P. membranifaciens*, and *D. hansenii* were the most common contaminants, isolated from Halloumi cheese and brine in high counts (up to10^5 CFU/g) (Mehyar et al., 2018).

Teleme is another traditional Greek cheese made with non-pasteurized milk, which is currently produced on an industrial scale, using yogurt commercial starters (Pappa et al., 2006). In Teleme processing, the curd is subjected to pressure to release whey and placed into the brine (18% w/v NaCl) for 20 h (Litopoulou-Tzanetaki and Tzanetakis, 2011). Laslo and

György (2018) reported that the total counts of yeasts in Teleme comprised up to 8×10^3 CFU/g. To our knowledge, there are no published studies on yeast identification in Teleme cheese.

White-Brined Cheeses From Other European Countries

In many European countries, white-brined cheeses are produced from pasteurized cow's milk and salted in brine without the use of dry-salting. Westall and Filtenborg (1998) isolated and identified spoilage yeasts in Feta-type cheese at three different dairies (A, B, and C) in Denmark (**Table 1**), showing high variation between the production sites. Predominant yeasts from the dairy A were *Torulaspora delbrueckii*, *G. candidum*, and *Y. lipolytica* (10^2–10^6 CFU/g), whereas *Candida boidinii*, *C. intermedia*, *C. zeylanoides*, *D. hansenii*, *K. lactis*, *K. marxianus*, *Pichia guilliermondii* (current name *Meyerozyma guilliermondii*), *P. membranifaciens*, and *Naumovozyma dairenesis* (formerly *Saccharomyces dairenesis*) were found in low counts (less than 10^2 CFU/g). Yeast species isolated from the dairy B, belonged to *D. hansenii*, *Y. lipolytica*, and *Candida sake* (10^2–10^6 CFU/g), while *T. delbrueckii*, *C. zeylanoides*, *G. candidum*, *K. lactis*, and *P. guilliermondii* were sporadically found. Less diversity was observed among the yeast species from the dairy C, most of them belonging to *Candida aaseri* (formerly *Candida butyri*) and *Y. lipolytica* (10^3–10^5 CFU/g) (Westall and Filtenborg, 1998). Šuranská et al. (2016) characterized diversity and composition of yeast consortium in various Serbian artisanal white-brined cheeses (**Table 1**) with the use of traditional culturing and molecular techniques (ITS-RFLP, 26S rRNA amplicon sequencing and ITS–clone library restriction analysis). The total yeast counts in cheese increased during the ripening period of 10 days (from 10^4 to 10^6 CFU/g). Yeasts *D. hansenii*, *C. zeylanoides*, and *K. lactis* were isolated in high numbers (up to 10^6 CFU/g) from most of the samples, followed by less abundant *T. delbrueckii*, *Trichosporon ovoides*, *Candida pararugosa*, *Y. lipolytica* and *G. geotrichum* (up to 10^3 CFU/g). Additionally, yeast species not associated with the cheese making, e.g., *Cryptococcus albidus*, *Hanseniaspora uvarum*, and *Filobasidium globisporum*, were rarely found in a few samples. Another study with Serbian white-brined cheeses identified 17 yeast species, among them, *D. hansenii*, *C. zeylanoides*, and *T. delbrueckii* were predominant (Golić et al., 2013). The authors pointed out that the presence of rare species, such as *Trichosporon gracile*, *T. ovoides*, and *Torulaspora quercuum*, was due to the bad hygienic conditions during cheese production. Chipilev et al. (2016) analyzed white-brined cheeses from Bulgarian dairies produced with pasteurized cow milk and vacuum packaged after 45 days ripening period. More than 63% of cheeses were contaminated with yeasts, mostly *Candida* spp., *Rhodotorula* spp., and *Saccharomyces* spp., in total counts of 10^2–10^6 CFU/g.

White-Brined Cheeses From Outside Europe

Serro Minas cheese is a traditional semi-hard cheese with acidic flavor, produced in Brazil from non-pasteurized bovine milk (**Table 1**). Whey of an older cheese covered with salt (known as "pingo") is used as a natural starter (back slopping) in cheese-making (Cardoso et al., 2015). Serro Minas is ripened for 3 days at room temperature and, then, for 60 days at 10°C (Ministério da Agricultura Pecuária e Abastecimento (MAPA), 2000). Cardoso et al. (2015) reported higher counts and diversity of yeast species in Serro Minas cheese during the rainy season compared to the dry season (2.6×10^7 CFU/g vs. 6.6×10^6 CFU/g, respectively, after 15 days of ripening), indicating that the climate conditions and seasonal variation had an impact on the cheese microbiota. *D. hansenii* and *Kodamaea ohmeri* (formerly *Pichia ohmeri*) were predominant in Serro Minas cheese (1.3×10^8 CFU/g and 7.9×10^6 CFU/g, respectively, after 15 days of ripening in the rainy season). Other species, such as *Trichosporon montevideense*, *S. cerevisiae*, *G. candidum*, and *Rhodotorula mucilaginosa*, were referred as temporary contaminants, occasionally occurred in high numbers (up to 10^5 CFU/g) throughout the ripening period. Furthermore, *C. parapsilosis*, *Candida tropicalis*, *G. candidum*, and *R. mucilaginosa* were only detected in the rainy season, while *Candida atlantica*, *C. intermedia*, *Candida phangngensis*, and *Candida silvae* were identified during the dry season (Cardoso et al., 2015).

In Africa, white-brined cheeses are mainly produced in the northern part. The most popular white-brined cheese in Egypt is Domiati, manufactured from buffalo or cow milk or their mixture (**Table 1**). Unlike the Greek Feta cheese, NaCl is added into the milk before coagulation and fermentation (Egyptian Organization for Standards and Quality (EOS), 2005; Ayad, 2009). According to Sharaf et al. (2014), 10 samples of Domiati cheeses out of 45 samples collected from the markets in Cairo, were contaminated with yeasts (up to 6×10^3 CFU/g). Even higher yeast contamination levels (66% of samples, average counts of 2.63×10^5 CFU/g) were recorded for Domiati in a later study (Hameed, 2016). The main yeast species isolated from Domiati were classified as *Candida albicans*, *Candida krusei*, and *D. hansenii*. Among them, *C. albicans* (detection frequency in Domiati cheese of 24%) is recognized as a human pathogen, commonly associated with poor sanitation and handling in cheese making (Hameed, 2016).

Mihalic cheese and the Turkish White cheese (Beyaz peynir) are the most consumed white-brined cheeses in Turkey, in which spoilage yeast consortia have been characterized (**Table 1**). Mihalic cheese is a semi-hard salty cheese with a pale creamy color and eye formation on the interior surface (Solak and Akin, 2013; Aday and Karagul Yuceer, 2014). It is traditionally manufactured from raw ovine, caprine, or cow milk without addition of starter cultures and ripened in wooden barrels filled with brine (16-18% v/v NaCl) for three months at 15-25°C (Solak and Akin, 2013; Karasu-Yalcin et al., 2017). Karasu-Yalcin et al. (2017) classified 72 yeast isolates from Mihalic cheese (29 samples in total), mainly belonging to the genera *Candida*, *Geotrichum*, and *Trichosporon*. The predominant species were halophilic *D. hansenii* (42% of the total isolates), along with *Candida cylindracea*, *C. inconspicua*, *Candida paludigena*, and *C. tropicalis*, found in lower numbers. In addition, *C. krusei*, *C. zeylanoides*, *G. candidum*, *Candida bertae*, *Candida catenulata*,

K. ohmeri, *S. cerevisiae*, and *Trichosporon asahii* were rarely identified (Karasu-Yalcin et al., 2017). According to the recent report by Togay et al. (2020), the total yeast counts in Mihalic cheese of 4.1×10^3–2.3×10^4 CFU/g were dominated by *D. hansenii* and *Clavispora lusitaniae*. Production of Beyaz Peynir comprises 60–80% of the total cheese production in Turkey (Turkish Standards, 1995). It is a semi-hard cheese with acidic and/or salty flavor, produced from ovine, caprine, cow milk or mixture of them. The cheese blocks are commonly ripened in brine (14–16% NaCl) for 30–60 days at 12–15°C (Turkish Standards, 1995; Atasever et al., 2002; Hayaloglu et al., 2002, 2008). Yeasts species belonging to *Candida* spp., *K. lactis*, *Starmera amethionina* (formerly *Pichia amethionina* var. *amethionia*), *D. hansenii*, *C. zeylanoides*, and *T. delbrueckii* have been isolated from the Turkish White cheese in total counts of 2.45×10^3 CFU/g (Öztürk and Şahin, 2000; Togay et al., 2020).

Akawi cheese belongs to the white-brined cheeses produced traditionally in Lebanon and Syria from pasteurized bovine and/or ovine milk (Hayaloglu et al., 2008; Ayyash et al., 2012; Hayaloglu, 2017). The curd is pressed in order to release the whey (Toufeili and Özer, 2006). High salt concentration in brine (20% w/w NaCl) and in the final product (9% w/w NaCl) ensures long shelf life of Akawi cheese (Pachlová et al., 2016). Halotolerant yeast strains of *D. hansenii* and *Candida guilliermondii* (anamorph form of *M. guilliermondii*, formerly *P. guilliermondii*) have been isolated from Akawi cheese produced traditionally in the Czech Republic (Pachlová et al., 2016). Khalil et al. (2018) isolated and identified yeast species from a traditional soft white-brined cheese Jiben-Al-Arab, manufactured from sheep milk in Mosul, Iraq. The cheese is typically stored in tins with brined whey at room temperature. The authors reported that most of the cheese samples were contaminated with low levels ($<10^2$ CFU/g) of *Candida* spp. (*C. parapsilosis*, *C. albicans*, *C. tropicalis*, and *C. krusei*), *G. candidum*, and *Rhodotorula* spp. (Khalil et al., 2018).

Spoilage yeasts have been detected and enumerated in other types of white-brined cheeses without taxonomic identification. Among them, cheese Ezine has a Geograpical Indication status and registered as a unique trademark in Turkey (Türk Patent Enstitüsü, 2006; Uymaz et al., 2019). A seasonal (March – July) mixtures of pasteurized ewe milk (approximately 45-55%), goat milk (at least 40%), and cow milk (the most 15%) are used for the cheese making with addition of aromatic herbs (oregano, mint, thyme, etc.), which contribute to the characteristic flavor (Hayaloglu et al., 2008). Ezine is produced without addition of starter cultures and ripened for a long period (8–12 months). Fungal average counts of 3.2×10^3 CFU/g have been reported during ripening of Ezine cheese from three different Turkish regions (Uymaz et al., 2019). Another example is a traditional white-brined cheese Nabulsi, popular in Jordan and the Middle East. During production, the rennet is added into non-pasteurized milk (cow, sheep, goat milk, or their mixture) without the use of starter culture, and the curd is allowed to set for 35–40 min before cutting. Then, the curd is boiled in the brine solution (18–20% w/v NaCl), filled in cans with brine, and stored at room temperature for up to 2 years (Al-Dabbas et al., 2014; Sabbah et al., 2019). Yeast counts of 3.6×10^5 CFU/g in Nabulsi cheese have been reported (Al-Dabbas et al., 2014).

PROPERTIES AND SPOILAGE POTENTIAL OF YEASTS

Proliferation of Spoilage Yeasts

Proliferation of spoilage yeasts in dairy products depends on a range of extrinsic factors, as temperature and humidity, particularly during storage, as well as intrinsic factors, such as milk composition, water activity, NaCl content, pH, and antimicrobials (Carrasco et al., 2006). In addition, the co-existing starter cultures and commensal microorganisms might influence the growth of spoilage yeasts. Thus, glucose and galactose, generated from lactose degradation by LAB, can be subsequently utilized by yeasts, otherwise incapable to ferment lactose, and promote their growth (Álvarez-Martín et al., 2008). In white-brined cheeses, yeasts may have detrimental effects on the final products by releasing off-flavors (bitterness, fruity, rancid, soapy), producing gas (CO_2), or causing discoloration and textural changes (Soliman and Aly, 2011; Padilla et al., 2014; Gonçalves Dos Santos et al., 2017; Al-Gamal et al., 2019). It should be noted, however, that yeasts in low numbers are rarely a problem, since the counts need to exceed 10^4-10^6 CFU/g, before the quality defects can be detectable (Laslo and György, 2018; Tokak et al., 2019).

Miloradovic et al. (2018) investigated the effect of packaging conditions on yeast growth in white-brined cheese produced in Serbia with goat milk. Before packaging, the cheese was ripened in two different salt concentrations (3% and 6% w/v NaCl) for 10 days, and, afterward, it was either vacuum packaged or kept in a modified atmosphere (MAP) (60% CO_2, 40% N_2) for 40 days at storage temperature. The brine strength had no effect on yeast counts, determined at the end of the brining period (10th day). Concurrently, the MAP was more effective for reduction of yeast numbers at the end of storage, resulting in lower yeast counts of 10^3–10^4 CFU/g, compared to 10^5 CFU/g in the vacuumed samples.

Using mathematical models, Vivier et al. (1994) described the species variation and the impact of pH, temperature, NaCl concentration, and water activity on the growth of *T. blattae*, *C. sphaerica*, and *L. thermotolerans*, isolated from brine of Feta cheese. All tested yeast species were able to grow at salt content up to 16%, low a_w of 0.94 and in the temperature range of 4–37°C. Among the yeast species, *C. sphaerica* was the most salt-tolerant (grown at 16% NaCl), while *L. thermotolerans* was the most resistant to high temperatures (grown at 41°C). In another comparative study with dairy yeasts, Praphailong and Fleet (1997) demonstrated that *D. hansenii* and *Pichia anomala* were the most halotolerant, able to grow at 15% NaCl, whereas growth of *K. marxianus* and *S. cerevisiae* was inhibited at 7.5% and 10% NaCl, respectively. It was further reported that pH variations within a range of pH 3.0 to 7.0 had insignificant impact on the growth of dairy-related yeasts *C. sphaerica*, *D. hansenii*, *Y. lipolytica*, *K. marxianus*, *L. thermotolerans*,

P. membranifaciens, *P. anomala*, *S. cerevisiae*, and *T. blattae* (Vivier et al., 1994; Praphailong and Fleet, 1997).

Proteolytic and Lipolytic Activity

Yeast's proteolytic and lipolytic activities (**Figure 1**) are the main factors, influencing the organoleptic characteristics and causing deterioration of white-brined cheeses (Sørensen et al., 2011; Karasu-Yalcin et al., 2017; Tokak et al., 2019). Yeasts have the ability to convert milk proteins and fat into amino acids and free fatty acids (FFA), the precursors of aroma and flavor compounds (Chen et al., 2010). In particular, the short-chain fatty acids (butyric and caproic) and the middle chain fatty acids (lauric and myristic) contribute to sour, rancid, sharp, and soapy flavor in cheese (Aday and Karagul Yuceer, 2014; Tokak et al., 2019). In addition to FFA, activity of yeast lipolytic enzymes (lipases, esterases, etc.) leads to release of the volatile aroma compounds, e.g., alcohols, aldehydes, ketones, and esters (Karasu-Yalcin et al., 2017). Formation of these compounds is associated with fruity, fusel, wood pulp, pomace, and butterscotch flavors (Sørensen et al., 2011). Sørensen et al. (2011) estimated production of volatile compounds by *Y. lipolytica* CBS 2075, *D. hansenii* D18335 and *S. cerevisiae* D7 grown in cheese medium at variable temperatures (12°C and 25°C) and NaCl concentration (0% and 3% w/v). The short-chain methyl-ketones (2-propanone, 2-butanone, 2-pentanone, and 3-methyl-2-pentanone) were primarily produced by *Y. lipolytica*. Concurrently, *D. hansenii* produced the highest levels of aldehydes (2-methylpropanal, 2-methylbutanal, and 3-methylbutanal) and alcohols (2-methyl-1-propanol and 2,3-methyl-1-butanol), while *S. cerevisiae* contributed to esters (ethyl-propionate and ethyl-butanoate). Interestingly, the authors observed that release of the volatile compounds by yeasts was highly influenced by the temperature and salt content in the growth medium (Sørensen et al., 2011).

Milk caseins are hydrolyzed through the synergistic action of acid phosphatase and proteolytic enzymes. High activity of acid phosphatase, converting phosphates to small peptides and free amino acids, contributes to flavor development in the acidic environment of matured cheeses (Karasu-Yalcin et al., 2017). Bitterness is the main flavor defect in cheese, commonly correlating with the proteolytic activity of yeasts and associated with the formation of low molecular weight hydrophobic peptides. Additionally, other metabolites, e.g., amino acids, amines, amides, long-chain ketones, and mono-glycerides, produced by *Candida* spp., *K. lactis*, *S. amethionina*, and *D. hansenii*, promote the bitter flavor in white-brined cheese (Öztürk and Şahin, 2000; Aday and Karagul Yuceer, 2014; Ganesan and Weimer, 2017). The cheese flavor is also affected by the volatile sulfur compounds, such as methanethiol and sulfides, generated from milk caseins through the amino acid metabolic pathways (methionine and

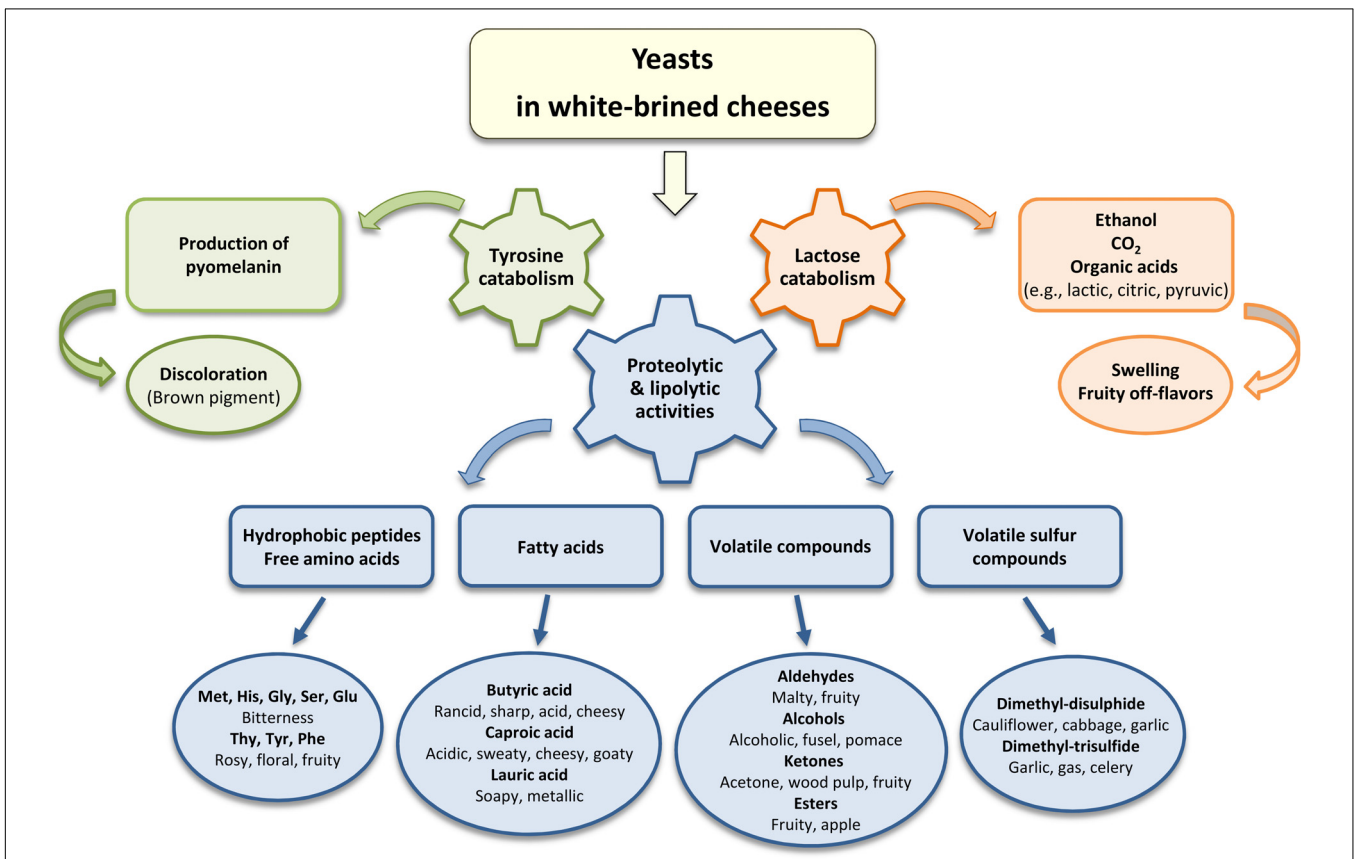

FIGURE 1 | Association between the metabolites produced by yeasts and the quality defects in white-brined cheeses, i.e., off-flavors, discoloration and swelling.

cysteine) (Aday and Karagul Yuceer, 2014; Laslo and György, 2018). Sulfides (dimethyl disulfide, DMDS, and dimethyl trisulfide, DMTS), released via metabolic activity of *Y. lipolytica*, correlated with cabbage and garlic flavors in cheese medium (Sørensen et al., 2011). The ability to produce different types of sulfides has been demonstrated for strains of *G. candidum*, *Y. lipolytica*, *D. hansenii*, and *S. cerevisiae* grown in a model cheese medium or laboratory medium, supplemented with amino acids (methionine and cysteine) (Arfi et al., 2002, 2004; López Del Castillo-Lozano et al., 2007).

Lipolytic and proteolytic properties have been characterized for several potential yeast spoilers and commonly reported as strain-dependent. Among the 199 yeasts from Serro Minas cheese (Brazil), only a minor part of isolates of *K. marxianus*, *D. hansenii*, and *K. ohmeri* (5–10%) displayed lipase and protease activity. Likewise, isolates of *G. geotrichum* and *R. mucilaginosa* showed either lipolytic or proteolytic activity, respectively (Cardoso et al., 2015). Extracellular lipase activity of *Y. lipolytica* was determined in Serbian white-brined cheeses by Golić et al. (2013). According to Westall and Filtenborg (1998), the lipolytic activity of *Y. lipolytica* and other yeast species found in high numbers (10^5 CFU/g), accounted for texture softening of the Danish Feta-type cheese. Among the isolates of *Candida* spp. from the white-brined cheese Jiben-Al-Arab (Iraq), *C. parapsilosis* and *C. tropicalis* were distinguished from *C. albicans* and *C. krusei* by high phospholipase activity, while *C. tropicalis* was the only species exhibiting esterase activity (Khalil et al., 2018). Karasu-Yalcin et al. (2017) reported that most of the yeast isolates (30 in total) from Mihalic cheese, belonging to *G. candidum*, *T. asahii*, and *Candida* spp., had esterase activity, while lipase activity was only detected for a few strains of *C. tropicalis*, *D. hansenii*, and *G. candidum*.

Lactose metabolism

Production of CO_2 and ethanol, the major by-products of yeast metabolism (**Figure 1**), might result in swelling of cheese cans (Bintsis et al., 2000). Besides, fruity flavor may develop when ethanol reacts with short-chain fatty acids to form a variety of esters (Laslo and György, 2018). Gas formation has been detected in cheese containers or packages, when lactose-fermenting yeasts reached typically more than 10^6 CFU/g (Bintsis et al., 2000; Öztürk and Şahin, 2000; Fadda et al., 2001; Sharaf et al., 2014). Several studies identified spoilage yeasts in white-brined cheeses in connection with the incidents of swollen packages. For instance, *Candida* spp., *K. lactis*, *S. amethionina*, and *D. hansenii*, determined in Turkish white-brined cheese, were capable to cause gas formation in the products even at high amounts of NaCl (12 % w/w) (Öztürk and Şahin, 2000). Fadda et al. (2001) reported that *Dekkera anomala* (a strongly fermenting yeast) and *K. lactis* were responsible for swelling of Sardinian Feta cheeses. Earlier industrial survey on Feta cheese demonstrated that yeast species of *T. blattae*, *C. sphaerica*, and *L. thermotolerans*, isolated from brine, caused gas formation in the packaged products (Vivier et al., 1994). Swelling of the packages of Feta-type cheese during storage at the Danish dairies was ascribed to lactate utilization and propagation of *T. delbrueckii* (Westall and Filtenborg, 1998).

Discoloration and Tyrosine Metabolism

Formation of brown pigments on cheese surfaces is commonly attributed to *Y. lipolytica* (**Figure 1**). To our knowledge, at least one publication reported discoloration of the white-brined cheese Domiati (Sharaf et al., 2014), though similar defects have been observed in other types of cheeses, e.g., soft surface-ripened cheeses, Mozzarella, Ricotta, and Gouda (Casalinuovo et al., 2015; Chierici et al., 2016; Igoshi et al., 2017). Cheese presents a suitable substrate for pigment production, as it contains lactic acid, amino acids (e.g., glycine, asparagine, glutamine), glucose, and manganese, promoting the browning effect. On the opposite, the increased content of glucose correlated with a delay of browning in tyrosine-containing laboratory medium (Carreira et al., 2001a,b). The brown pigments, known as melanins, are produced by yeasts from L-tyrosine via dihydroxyphenylalanine (DOPA) pathway or through accumulation and autoxidation of a pigment precursor, homogentisic acid (HGA) (Carreira et al., 2001b; Schmaler-Ripcke et al., 2009; Ben Tahar et al., 2020). In *Y. lipolytica*, production of pigments includes synthesis and accumulation of HGA, followed by convertion of HGA into benzoquinone acetic acid by autoxidation, which is further turned into pyomelanin through self-polymerization (**Figure 2**) (Ben Tahar et al., 2020). Recently, Ben Tahar et al. (2020) verified production of melanin pigments in *Y. lipolytica* W29 via L-tyrosine catabolism. Interestingly, these pigments exhibited both antioxidant and antimicrobial activities, indicating perspectives for biotechnological applications of *Y. lipolytica* (Ben Tahar et al., 2020).

Production of Biogenic Amines

Biogenic amines (BA) are toxic metabolites, commonly arising in fermented foods from decarboxylation of free amino acids due to microbial activity. The most abundant BA in cheese are histamine, tyramine, cadaverine, putrescine, tryptamine, and phenylethylamine, produced mainly by lactic acid bacteria, *Enterobacteriaceae*, and, to a lesser extent, by *D. hansenii*, *Y. lipolytica*, *K. marxianus*, *S. cerevisiae*, and *Candida* spp. (Benkerroum, 2016). BA are indicators of cheese quality and safety, since, in significant amounts, they can cause adverse effects on human health, e.g., fluctuation of blood pressure, headache, vomiting, and diarrhea (Ruiz-Capillas and Herrero, 2019). According to the European Food Safety Authority (EFSA), consumption of 50 mg histamine and 600 mg tyramine (per person per meal) are considered safe for healthy individuals (EFSA, 2011). The United States Food and Drug Administration (FDA) set the maximum levels of histamine to 50 mg/kg in fishery products (FDA, 2020). Safety criteria for other BA are not covered by specific legislation.

Biogenic amines have been found in Feta (Valsamaki et al., 2000), Akawi cheese (Pachlová et al., 2016), Beyaz peynir (Durlu-Özkaya et al., 1999), Iranian white brined cheese (Aliakbarlu et al., 2011; Rohani et al., 2013), and other varieties of white-brined cheeses and ripened cheeses collected from the European dairies and small-scale farms (Bunkova et al., 2013;

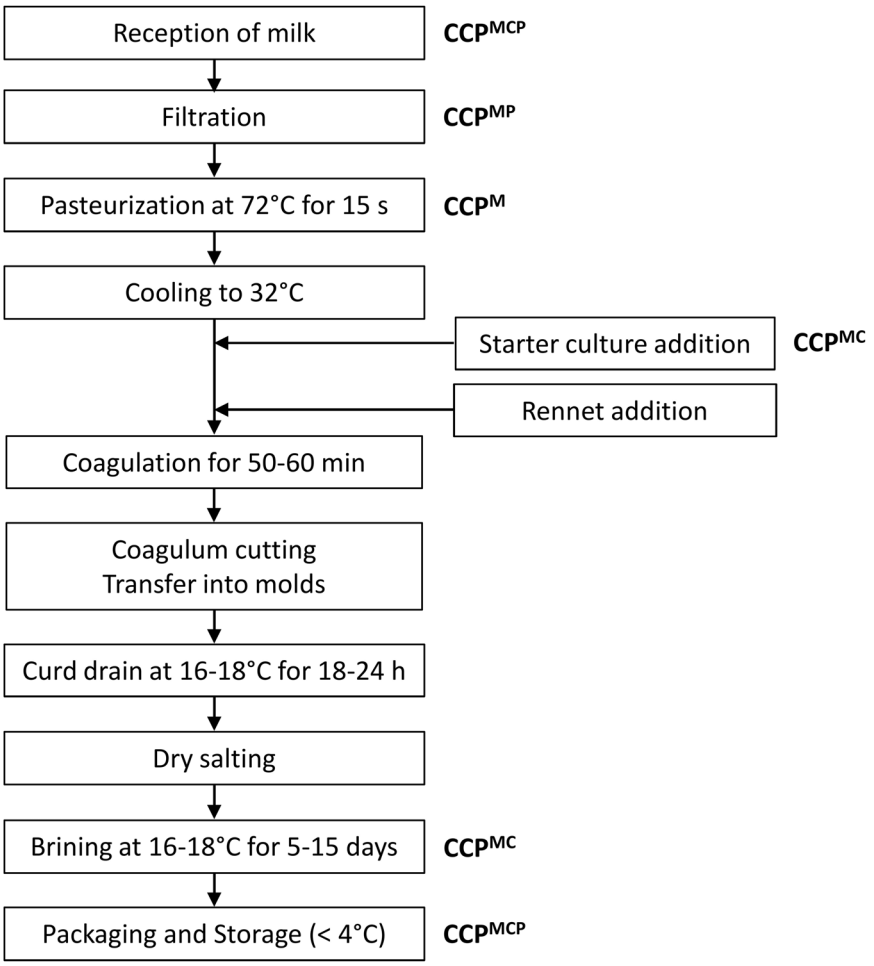

FIGURE 2 | Flow-chart of Feta cheese production with indicated Critical Control Points (CCP) of the microbiological[M], chemical[C] and physical[P] hazards [modified from Mauropoulos and Arvanitoyannis (1999); Tamime et al. (2007) and El-Hofi et al. (2010)].

Combarros-Fuertes et al., 2016; Bonczar et al., 2018; Espinosa-Pesqueira et al., 2018; Mayer and Fiechter, 2018). Based on diverse research, EFSA published the mean values of histamine (21–62 mg/kg), tyramine (68–104 mg/kg), putrescine (25–65 mg/kg), and cadaverine (72–109 mg/kg) recorded in cheese (EFSA, 2011). Pachlová et al. (2016) reported that the total BA (putrescine, histamine, tyramine and cadaverine) in several batches of traditionally manufactured cheese Akawi (Czech Republic) exceeded 120 mg/kg. Yeast strains of *D. hansenii* and *C. guilliermondii*, isolated from Akawi, were tested positive for decarboxylase activity, indicating that they might contribute to BA accumulation during the long storage periods. The authors further pointed out that decarboxylase activity in yeasts was strain-dependent and, particularly, putrescine and cadaverine were predominant BA produced in high levels (50–1000 mg/L) in laboratory medium (Pachlová et al., 2016). Strain specificity in BA production by microorganisms is well recognized (Benkerroum, 2016). Bäumlisberger et al. (2015) demonstrated that certain strains of *D. hansenii* and *Y. lipolytica* had an ability to metabolize a broad spectrum of BA due to a peroxisomal amine oxidase activity, and suggested that BA non-producer strains had a potential to reduce BA in fermented foods (Bäumlisberger et al., 2015).

In cheese, production of BA is highly influenced by amino acid availability, the presence of contaminating microorganisms, as well as processing and storage conditions. Occurrence of BA can be reduced or prevented by proper hygiene practices, milk pateurization, low pH, optimized ripening conditions (e.g., high salt content), controlled packaging (e.g., vacuum pakaging), and low storage temperatures (Valsamaki et al., 2000; Novella-Rodríguez et al., 2002; Aliakbarlu et al., 2011; Andiç et al., 2011; Bunkova et al., 2013; Gardini et al., 2016). Aliakbarlu et al. (2011) performed modeling of the BA content in Iranian white brined cheeses as a function of processing variables, such as ripening time (25–75 days), temperature (4–14°C), and brine concentration (10–13%). At low level of ripening time, the BA content decreased with increasing levels of brine concentration, while at high level of ripening time, brine concentration had inverse effect, which was explained by the softening of the texture and diffusion of BA to brine (Aliakbarlu et al., 2011).

BIO-PROTECTIVE CULTURES AND OTHER YEAST INTERACTIONS

Bio-Protective Cultures

The dairy market alongside with consumer's demands continues to move towards more natural products, i.e., less processed and free from artificial ingredients ("clean label") (Román et al., 2017). Finding clean-label solutions for the preservation of white-brined cheeses is particularly important, regarding their susceptibility to yeast spoilage. In this connection, bio-protective cultures offer a promising alternative to chemical preservatives (Crowley et al., 2013). Bio-protective cultures are referred as food cultures, deliberately applied as live microorganisms to control the microbiological status in food (EFFCA, 2018; Ben Said et al., 2019). As food additives, bio-protective cultures should have "generally recognized as safe" (GRAS) status and be included in the qualified presumption of safety (QPS) list in Europe (Ben Said et al., 2019). In addition to yeast inhibition, bio-protective cultures should be able to proliferate in dairy products without affecting the performance of other starter and adjunct cultures, and without changing the technological and sensory quality of the products (Schnürer and Magnusson, 2005). Lactic acid bacteria (LAB) associated with milk fermentation, specifically within the genus *Lactobacillus*, are well known for their preserving effects (Bernardeau et al., 2008). Various commercial protective cultures, consisting of selected strains of LAB, have been developed and applied in dairy production, among them, FRESHQ® (Chr. Hansen) and HOLDBAC® YM (DuPont) targeting yeasts and molds in cheeses and other fermented dairy products.

Interactions Between Spoilage Yeasts and LAB

Inhibitory properties of LAB against potential spoilage yeasts have been examined for several fermented milk products, mainly yogurts, sour cream, and surface-ripened cheese (Corsetti et al., 2001; Crowley et al., 2013; Delavenne et al., 2013; Laèanin et al., 2017; Fröhlich-Wyder et al., 2019; Garnier et al., 2019). The studies commonly pointed out that the antifungal activity of LAB was strain-specific and depended on the target yeast species and/or strains. **Table 2** presents examples of the LAB strains, mixed cultures and yeasts, producing antifungal metabolites and inhibiting potential yeast spoilers in white-brined cheeses. In the following, new taxonomic names of LAB (shown in brackets) are referred in accordance with Zheng et al. (2020). Leyva Salas et al. (2018) performed a large-scale screening assay of antifungal activity of LAB in yogurt and cheese matrix, among them, *Lactobacillus harbinensis* (*Schleiferilactobacillus harbinensis*), *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*), *Leuconostoc mesenteriodes*, *Lactococcus lactis*, *Lactobacillus rhamnosus* (*Lacticaseibacillus rhamnosus*), and *Propionibacterium jensenii* (*Acidipropionibacterium jensenii*), against potential spoilage yeasts (*G. geotrichum* and *Y. lipolytica*). The authors developed two antifungal combinations of mixed cultures, consisting of *L. plantarum* L244, *S. harbinensis* L172 and/or *L. rhamnosus* CIRM-BIA1113, suitable for application as adjunct cultures in sour cream and semi-hard cheese. According to Delavenne et al. (2013), *S. harbinensis* had the strongest antifungal effect in milk and yogurt, compared to *Lactbacillus casei* (*Lacticaseibacillus casei*), *Lactobacillus paracasei* (*Lacticaseibacillus paracasei*), and *L. rhamnosus*, completely inhibiting all tested yeast strains of *D. hansenii*, *K. lactis*, *K. marxianus*, *R. mucilaginosa*, and *Y. lipolytica*.

Major mechanisms behind the antimicrobial activities of LAB in dairy products include competition for the limited amount of nutrients (competitive exclusion), decrease of pH due to lactic acid production, and release of antifungal compounds, such as organic acids, fatty acids, peptides, and hydrogen peroxide (Álvarez-Martín et al., 2008; Siedler et al., 2019, 2020; Garnier et al., 2020). Recently, Siedler et al. (2020) demonstrated that competitive exclusion through depletion of the essential trace element manganese was the main factor, accounting for inhibition of *D. hansenii*, *T. delbrueckii*, and *R. mucilaginosa* by *L. rhamnosus* and *L. paracasei*. The authors established a link between the differences in expression of the manganese transporter (MntH1) and the protective effect of the tested LAB, and proposed that manganese scavenging might be a common trait within *Lactobacillus* spp. Al-Gamal et al. (2019) reported that inhibitory effect of *L. plantarum* DSA20174 and *Lactobacillus helveticus* CNRZ32 on the growth of *C. parapsilosis*, isolated from Egyptian Feta-type cheeses, was related to excessive production of organic acids by LAB. The recent mechanistic studies in fermented milk and yogurt demonstrated that *S. harbinensis* K.V9.3.1Np produces both polyamines and organic acids (acetic, lactic, 2-pyrrolidone-5-carboxylic, hexanoic, benzoic, and 2-hydroxybenzoic), leading to membrane disruption and cell lysis of *Y. lipolytica* (Delavenne et al., 2015; Mieszkin et al., 2017; Mosbah et al., 2018). McNair et al. (2018) isolated and characterized a bioactive peptide (DMPIQAFLLY) derived from β-casein in sour cream added bioprotective cultures (*L. paracasei* CH127 and *L. rhamnosus* CH126). The peptide targeted *D. hansenii*, attenuating its growth rate and affecting cell morphology (smaller and denser colonies). Of particular interest is an extensive research by Garnier and co-workers, who characterized antifungal activities of LAB (430 strains in total) against yeasts (*G. geotrichum* and *Y. lipolytica*), using cheese mimicking model, and identified a broad spectrum of potential antifungal compounds in selected dairy fermentates of *L. rhamnosus* CIRM-BIA1952 and *A. jensenii* CIRM-BIA1774 (Garnier et al., 2019, 2020). Overall, more than 50 compounds (organic acids, fatty acids, volatile compounds, and peptides), produced by specific LAB, have been identified. Among the major organic acids, lactic and acetic acids were most abundant in *L. rhamnosus* CIRM-BIA1952 fermentate, while propionic and acetic acids were associated with *A. jensenii*. Furthermore, α_{s2}-casein-derived peptides (pepa4c177 or RLNFLKKIS) identified in *L. rhamnosus* CIRM-BIA1952 fermentate, possessed the ability to inhibit *R. mucilaginosa* (Garnier et al., 2020).

Other Microbial Yeast Interactions

Studies on interactions within yeast consortium have been preferably focused on surface-ripened cheeses and fermented milk products, however, similar interaction mechanisms are

TABLE 2 | Inhibitory activity of lactic acid bacteria, yeasts and their metabolites against yeast species, frequently occurring as contaminants in white-brined cheeses.

Microorganisms	Dairy matrix/Media	Targets	Metabolites	References
Lactic acid bacteria (LAB)				
Schleiferilactobacillus harbinensis K.V9.3.1 Np	Milk, yogurt, and fermented milk	*Debaryomyces hanseii* *Kluyveromyces lactis* *Kluyveromyces marxianus* *Rhodotorula mucilaginosa* *Yarrowia lipolytica*	Polyamides; organic acids (acetic, benzoic, lactic, 2-pyrrolidone-5-carboxylic, hexanoic, 2-hydroxybenzoic)	Delavenne et al., 2013, 2015; Mieszkin et al., 2017; Mosbah et al., 2018
Acidipropionibacterium jensenii CIRM-BIA1774	Sour cream and semi-hard cheese	*Galactomyces geotrichum* *Yarrowia lipolytica*	Propionic acid; Acetic acid	Garnier et al., 2019, 2020
Lacticaseibacillus rhamnosus CIRM-BIA1952	Sour cream and semi-hard cheese	*Galactomyces geotrichum* *Yarrowia lipolytica* *Rhodotorula mucilaginosa*	Lactic acid; Acetic acid Bioactive peptide (RLNFLKKIS)	Garnier et al., 2019, 2020
Mixed cultures				
Lactiplantibacillus plantarum L244 *Schleiferilactobacillus harbinensis* L172 *Lacticaseibacillus rhamnosus* CIRM-BIA1113	Sour cream and semi-hard cheese	*Galactomyces geotrichum* *Yarrowia lipolytica*	Not reported	Leyva Salas et al., 2018
Lacticaseibacillus rhamnosus *Lacticaseibacillus paracaseii*	Fermented milk	*Debaryomyces hanseii* *Torulaspora delbrueckii* *Rhodotorula mucilaginosa*	Not reported	Siedler et al., 2020
L. plantarum DSA 20174 *Lactobacillus helveticus* CNRZ 32	M17/MRS	*Candida parapsilosis*	Organic acids	Al-Gamal et al., 2019
Lacticaseibacillus paracasei CH127 *Lacticaseibacillus rhamnosus* CH126	Sour cream	*Debaryomyces hansenii*	Bioactive peptide (DMPIQAFLLY)	McNair et al., 2018
Yeasts				
Yarrowia lipolytica 1E07	Cheese model	*Geotrichum candidum* 3E17	Ammonium; Proline	Mounier et al., 2008
Debaryomyces hansenii 1L25	Liquid cheese medium	*Yarrowia lipolytica* 1E07	Not reported	Malek et al., 2018
Debaryomyces hansenii	YEPD-methylene blue agar, and commercial cheeses (Romano and blue cheese)	*Yarrowia lipolytica* *Wickerhamomyces anomalus* *Candida tropicalis* *Candida albicans*	Mycocins	Banjara et al., 2016; Al-Qaysi et al., 2017
Kluyveromyces lactis	YPD agar	*Saccharomyces cerevisiae*	Zymocin	Jablonowski et al., 2001; Lu et al., 2007; Lentini et al., 2018
Lindnera saturnus	Cheese	*Saccharomyces cerevisiae* VL1 *Kluyveromyces marxianus* ATCC8640	Not reported	Liu and Tsao, 2009

foreseen in white-brined cheeses (Fröhlich-Wyder et al., 2019). Interactions within the yeast communities are facilitated by several communication mechanisms referred as quorum sensing (QS), which regulate such fungal behaviors as growth, sporulation, biofilm production, secretion of virulence factors, etc. (Mehmood et al., 2019). Aromatic alcohols generated via the amino acids metabolic pathways, e.g., tryptophol, tyrosol, and phenylethanol, are the most common QS molecules identified in yeasts, e.g., *S. cerevisiae* (Avbelj et al., 2015, 2016). Gori et al. (2007) reported that dairy yeasts *D. hansenii*, *S. cerevisiae* and *Y. lipolytica* produced ammonium compounds, which acted as signaling molecules, affecting development of the neighboring yeast colonies of the same species on cheese agar plates.

Negative interactions between *Y. lipolytica* and other dairy yeasts, such as *G. candidum* and *D. hansenii* in model cheeses have been reported (Mounier et al., 2008). The yeast *Y. lipolytica* inhibited hyphal formation and caused morphological changes (spaghetti-like structures) in *G. candidum*, possibly, due to the high amounts of ammonium and proline produced by *Y. lipolytica*, along with reduced metabolic efficiency in *G. candidum* (Mounier et al., 2008). Recently, Malek et al. (2018) used transcriptomic analysis to elucidate the interactions between *D. hansenii* co-cultured with *Y. lipolytica*. The study demonstrated that growth inhibition of *D. hansenii* was related to a decrease in mitochondrial respiratory chain functioning, and, consequently, to a programmed cell death, rather than ammonia production or competition for nutrients.

Certain dairy-related yeast species are capable to secrete toxins exhibiting antagonistic activities against other sensitive yeasts (killer toxins or mycocins). Mechanisms behind the action of killer toxins include disruption of membrane integrity, blocking of the DNA synthesis, and/or mRNA translation (Mannazzu et al., 2019). Currently, four killer toxins (K1, K2, K28, and Klus) have been identified in *S. cerevisiae* and differentiated by receptor sites, killing mechanisms, and lack of cross-immunity (Orentaite et al., 2016; Becker and Schmitt, 2017; Gier et al., 2020). The killing activity of the most well-studied toxin K1 is based on binding to the β-1,6-D-glucan on a target cell and disruption of cytoplasmic membrane by forming the cation-selective ion channels (Gier et al., 2020). Dairy isolates of *D. hansenii* produced

mycocins, targeting *Y. lipolytica*, *Wickerhamomyces anomalus*, *C. tropicalis*, and *C. albicans* (Banjara et al., 2016; Al-Qaysi et al., 2017; Çorbacı and Uçar, 2017). Studies with *C. albicans* mutants defective in MAPK kinase pathways suggested that, specifically Hog1 phosphorylation site and the kinase activity, were implicated in the resistance of *C. albicans* to *D. hansenii* killer toxins (Morales-Menchén et al., 2018). Lethality of toxin zymocin, secreted by the dairy yeasts *K. lactis*, involves cleavage of tRNA in *S. cerevisiae* (Lu et al., 2007; Lentini et al., 2018). Liu and Tsao (2009) reported that yeast species *Lindnera saturnus* (former name *Williopsis saturnus)* reduced the growth of lactose and/or galactose fermenting spoilage yeasts *S. cerevisiae* VL1 and *K. marxianus* ATCC8640 in cheese, possibly, via competitive exclusion or production of killer toxins.

MEASURES TO PREVENT CONTAMINATION AND PROLIFERATION OF SPOILAGE YEASTS IN WHITE-BRINED CHEESES

Quality Assurance

To assure the quality and safety of dairy products, several preventive and control strategies can be applied during processing, storage and handling of the finished product [reviewed by Garnier et al. (2017a)]. These strategies rely on the universal management systems, such as the Hazard Analysis Critical Control Points (HACCP) system, Good Manufacturing Practice (GMP), Good Hygienic Practice (GHP), and the International Organization for Standardization (ISO) 22000 (Papademas and Bintsis, 2010; Tamime et al., 2011; FACEnetwork, 2016; ISO, 2018; Awasti and Anand, 2020). Preventive strategies aim to avoid yeast cross-contamination during production, using such technologies as milk pasteurization, air filtration, sanitation, aseptic packaging conditions, etc. Control strategies are based on the inhibition of yeast growth by chemical preservation, the addition of protective cultures, refrigeration, or modified atmosphere packaging. Based on the principles of HACCP and GMP, dairies have developed site-specific regulations and sanitation procedures. Implementation of HACCP analyzes in the production of Feta-type cheeses have been reported (Mauropoulos and Arvanitoyannis, 1999; Tamime et al., 2007; El-Hofi et al., 2010; Carrascosa et al., 2016; Kapshakbayeva et al., 2019). For instance, Kapshakbayeva et al. (2019) performed a systematic analysis of hazards, their causes, consequences, and preventive measures throughout the production line of the white-brined cheese type Halloumi, and identified 10 risk factors and the Critical Control Points (CCP) in the individual production step (equipment, raw milk, pasteurization, enzyme application, curd processing, molding, salting, and packaging). **Figure 3** shows the flow chart of Feta cheese production, in which the main procedures with potential critical hazards (microbiological, chemical and physical) are indicated as CCP [modified from Mauropoulos and Arvanitoyannis (1999)]. Microbiological hazards may be caused by the growth of psychrotrophic microorganisms and pathogens (raw milk), presence of bacteriophages (from starter cultures and/or environment), and cross-contamination with spoilage microorganisms after milk pasteurization. Chemical hazards may result from the presence of toxins, residues of detergents, disinfectants, and other chemical substances, while physical hazards are typically associated with penetration of extraneous material from the environment, e.g., parts of the equipment and packaging material (Tamime et al., 2007; El-Hofi et al., 2010).

In the case of documented yeast-spoilage problems in the dairy industry, a root cause analysis (RCA) need to be conducted as a part of the industrial process control (BRC Global Standards, 2012). The purpose of RCA is to trace the origin(s) of the yeast contamination in order to implement appropriate preventive measures to address the spoilage problem and food safety in general. The next step is to employ long-term corrective actions to address the root cause, identified during RCA, and to ensure that the problem does not recur. In practical circumstances, however, there may be more than one root cause for a given spoilage incident (e.g., related to faulty equipment, improper processing, personnel faults, improper ingredients or environmental issues), and this multiplicity can hamper the establishment of the causal graph.

Routes of Contamination

Depending on the design of production plants and in case of open facilities, yeast contamination of heat-treated milk can occur if sub-optimal air quality conditions prevail in the production environment. For instance, due to the pressure differences, contaminated air from the surrounding environment can enter product vessels via the incubation tanks. In practice, two principal approaches can minimize this risk: (a) optimally, the entire processing environment should be rendered a "clean area" via the use of High-Efficiency Particulate Air (HEPA) filters. In terms of clean-room classification, these HEPA filters should be of at least ISO 8 class number, according to ISO 14644-1; (b) in case the former is not a feasible option due to constructive restrains or financial limitations, the establishment of 'local' clean areas around exposed equipment (i.e., the establishment of "clean" mini-environments that protect equipment such as filling and packaging equipment) should be implemented (ISO, 2015).

Pasteurization of milk will, in principle, destroy all yeast cells, including yeast spores, as they are generally not heat resistant. However, in some countries, e.g., Greece, good quality raw milk can be used for the manufacture of Feta cheese, provided that the cheese undergoes proper ripening for at least two months. However, to our knowledge, raw-milk Greek Feta cheese manufacture is only practiced by small artisanal cheesemakers. In contrast, large dairy companies pasteurize the milk before cheese making (75°C for 15 s), which effectively controls microbial food safety hazards (Tamime et al., 2007). Hence, besides the inactivation of pathogens, the heat-treated milk should be essentially devoid of viable vegetative cells (bacterial and fungal). Consequently, with respect to white-brined cheeses, the problems with yeast spoilage mainly originate from environmental contamination(s) at some production stage(s) after the heat-treatment step. This has been stated by several studies in dairy plants, which

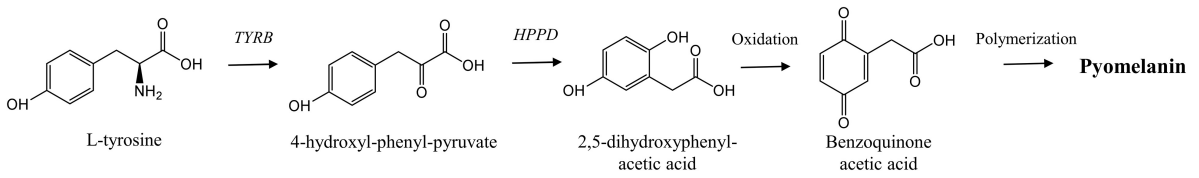

FIGURE 3 | Metabolic pathways of pyomelanin production by *Yarrowia lipolytica*. Abbreviations: *TYRB*, tyrosine aminotransferase; *HPPD*, 4-hydroxyl-phenyl-pyruvate dioxygenase [modified from Schmaler-Ripcke et al. (2009), Ben Tahar et al. (2020)].

pointed out that production facilities and, specifically, the contaminated air are major sources of yeast contamination of white-brined cheeses (Temelli et al., 2006; Beletsiotis et al., 2011; Stobnicka-Kupiec et al., 2019; Kandasamy et al., 2020). Stobnicka-Kupiec et al. (2019) identified *C. albicans*, *Candida glabrata*, *C. rugosa*, *D. hansenii*, *G. candidum*, *Rhodotorula* spp., and *Y. lipolytica* from the air and surface samples of commercial and traditional Polish dairy plants. The highest concentration of yeast and molds was found in worktops in milk reception, cheese production area, and in the air samples (50–480 CFU/m^3). Another one-year prospective study in a Greek dairy plant showed that an average fungal load, presented by *Cladosporium* spp., *Penicillium* spp., and unidentified yeasts, comprised 362.3 CFU/m^3 in outdoor air and up to 266.2 CFU/m^3 in the indoor locations (Beletsiotis et al., 2011). Substantial reduction of the fungal indoor spores (by 20-fold) could be further achieved by the installation of HEPA filters.

Sanitation and Cleaning Procedures

Besides air filtration, UV irradiation of the air or ozonation is recommended for the inactivation of airborne microorganisms in food processing environments (Varga and Szigeti, 2016). Implementation procedures, advantages and disadvantages of these methods have been reviewed (Masotti et al., 2019a). Inactivation of airborne microorganisms by ozonation and aerosolization with hydrogen peroxide has recently been tested in a dairy factory in Northern Italy (cheese making, storage, and packaging areas). The initial levels of yeasts in cheese-making facilities and packaging area (137 ± 439 CFU/m^3) were eliminated by both treatments, most effectively by hydrogen peroxide (Masotti et al., 2019b). The occurrence of yeasts in dairy facilities suggests that regular monitoring of microbial counts, with subsequent cleaning procedures, should be implemented at dairies to reduce fungal contamination and to satisfy the GMP requirements. Cleaning and disinfection are specifically important in humid areas of the dairy production, such as wall corners, floor and floor drains, ceiling, and parts of equipment, where yeast species are able to survive and even proliferate (Awasti and Anand, 2020). Cleaning-in-Place (CIP) circular washing with different detergents and hot water is applied as a current standard GMP method of cleaning of tanks and piping in dairy industries, eliminating impurities and reducing the levels of microbial contamination (Memisi et al., 2015).

CONCLUSION AND FUTURE PERSPECTIVES

From the present survey, it is clear that the very fast development within biotechnological methodologies for identification and detection of spoilage yeasts is only sparely reflected in the dairy industry. Especially, as the spoilage potential vary significantly between different species of spoilage yeasts, correct identification is of outmost importance. With an increased focus on food waste and extended shelf life of dairy products being exposed to increased storage temperatures, there is no doubt that control of spoilage microorganisms is more urgent than ever. Therefore, there is an urgent need for technologies that easily can be transferred to the food sector being affordable, with a high level of precision and easy to handle.

While molds are recognized as hazardous contaminants on the surface of solid and semi-solid surface-ripened cheeses, yeasts are due to their fermentative capacity recognized as the main spoilage microorganisms of white-brined cheeses. Even though a huge variety of yeast species have been identified from these types of cheeses, it is worth noticing that only a limited number of yeast species will be able to proliferate in the products, and that there is a clear link between contaminants with spoilage potential and product specifications. The most predominant yeast species causing quality defects seem to be the ones normally associated with dairy products, e.g., *D. hansenii*, *K. marxianus*, *K. lactis*, *Y. lipolytica*, and *G. candidum*. Due to its halophilic nature, the marine associated yeast species *D. hansenii* might often be present in brined dairy products being introduced with the NaCl (Haastrup et al., 2018). Even though this yeast species thoroughly has been reported to have beneficial effects for surface ripened red smear cheeses (Gori et al., 2007; Ryssel et al., 2015; Haastrup et al., 2018), its role in white-brined cheeses is not really understood. For yeast species as *K. marxianus* and, especially, *K. lactis*, gas production seems to be a significant problem due to their ability to ferment lactose and/or galactose, which have not been assimilated by the initial starter cultures. Also, other fermentable carbohydrates added together with ingredients, e.g., spices and vegetables, might enhance the growth of spoilage yeasts. Off-flavors are often linked to yeast species, having high enzymatic activities, such as *D. hansenii*, *G. candidum*, *K. lactis*, and *Candida* spp. Pigmentation might additionally occur due to the production of pyomelanins by *Y. lipolytica.* Even within these yeast species, significant intraspecies variations occur and the risk of persistent yeast strains, being able to establish in the

production plant, should always be considered. Accumulation of BA due to the activity of *D. hansenii*, *Y. lipolytica*, *K. marxianus* and *S. cerevisiae* and other microorganisms might rise safety concerns during prolonged storage of the white-brined cheeses. The role of specific yeast strains, originated from varieties of white-brined cheeses, in production of BA is not clear and needs to be further investigated in relationship to the processing and storage conditions.

Despite the relatively simple processing technology, white-brined cheeses might globally be produced within many different flow charts, slightly differentiating them from each other. However, even small changes might have an impact on the extrinsic and intrinsic parameters and, thereby, on the yeast species being able to proliferate in this particular type of white-brined cheese. As a consequence, most producers of white-brine cheeses are not aware of the yeast species being the most harmful for their particular type of cheese, as well as the defects they might cause. Unfortunately, research within this scientific field is lagging far behind. Practical knowledge exists on the use of bio-protective cultures for white-brined cheeses; however, scientific knowledge on the interaction mechanisms is still missing, especially on the production of antifungal peptides for inhibition of spoilage yeasts. Likewise, other microbial interaction mechanisms and their influence on the proliferation of spoilage yeasts in white brined cheeses are not investigated in detail, i.e., it is unknown how the primary starter cultures and their lysis influence the growth of spoilage yeasts. As alternatives to costly bio-protective cultures, the possibility of development of new types of starter cultures, having potential to inhibit spoilage yeasts, should be explored. Implementation of good manufacturing practices is an integral part of the entire food chain, being a very important tool to prevent food waste and to optimize income generation. The careful design of processing plants should also be seen as an absolute requirement for safe food processing. Prevention of airborne contamination is often a neglected area, when it comes to yeasts. However, it is clear that yeasts can be transported in dairy plants through small aerosols. In this case, airborne yeasts need to be identified in order to evaluate their spoilage potential. Often yeast species in air samples are not capable of growing in the cheese, though their presence should still be avoided.

In conclusion, white-brined cheeses are globally produced in a variety of brands; even more diverse are the yeast species capable of causing quality defects of these cheeses. Unfortunately, detailed knowledge on spoilage potential and variations at species and strain level is still missing. Implementation of advanced technologies for species and strain identification, being simple to handle and of low cost, is still important to prevent food waste and enhance the sustainability of these types of cheeses. Gathering of scientific knowledge on yeast interactions with other dairy-related microorganisms as well as the cheese matrix, will add to an optimized production of white-brined cheese of enhanced quality.

AUTHOR CONTRIBUTIONS

LJ and AG designed the manuscript. AG, TS, GT, NL, and LJ wrote the manuscript. KR critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Influence of Autochthonous Putative Probiotic Cultures on Microbiota, Lipid Components and Metabolome of Caciotta Cheese

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The present study was undertaken to produce probiotic Caciotta cheeses from pasteurized ewes' milk by using different combinations of autochthonous microbial cultures, containing putative probiotic strains, and evaluate their influence on gross composition, lipid components, sensory properties and microbiological and metabolite profiles of the cheeses throughout ripening process. A control cheese was produced using commercial starter cultures. The hydrophilic molecular pools (mainly composed by amino acids, organic acids, and carbohydrates) were characterized by means of ^{1}H NMR spectroscopy, while the cholesterol, α-tocopherol and fatty acid composition by HPLC-DAD/ELSD techniques. Conventional culturing and a PCR-DGGE approach using total cheese DNA extracts were used to analyze cheese microbiota and monitor the presence and viability of starters and probiotic strains. Our findings showed no marked differences for gross composition, total lipids, total cholesterol, and fatty acid levels among all cheeses during ripening. Differently, the multivariate statistical analysis of NMR data highlighted significant variations in the cheese' profiles both in terms of maturation time and strains combination. The use of autochthonous cultures and adjunct probiotic strains did not adversely affect acceptability of the cheeses. Higher levels of lactobacilli (viability of 10^{8}–10^{9} cfu/g of cheese) were detected in cheeses made with the addition of probiotic autochthonous strains with respect to control cheese during the whole ripening period, suggesting the adequacy of Caciotta cheese as a carrier for probiotic bacteria delivery.

Keywords: probiotic cheese, autochthonous cultures, lipids, metabolomics, NMR

INTRODUCTION

A "Functional Food" may be defined as any food with a positive impact on the consumer's health, physical performance or state of mind in addition to its nutritious value (Karimi et al., 2012). The concept of biofunctional foods is generally used when the beneficial physiological effects are conferred by microorganisms (Gobbetti et al., 2010). These effects may derive from the interactions of ingested living microorganisms with the host (probiotic effect), and/or the ingestion of the microbial metabolites produced during fermentation (bioactive effect) (Linares et al., 2017). Lactic

acid bacteria (LAB) play a multifunctional role in fermented foods that is not limited to the transformation of substrate and product preservation, but it is also related to the improvement of nutritional value and organoleptic characteristics as well as functional properties because of bioactive molecule production. LAB are generally considered beneficial microorganisms with some strains considered "probiotics."

Probiotics, as part of functional foods, are a growing area of scientific interest for their role in maintaining a healthy intestinal ecosystem. They are bacteria, generally lactobacilli and bifidobacteria, but also enterococci and yeasts, associated with a plethora of health beneficial effects (Franz et al., 2011; Albenzio et al., 2013; Saad et al., 2013; Perotti et al., 2014; Merchàn et al., 2020). Probiotic food is a processed product which contains in a suitable matrix and in sufficient concentration viable probiotic microorganisms that must be able to survive in the gastrointestinal tract (Saxelin et al., 2003). Populations of at least 10^7 CFU/g in the final product have been suggested as therapeutic quantities of probiotic cultures in different processed foods (Talwalkar and Kailasapathy, 2004; Karimi et al., 2012).

In the last decade, the interest in dairy products containing specific microbial strains with potential health-promoting properties has heightened. Cheese is one of the most common carriers used to efficiently deliver living probiotic microorganisms because it shows a good buffering capacity, generating a more favorable environment for probiotic survival throughout gastric transit (Castro et al., 2015; Homayouni et al., 2020). Several studies have addressed the development of probiotic cheeses including Cheddar (Zhang et al., 2013), Argentinean ovine cheese (Perotti et al., 2014) Scamorza (Albenzio et al., 2013) and Ricotta (Sameer et al., 2020).

Among the major challenges associated with the use of probiotic cultures for the production of functional cheeses are their ability to survive the technological hurdle during processing and ripening and their safety for human (Karimi et al., 2012; Castro et al., 2015). Furthermore, their introduction should not negatively affect the expected sensory characteristics (flavor, texture, and appearance) and biochemical composition of cheeses (Karimi et al., 2012; Sabikhi et al., 2014). In general, the incorporation of probiotic bacteria should not imply a loss of overall quality of the product.

Lactobacillus strains used as probiotics are generally of human or animal origin. However, several studies showed that strains with potential probiotic properties are also found in dairy products and other non-dairy fermented foods (Zielińska and Kołożyn-Krajewska, 2018). So far, many lactobacilli isolated from good quality artisanal raw milk cheeses have been selected on the basis of their functional properties and used along with standard or autochthonous starter cultures in the manufacturing process of different cheese types to improve and enhance their sensory quality (Randazzo et al., 2008; Guarrasia et al., 2017).

Presently, the use of probiotic yeasts is still scarce, despite their common presence in several fermented foods where they are actively involved in the production of aroma components and inhibition of spoilage bacteria (Fleet, 2007; Tofalo et al., 2019). On this basis, several yeast species have been extensively used as adjunct cultures to enhance flavor formation in certain cheese varieties (Centeno et al., 2017). The most important role of yeasts in cheese ripening is related to the increase of pH due to the metabolization of lactate and alkaline metabolite formation.

The technological, probiotic and bioactive potential of these putative probiotic strains should be evaluated *in situ* by analyzing their interactions in terms of cheese composition, physical chemical characteristics, nutrient availability, viability and biomolecules production.

Caciotta is as a semi-soft cheese with a short-medium ripening time produced from pasteurized whole cow's, ewe's or goat's milk, and represent one of the most common traditional Italian cheese variety. "Caciotta sarda" is produced from December to July throughout the Sardinian territory using Sardinian sheep's milk. The shape is cylindrical and the weight varies from 1 to 2.2 Kg. The milk is pasteurized, cooled to 36°C and coagulated with the addition of calf rennet and commercial microbial starter.

The organoleptic characteristics of this cheese type can vary as a function of the production area, the ripening time and the milk used (Gobbetti et al., 2018). Since the ripening time generally varies from 15–20 days to 2–6 months, milk pasteurization is needed to eliminate any pathogenic bacteria possibly present in raw milk (Aquilanti et al., 2011). On the other hand, pasteurization causes the loss of indigenous LAB species relevant for fermentation and ripening process. Therefore, in order to standardize the production while preserving at the same time the unique characteristics of this typical cheese variety, the addition of selected autochthonous adjunct cultures appears as a most efficient tool, as reported in other studies (Fusco et al., 2019; Bancalari et al., 2020; Calasso et al., 2020). The use of autochthonous starter cultures has already been proved to be an effective mean for standardizing the production of raw milk cheeses at the same time positively affecting their quality (Silvetti et al., 2017; De Pasquale et al., 2019; Dolci et al., 2020). When applying pasteurization to reduce cheese defects, the addition of selected LAB strains is of particular relevance for reconstituting the original microflora responsible for the typicality of traditional products, as shown by other authors for Caciotta cheese (Turchi et al., 2011; Bancalari et al., 2020).

In this work, we used a multidisciplinary approach to investigate the influence of adjunct cultures, containing autochthonous putative probiotic *Lactobacillus* and yeast strains, on the chemical and microbiological composition and sensory properties of industrially produced ewe's milk Caciotta cheese throughout a ripening period of 60 days. For the sake of comparison all analyses were performed also on Caciotta made with commercial starter cultures used as control.

MATERIALS AND METHODS

Microbial Strains and Cheese Manufacture

As reported in **Table 1**, three types of experimental Caciotta cheeses (C1, C2, C3) were made using three different combinations of autochthonous adjunct cultures, containing putative probiotic *Lactobacillus* and yeast strains coupled with starter cultures represented by autochthonous *L. lactis* subsp.

TABLE 1 | Starter and putative probiotic cultures used in the manufacturing of ovine Caciotta cheeses.

Caciotta types	Cultures	Origin
CC	*Lactococcus lactis subsp. lactis* Lyoto MO540	Commercial starter cultures
	Lactococcus lactis subsp. lactis Lyoto MO536	Commercial starter cultures
C1	*Lactococcus lactis subsp. lactis* 6MRSLS5	Raw sheep milk
	Lactobacillus plantarum 19/20711	Raw sheep milk
	Debaryomyces hansenii (FS6 DH1)	Fiore Sardo cheese
C2	*Lactococcus lactis subsp. lactis* 1FS171M	Fiore Sardo cheese
	Lactobacillus plantarum 62LP39b	Raw sheep milk
C3	*Enterococcus faecalis* 3M17LS5	Raw sheep milk
	Lactobacillus plantarum 11/20966	Raw sheep milk
	Kluyveromyces lactis 17bKL2	Fiore Sardo cheese

lactis or *Enterococcus faecalis* strains, selected on the basis of their technological characteristics and safety properties needed for the application as functional starter cultures (Cosentino et al., 2002, 2004; Pisano et al., 2015). The *Lactobacillus* and yeasts strains used in this study were previously tested for some *in vitro* functional properties associated with probiotic features in view of their use in cheese production as adjunct cultures (Pisano et al., 2008, 2014; Fadda et al., 2004, 2017). The main features of the autochthonous strains used in this study have been reported in **Supplementary Table 1** in Supplementary Material. A control cheese (CC) was produced using a commercial starter culture (*L. lactis* Lyoto MO540 and MO536, Sacco, Italy). All cheeses were manufactured by a dairy plant (Argiolas Formaggi, Dolianova, Cagliari, Italy) according to their Caciotta production protocol.

The autochthonous LAB strains were maintained at −20°C in MRS or M17 broth (Microbiol, Cagliari, Italy) with sterile 15% (v/v) glycerol (Microbiol) as cryoprotector in the microbial collection of the Department of Medical Sciences and Public Health. Fresh cultures were prepared in autoclaved sterile skimmed milk after two consecutive transfers in MRS or M17 broth (1% inoculum) incubated at 30°C in aerobic conditions for 18 h. Yeasts strains were maintained in YEPD (Yeast Extract Peptone Dextrose, Microbiol) with 20% (w/v) glycerol. The strains used in combination did not produce antibacterial substance against each other (data not shown).

Overall, four Caciotta cheese-making trials were carried out in four different days. In each trial two cheese batches were simultaneously produced from two vats of the same pasteurized ewes' milk using two different starter combinations (commercial starter or autochthonous starter containing putative probiotic strains as adjunct) for a total of eight cheeses batches, so two independent experiments were made in different days for each strains combination (**Supplementary Figure 1** in Supplementary Material). To prepare the inoculum for cheese making, the strains were inoculated in pasteurized ovine milk incubated at 30°C for 18–24 h and added to vat milk at a level of 10^7, 10^5 and 10^4 cfu/mL for LAB, *Kluyveromyces* and *Debaryomyces* species, respectively.

The chemical composition of raw ewe's milk used for cheese-making was 6.43% fat, 5.58% protein, 4% lactose, and the pH measured at 6.7. After pasteurization (72°C for 20 s), milk in each vat (150 L) was cooled at 37°C and inoculated with starter and adjunct cultures. After 30 min of resting time, rennet was added to the milk (1:50000) and coagulation took place at 37°C within 20 min. The curds were cut and left to rest for 10 min. Then, the curd pieces were hand pressed into molds for whey drainage (30°C, 85–90% of humidity). After brine salting for 12–16 h (23% °Be), the cheeses were transferred to a ripening chamber and stored 2 months at 12–15°C and 85% relative humidity. The weight of the cheeses was about 1.5 kg, diameter was 20 cm, and height was 15 cm. Samples were taken for all analysis at 1, 15, 30, and 60 days after production. The cheeses were sent to the laboratory under refrigeration (ca. 4°C) and were either analyzed immediately or frozen, depending on the assays.

Compositional Analysis

Chemical composition (fat (wet weight), fat (dry weight), protein, salt and moisture) was determined by a FoodScan™ Lab Dairy Analyser type 78810 (Foss, Hillerød, Denmark) by the dairy plant laboratory. The pH of the milk and the homogenized cheese samples (10 g in 10 ml of distilled water) was measured with a HI8520 pH meter (Pool Bioanalysis Italiana, Milan, Italy). Water activity (a_w) was determined using a PAWKIT water activity meter (Decagon Devices, Pullman, WA, United States) in accordance with the manufacturer's instructions. All determinations were made in duplicate.

Microbiological Analyses

At each sampling point a slice from a full piece of cheese (ca. 200 g) was sampled, then triplicate aliquots of 10 g were added to 90 mL of 2% (w/v) sodium-citrate sterile solution and homogenized in a Stomacher Laboratory Blender (Pool Bioanalysis Italiana, Milan, Italy) for 2 min at normal speed. Decimal dilutions were prepared in sterile 0.1% (w/v) peptone solution and spread onto the surface of the different agar media. Total mesophilic aerobic microbiota was enumerated using the pour plate method on Plate Count agar (PCA, Microbiol, Cagliari, Italy) incubated at 30°C for 48–72 h. *Enterobacteriaceae* and *E. coli* were determined on Violet Red Bile Glucose agar (VRBGA, Microbiol) and Triptone Bile X-gluc agar (TBX, Microbiol), respectively, using the pour plate method. VRBG and TBX plates were covered with a layer of the same culture medium before incubation at 37°C and 44°C for 24 h, respectively. Lactococci and enterococci were counted in M17 and KF agar (Microbiol) supplemented with 1% TTC, incubated at 30 and 37°C for 48 h, respectively. For the enumeration of lactobacilli, MRS agar acidified at pH 5.4 with glacial acetic acid incubated at 30°C in microaerophilic conditions for 48 h was used. Coagulase positive staphylococci were counted on Baird-Parker egg-yolk-tellurite agar (Microbiol) incubated at 37°C for 48 h. Black

colonies with or without the typical clearing of the egg yolk were tested for catalase-positivity, mannitol fermentation and coagulase production by the tube coagulase test. Yeasts and molds were enumerated in Potato Dextrose agar (PDA, Microbiol) plates containing 0.1 g/L cloramphenicol incubated at 25°C for 5 to 20 days.

DNA Extraction From Cheese Samples

Total DNA was extracted directly from samples collected during cheese making and ripening. At each sampling point a slice from a full piece of cheese (ca. 200 g) was sampled and a 25 g aliquot was homogenized in 225 mL of sodium citrate (pH 7.5), using a Stomacher for 3 min, then 1 mL of the homogenate was incubated for 30 min at 45°C in a dry Thermo-block heater (Asal, Milan, Italy). After the addition of equal volume of absolute ethanol, the samples were centrifuged at 10000 × *g* for ten min. The pellets were resuspended in 200 μL lysis buffer (200 mM Tris–HCl, pH 8.0, 250 mM NaCl, 25 mM EDTA, SDS 0.5% w/v) and 18 μL of proteinase K solution (2 mg/mL, Sigma) were added. The sample was vigorously vortexed and incubated for 60 min at 65°C before addition of 20 μL 2.5 M potassium acetate (Sigma). The resulting mixture was placed on ice for 2 h before centrifugation at 12.000*g* for 30 min. Two hundred microliters of the supernatant were transferred to a new tube and an equal volume of 2-propanol (Sigma) was added to precipitate nucleic acids. Nucleic acids were extracted in the aqueous phase by centrifugation at 12000 × *g* (5′, 4 °C) by adding 750 μL of SEVAG (Chloroform: isoamilic alcohol 24:1). The resulting DNA solution was precipitated with 70% ethanol by centrifugation at 12000 (4°C, 30 min), air-dried and resuspended in 100 μL of TE (10 mMTris–Cl, pH 8.0, 1 mM EDTA). DNA concentration and purity were checked by optical density at 260 nm and ratio O.D. 260 nm/280 nm determinations, respectively.

PCR Amplification of 16S and 26S rDNA Sequences and DGGE Analyses

For DGGE analysis, the universal primers F357 (5′-TACGGGAGGCAGCAG -3′) with the GC clump and R 518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993) were used to amplify the V3 region of 16S rRNA. Yeast DNA amplifications were carried out using primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′), with the GC clamp and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) (Cocolin et al., 2000). Amplification reactions were performed in a Mastercycler gradient 5331 (Eppendorf) in a final 50 μL reaction mixture volume each containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 mM primer, 2.5 U of *Taq* DNA polymerase (Sigma), and 10 μL of template DNA. PCR products were routinely checked for positive amplification on 1.8% w/v agarose gels prior to further DGGE analysis.

PCR products were analyzed by DGGE using a Bio-Rad D-code™ apparatus (Bio-Rad Laboratories) on 20 cm × 20 cm × 1 mm gel. Parallel electrophoresis was performed in 1 × TAE at 60 °C, employing 8% polyacrylamide gels with a denaturing range of 40–60%. The gels were run for 16 h at 75 V. Bands were visualized under UV light after staining with ethidium bromide (0.5 mg mL^{-1}), and photographed under UV trans-illumination. A ladder consisting of an amplicon mix of the different species used as starter and adjunct probiotic cultures in the manufacturing of caciotta cheeses was created in order to monitor their presence throughout ripening.

Sensory Analysis

Descriptive sensory profile of 60 days-ripened cheeses was carried out by a panel of six assessors trained in the sensory analysis of Caciotta cheeses produced in Sardinia. The experimental and control cheeses were evaluated for their general appearance, odor intensity, taste (acid, sweet, bitter, salty) aroma intensity, texture (intensity of elasticity, firmness, solubility, granularity) and overall acceptability, using 7-point category scale, ranging from 1 (low or poor) to 7 (high or excellent). The panelist had no information about the experimental design. All cheeses were kept at 20°C for 1 h before starting the test, coded with a three-digit code number and presented in random order.

Extraction and Saponification of Caciotta Cheeses Lipids

Total lipids (TL) were extracted from portions of grated cheese samples (100 mg, in triplicate) by the Folch procedure (Folch et al., 1957), using the mixture chloroform:methanol:water 2:1:1 (v/v/v) as previously reported (Rosa et al., 2015). The chloroform fraction (lipid extract) from each sample was separated from the methanol/water mixture and dried down under vacuum. The dried lipid fractions, dissolved in ethanol, were subjected to mild saponification as previously reported (Rosa et al., 2015, 2017) for the separation of lipid components. The unsaponifiable (total cholesterol and α-tocopherol) and saponifiable (FA) fractions were collected, the solvent evaporated, and a portion of the dried residues was injected into the high-performance liquid chromatograph (HPLC) (Rosa et al., 2015, 2017). An external standard mixture (containing 1 mg of triolein, trilinolein, and cholesterol) was processed as samples to calculate the fatty acid and cholesterol recovery during saponification.

Analyses of Fatty Acids, Cholesterol, and α-Tocopherol in Caciotta Cheeses

Analytical standards of FA, cholesterol, trilinolein, triolein, α-tocopherol, conjugated (9Z,11E)-linoleic acid (CLA), and all solvents used, of high purity, were purchased from Sigma–Aldrich (Milan, Italy). All the chemicals used in this study were of analytical grade.

Analyses of lipid components were carried out with a 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, United States) equipped with a diode array detector (DAD) and an Infinity 1260 evaporative light scattering detector (ELSD). Total cholesterol (detected at wavelength of 203 nm) and α-tocopherol (at 292 nm) were measured with an Inertsil ODS-2 column and methanol as the mobile phase (at flow rate of 0.7 mL/min) as previously reported (Rosa et al., 2015, 2017). Analyses of FA (unsaturated were detected at 200 nm and with ELSD, saturated with ELSD) were carried out with a XDB-C_{18} Eclipse, with a mobile phase of acetonitrile/water/acetic acid

(75/25/0.12, v/v/v), at a flow rate of 2.3 mL/min as previously reported (Rosa et al., 2015, 2017). The temperature of the column was maintained at 37°C. ELSD detector settings were: evaporator temperature 40°C, nebulization temperature 40°C, with nitrogen as the nebulizing gas at a flow of 1 L/min. The FA identification and quantification were performed using standard compounds and the Agilent OpenLAB Chromatography data system as previously described (Rosa et al., 2015, 2017). Calibration curves of the lipid compounds were found to be linear (DAD) and quadratic (ELSD), with correlation coefficients > 0.995.

NMR Analysis

A total of 96 samples were prepared for metabolomics analysis (2 trials × 4 added culture mixtures × 4 time points × 3 replicates). At each ripening time, a slice of cheese (ca. 300 mg) was sampled. Cheese was removed from 1 cm of the crust, freeze dried and then powdered in a ceramic mortar with a pestle. A methanol-chloroform-water extraction was performed for each cheese sample in triplicate as previously reported (Piras et al., 2013). The methanol/water fraction was separately collected, dried in vacuum at room temperature by using an Eppendorf concentrator 5301 (Eppendorf AG, Hamburg, Germany), and then stored at −80 °C. Prior to ^{1}H NMR analysis, the hydrophilic metabolites were dissolved in 700 μL of a D_2O solution containing 0.80 mM sodium 3-trimethylsilyl-(2,2,3,3-d4)-1-propionate (TSP) used as internal standard. The pH of the final sample was adjusted to 4.00 ± 0.05 by adding small amounts of NaOD or DCl. Then, an aliquot of 650 μL was transferred into a 5 mm NMR tube.

^{1}H NMR spectra were recorded on a Varian UNITY INOVA 500 spectrometer operating at 499.839 MHz. Experiments were carried out using the standard Varian PRESAT pulse sequence. For each experiment, 256 scans were collected into 32 k data points at 300 K over a spectral width of 6000.6 Hz with a 90° pulse, an acquisition time of 2.5 s, and a relaxation delay of 4 s. A presaturation technique with low power radiofrequency irradiation for 2 s was applied to suppress the residual water signal. The FIDs were multiplied by an exponential weighting function equivalent to line broadening of 0.3 Hz prior to Fourier transformation. ^{1}H NMR chemical shifts were referenced to TSP (δ 0.0 ppm). The assignment of NMR peaks was performed according to literature data (Piras et al., 2013), the Food Database,[1] and Chenomx NMR suite 8.1 software (evaluation version, Chenomx, Edmonton, AB, Canada).

All spectra were phased and baseline corrected using MestReNova (Version 14.0, Mestrelab Research SL, Santiago de Compostela, Spain). Correction for minimal misalignments in chemical shift, mainly due to pH-dependent signals, was done before deleting the regions containing the residual water and internal standard TSP signals, and segmenting the NMR spectra into intervals (bins) with equal width of 0.0025 ppm over a chemical shift range of 0.5–9.5 ppm. Bins were then normalized to the sum of total spectral area to compensate for the overall concentration differences.

[1]http://www.fdb.ca

Statistical Analyses

Data on compositional, microbiological and sensory analyses were analyzed using the software GraphPad Prism Statistics software package version 5.00 (GraphPad Prism Software Inc., San Diego, CA, United States). Means were compared by one-way analysis of variance (One-way ANOVA) using Bonferroni Multiple Comparisons Test. The data on sensory analyses were analyzed by Kruskal-Wallis test in order to detect any significant differences between the organoleptic profiles of the cheeses. A difference of $P < 0.05$ was considered significant.

Multivariate statistical analysis (MVA) of the NMR-data set was conducted by using SIMCA version 16.0 (Umetrics, Umea, Sweden). Prior to MVA, data were Pareto scaled. An explorative principal component analysis (PCA) was first performed to overview the variability of data and possible trends of groups (Bro and Smilde, 2014). The performance of the PCA model was evaluated using the coefficients R^2 and Q^2, defined as the proportion of variance in the data explained and predictable by the model, respectively. To investigate the relationship between the Nuclear magnetic resonance (NMR) spectral profile of cheeses and the ripening time, orthogonal partial least squares (OPLS) was applied (Trygg and Wold, 2002). OPLS is an extension of PLS that splits the systematic variation in the X block into two parts, one that models the correlation between X and Y (predictive) and another that shows the systematic X variation not related (orthogonal) to Y. The complexity of the model is reduced by orthogonalization since the systematic variation in the X matrix not correlated with Y is removed. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) was employed in a pair-wise comparison to discriminate groups (classes) of cheeses at 60 days of ripening (Bylesjo et al., 2006). The quality of OPLS and OPLS-DA models was evaluated by R^2Y and Q^2Y, i.e. the fraction of the variation of Y-variable and the predicted fraction of the variation of Y-variable, respectively. Permutation test was used to assess the reliability of models and CV-ANOVA (Cross-Validation Analysis Of Variance) test to evaluate their significance. Potential variables that are statistically significant were selected by analyzing the S-line correlation coefficient plot that is tailor-made for NMR spectroscopy data: peaks with positive (negative) intensity belong to the most abundant metabolites in the group on the right (left) side of the scores plot; peak in warm colors belong to metabolites that contribute more significantly to the class separation than do the metabolites associated with signals in cool colors. Variables were selected according to a $p(\text{corr}) \geq |0.5|$ and $p(\text{cov}) \geq |0.05|$ The correlation coefficient p(corr) value refers to the credibility of the contribution of the variable in the mathematical model, while p(cov) represent the modeled covariation.

RESULTS AND DISCUSSION

Physico-Chemical Composition of Caciotta Cheese During Ripening

The physico-chemical compositions (pH, a_w, moisture, fat, fat DM, salt, protein) of experimental and control Caciotta cheeses

at 1, 15, 30, and 60 days of ripening are reported in **Table 2**. The pH evolution over the 60 day of ripening varied among cheese types: the mean values initially decreased up to 15 (in C1 and C3) or 30 (in CC and C2) days of ripening, then increased at 60 days in all cheeses. Although no significant differences were observed among cheese types, a faster increase in pH (from 30 to 60 days of ripening) was recorded in cheeses produced with adjunct probiotic strains compared to the control (ΔpH CC: 0.1; ΔpH C1, C2, C3: 0.41, 0.69, 0.91, respectively). The highest pH value observed in C3 at the end of maturation could be related to both the proteolysis during ripening and the lactic acid utilization by the yeasts strain *K. lactis* used as adjunct in the manufacturing of the cheese (Fadda et al., 2004). The mean water activity (a_w) of all cheeses decreased during ripening, achieving values around 0.94–0.96 at 60 days, with no significant differences ($P > 0.05$) among the batches. A_w is a key factor in maintaining probiotic viability in the final products and the values observed in our study are unlikely to affect the viability and growth of the potential probiotic strains, since *Lactobacillus*, *Debaryomyces hansenii* and *Kluyveromyces lactis* species have been shown to possess stability in low water activity and maintain their viability in different low moisture foods (Tapia et al., 2007; Marcial-Coba et al., 2019).

With regard to the compositional parameters, no significant differences ($P > 0.05$) were detected between the control and the experimental cheeses. The mean values of fat, protein and salt increased during ripening, coupled with a decrease in the moisture mean content in all cheese types, in agreement with previous results (Pisano et al., 2007; Buccioni et al., 2012). At day 60, the composition of cheeses was found within the range of other Italian ewes' milk cheeses (Coda et al., 2006). It has been previously observed that the addition of probiotic microorganisms did not affect the main compositional parameters (moisture, salt, fat and protein contents) of different cheese types (Zhang et al., 2013; Perotti et al., 2014; Terpou et al., 2018), and did not negatively influence the ripening process of Edam cheese (Sabikhi et al., 2014).

Microbiota Analysis

In all the analyzed cheese samples, the overall hygienic quality was evaluated by the determination of microbial hygiene indicators *Enterobacteriaceae* and coagulase positive

TABLE 2 | Physico-chemical characteristics of ovine Caciotta cheeses during ripening.

Parameter	Caciotta type	Ripening time (days)			
		1	15	30	60
pH	CC	5.28 ± 0.41	4.92 ± 0.42	4.90 ± 0.31	5.07 ± 0.15
	C1	6.06 ± 0.39	5.10 ± 0.40	5.12 ± 0.39	5.59 ± 0.80
	C2	5.30 ± 0.37	5.02 ± 0.31	4.97 ± 0.25	5.66 ± 0.99
	C3	5.82 ± 0.57	4.81 ± 0.08	4.88 ± 0.09	5.80 ± 0.57
a_w	CC	0.99 ± 0.01	0.98 ± 0.00	0.98 ± 0.01	0.96 ± 0.00
	C1	0.99 ± 0, 01	0.97 ± 0.01	0.97 ± 0.00	0.96 ± 0.01
	C2	0.99 ± 0, 02	0.97 ± 0.00	0.97 ± 0.00	0.94 ± 0.02
	C3	1.00 ± 0, 02	0.98 ± 0.01	0.97 ± 0.02	0.95 ± 0.01
Moisture (%)	CC	49.68 ± 2.26	47.28 ± 2.24	46.04 ± 1.03	40.38 ± 2.45
	C1	50.84 ± 0.11	47.34 ± 2.90	46.96 ± 4.17	43.64 ± 2.99
	C2	47.71 ± 1.24	44.58 ± 2.45	44.26 ± 2.46	37.94 ± 4.03
	C3	49.74 ± 0.92	48.87 ± 0.06	46.37 ± 2.76	43.67 ± 2.37
Fat (%)	CC	25.16 ± 0.25	26.50 ± 0.17	27.14 ± 0.90	31.18 ± 2.33
	C1	24.12 ± 0.79	26.03 ± 2.01	26.69 ± 2.23	28.59 ± 2.11
	C2	26.58 ± 1.90	28.63 ± 2.67	30.555 ± 3.86	32.66 ± 6.15
	C3	25.06 ± 1.48	24.65 ± 0.07	26.41 ± 2.67	27.85 ± 2.26
Fat (%DM)	CC	50.06 ± 2.75	50.32 ± 2.47	50.31 ± 2.64	51.20 ± 3.25
	C1	49.07 ± 1.70	49.40 ± 1.10	49.81 ± 0.94	50.23 ± 0.38
	C2	50.82 ± 2.43	51.62 ± 2.52	52.82 ± 4.43	53.91 ± 4.40
	C3	49.85 ± 2.05	48.20 ± 0.08	49.18 ± 2.45	49.39 ± 1.93
Salt (%)	CC	1.02 ± 0.71	1.27 ± 0.52	1.65 ± 0.42	2.06 ± 0.03
	C1	1.32 ± 0.29	1.54 ± 0.10	1.76 ± 0.08	2.24 ± 0.16
	C2	1.32 ± 0.29	1.54 ± 0.10	1.76 ± 0.08	2.24 ± 0.16
	C3	0.95 ± 0.17	1.55 ± 0.09	1.80 ± 0.02	2.19 ± 0.25
Protein (%)	CC	20.93 ± 0.42	21.77 ± 0.09	22.15 ± 0.37	24.85 ± 1.56
	C1	19.86 ± 0.52	21.60 ± 1.32	21.71 ± 1.48	22.96 ± 1.01
	C2	21.02 ± 0.03	21.87 ± 0.08	22.54 ± 0.71	24.14 ± 0.56
	C3	20.99 ± 0.80	21.20 ± 0.05	21, 95 ± 1.27	23.62 ± 1.31

Data are the mean ± standard deviation (SD) of two cheese-batches. No significant differences ($P > 0.05$) were detected among cheese types for all parameters analyzed.

staphylococci, which were always under the detection limit (< 10 cfu/g and < 100 cfu/g, respectively) (data not shown). It has been demonstrated that efficient starter culture and/or an enforcement by an adjunct is essential to achieve a fast pH decrease, thus contributing to control these undesired microorganisms (Terpou et al., 2018).

The viable counts of LAB and yeasts are shown in **Table 3**. The numbers of presumptive lactococci were high throughout the ripening and did not significantly differ ($P > 0.05$) among all samples, presumably due to the use of the same type and amount of starter cultures. The presence of lactococci in C3 cheeses (where no lactococcal strain was included in the starter) may be due to secondary contamination considering that they are widely used as starter in cheese productions and taking into account their ability to colonize and adapt to different substrates in the dairy plant (Calasso et al., 2016). As expected, in experimental cheeses, the use of *Lactobacillus* strains as adjunct cultures significantly increased the population of lactobacilli as compared to the control. Moreover, lactobacilli increased their numbers until 60 days of ripening. Similar results were observed in studies involving the use of autochthonous LAB in cheese making (Piras et al., 2013; De Pasquale et al., 2019; Fusco et al., 2019). The counts of presumptive enterococci were higher in C3 cheese, where *E. faecalis* strain was present in the starter culture, with mean values ranging from 10^5 to 10^7 cfu/g, while in the other cheese types they showed mean concentrations from 10^2 cfu/g in curd to about 10^5 cfu/g at the end of ripening, in agreement with other studies (Giraffa, 2003; Piras et al., 2013; Fuka et al., 2017; Hanchi et al., 2018). With respect to yeast viable counts no marked differences were observed between the cheeses with the exception of C3, produced with an adjunct autochthonous probiotic *K. lactis* strain, that showed during manufacturing and ripening counts higher than both CC and C2 (produced without probiotic yeast) and C1 cheese, even though the latter was produced with the addition of the yeast *D. hansenii*. The yeast counts found in C3 cheese throughout maturation are in line or lower than those reported in others studies where yeast strains have been used as co-starter or adjunct in the production of cheeses (Lanciotti et al., 2005; De Freitas et al., 2008; Pereira Andrade et al., 2019). The similar yeast counts observed in control, C1 and C2 cheese could be related to the addition of the species *Geotrichum candidum* routinely used together with *Penicillium candidum* by the dairy plant as protective cultures in the cheese-making, in order to prevent or limit the growth of spoilage fungi in the cheese rind. To the best of our knowledge this is the first study reporting the use of autochthonous yeast strains with potential probiotic properties as an adjunct for the production of cheese.

The colonies counted as presumptive lactococci, lactobacilli, enterococci and yeasts were randomly selected and identified by biochemical and molecular means, confirming their belonging to the inoculated species, the exception being *D. hansenii* that was not recovered in any of C1 samples and the presence of *E. faecalis* in CC and *E. faecium* in the other cheese types (data

TABLE 3 | Viable counts (Log ufc/g) of presumptive lactococci, lactobacilli, enterococci and yeasts during manufacturing and ripening of control and experimental Caciotta cheeses.

Cheese type	Days of ripening				
	Curd	1	15	30	60
Presumptive Lactococci					
CC	8.05 ± 0.35^{a}	8.30 ± 0.82^{a}	7.75 ± 0.75^{a}	8.55 ± 0.15^{a}	8.17 ± 0.13^{a}
C1	7.90 ± 0.58^{a}	7.45 ± 1.56^{a}	7.80 ± 1.50^{a}	8.81 ± 0.11^{a}	9.72 ± 0.38^{a}
C2	9.39 ± 1.09^{a}	8.89 ± 0.41^{a}	9.20 ± 0.19^{a}	9.09 ± 0.31^{a}	9.05 ± 0.45^{a}
C3	7.48 ± 1.12^{a}	8.38 ± 0.38^{a}	7.90 ± 0.93^{a}	9.10 ± 0.10^{a}	7.74 ± 1.04^{a}
Presumptive Lactobacilli					
CC	1.50 ± 0.50^{b}	2.65 ± 0.35^{a}	4.15 ± 0.15^{a}	5.54 ± 0.06^{a}	6.16 ± 0.16^{a}
C1	5.98 ± 0.98^{ab}	7.42 ± 0.98^{b}	8.50 ± 0.50^{b}	8.61 ± 0.43^{b}	9.15 ± 0.15^{b}
C2	7.07 ± 0.77^{a}	7.75 ± 1.15^{b}	8.68 ± 0.68^{b}	8.10 ± 0.85^{b}	8.85 ± 0.35^{b}
C3	7.09 ± 0.91^{a}	7.75 ± 0.55^{b}	8.78 ± 0.30^{b}	8.93 ± 0.15^{b}	8.93 ± 0.33^{b}
Presumptive Enterococci					
CC	3.15 ± 1.15^{a}	2.65 ± 0.65^{a}	3.86 ± 0.74^{a}	5.02 ± 0.18^{a}	4.92 ± 0.67^{a}
C1	2.75 ± 0.45^{a}	2.75 ± 0.75^{a}	4.15 ± 1.15^{a}	5.42 ± 0.58^{a}	5.3 ± 0.70^{a}
C2	2.15 ± 0.15^{a}	3.65 ± 0.15^{a}	3.13 ± 1.06^{a}	4.60 ± 2.00^{a}	5.00 ± 2.00^{a}
C3	5.65 ± 0.35^{a}	6.58 ± 0.71^{a}	6.14 ± 0.97^{a}	7.24 ± 1.24^{a}	7.74 ± 0.74^{a}
Yeasts					
CC	3.50 ± 0.50^{a}	3.16 ± 0.16^{a}	3.79 ± 0.39^{a}	4.23 ± 0.76^{a}	3.65 ± 1.35^{a}
C1	3.35 ± 0.65^{a}	2.89 ± 0.19^{a}	2.34 ± 1.00^{a}	3.98 ± 0.80^{a}	3.63 ± 0.37^{a}
C2	3.15 ± 0.15^{a}	3.04 ± 0.04^{a}	3.28 ± 0.24^{a}	4.99 ± 0.88^{a}	3.60 ± 0.40^{a}
C3	5.39 ± 0.69^{a}	5.34 ± 0.56^{b}	4.35 ± 0.35^{a}	6.16 ± 0.82^{a}	5.04 ± 0.56^{a}

Values are the mean ± standard error of triplicate samples of two batches of the same cheese type.
Different superscripts in the same column indicate significant differences ($P < 0.05$).

not shown). In general, the isolation of a varied microbiota in cheese is not unexpected since cheeses and related dairy environment (such as equipment, tanks, ripening rooms) are characterized by a complex microbiota that inevitably interact with the bacteria from milk and starter culture during cheese production (Montel et al., 2014).

In addition to the cultural analyses, a culture-independent approach using PCR-DGGE was used to monitor the presence of probiotic adjunct strains using the identification ladder indicated in MM (**Figure 1**). Although this molecular approach is not able to provide a complete picture of diversity and community composition of microbial ecosystems as that given by high-throughput sequencing methods, it can still be considered a helpful technique to monitor dominant LAB community changes in environmental and intestinal ecosystem (Yunita and Dodd, 2018).

In general, a good concordance was observed between PCR-DGGE and plate culture isolation, although a few inconsistencies were seen, as reported by other studies combining culture-dependent and –independent analyses (Pangallo et al., 2014; Silvetti et al., 2017). DGGE profiles of cheese samples consisted of just few bands mainly corresponding to the species used as starter and adjunct cultures, and they revealed the dominance of a band corresponding to *L. lactis* species in all cheeses throughout ripening. A weaker band corresponding to *L. plantarum* species was observed in all experimental probiotic cheeses. As the presence of *L. plantarum* probiotic strains during cheese making and maturation of probiotic cheeses was confirmed by cultural analysis and genotypic characterization by rep-PCR, the detection of weak bands in DGGE analysis could be due to different affinity of the primers to template DNA for different species, as observed by other authors (Bae et al., 2006; Mayrhofer et al., 2014). In additions to *L. lactis* and *L. plantarum*, other bands (indicated with the letter *h* and *i* in **Figure 2**) that could not be identified using our ladder were detected in all cheeses. These bands were excised and sequenced and were found to be homologous to *St. thermophilus* and *E. faecium* (*h* and *i*, respectively), typical adventitious LAB in cheese (Carafa et al., 2019).

DGGE analysis of 26S rDNA was used to investigate the presence of adjunct probiotic yeast strains *D. hansenii* and *Kl. lactis*. While *Kl. lactis* could be clearly detected in C3 cheeses at each stage of ripening, the band corresponding to *D. hansenii* was not seen in the DGGE gel of C1 cheeses, confirming the result of culture analysis. Since a detection limit of 10^3 cfu/mL for DGGE analysis has been reported (Cocolin et al., 2000) and our cultural method has a detection limit of 10^2 cfu/mL, on the basis of the metabolome results (see section "Metabolic Profile of Caciotta Cheeses During Maturation") we have speculated that *D. hansenii* may be present in the cheese in a viable but not detectable state. DGGE analysis of 26S rDNA also showed a constant presence of the species *Geotrichum candidum* and *Penicillium candidum* in all cheeses throughout ripening.

Sensory Analysis

A sensory evaluation of probiotic and control caciotta cheeses at 60 days of ripening was performed by six panelists. Mean

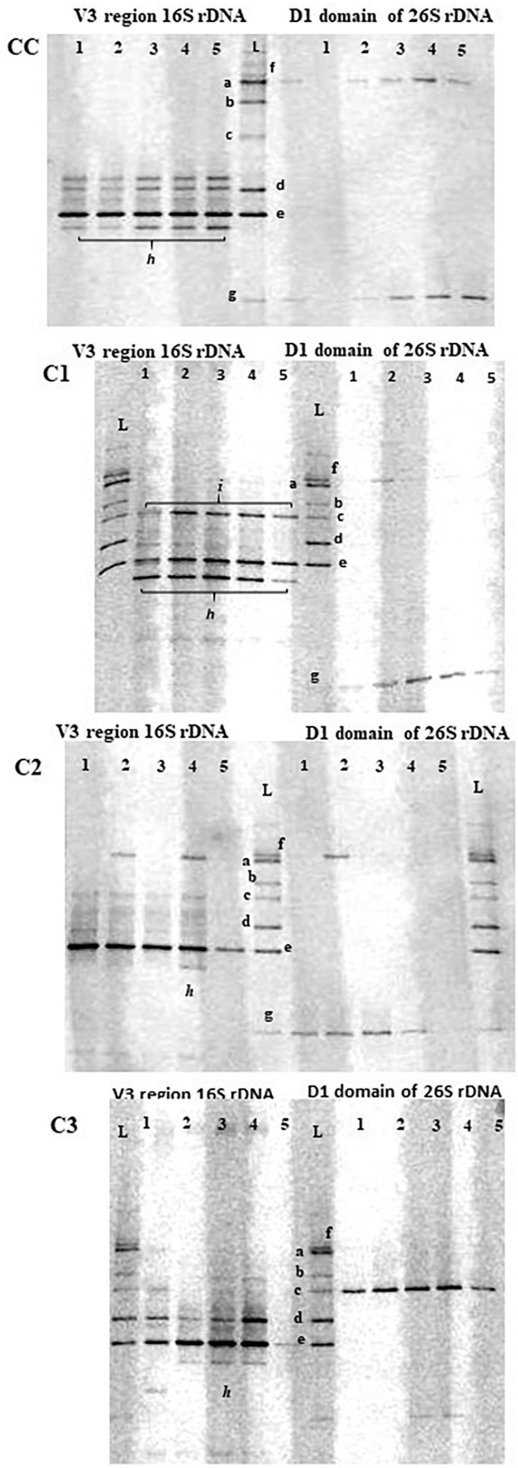

FIGURE 1 | PCR-DGGE profiles of V3 16S and 26S rDNA regions in control (CC) and experimental (C1, C2, C3) cheese samples at different times of ripening. Lane 1, curd; lane 2, 1d-cheese; lane 3, 15d-cheese; lane 4, 30d-cheese; lane 5, 60d-cheese; L, identification ladder. a = *L. plantarum* ATCC 20174; b = *Debaryomyces hansenii* ATCC36239; c = *Kluyveromyces lactis* ATCC 56498; d = *E. faecalis* ATCC 29212; e = *L. lactis* ATCC 19435; f = *Geotrichum candidum*; g = *Penicillium candidum*; *h* = *S. thermophilus*; *i* = *E. faecium*.

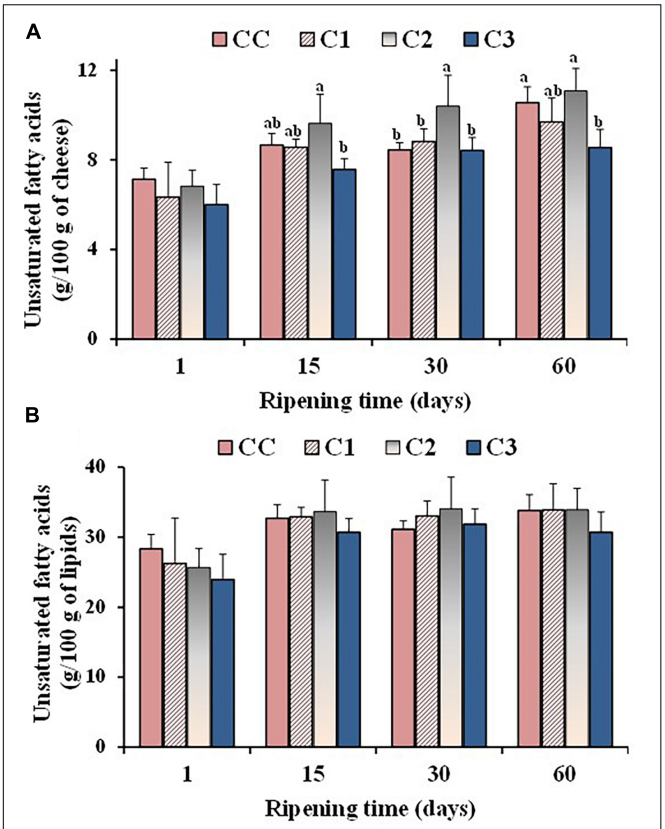

FIGURE 2 | Values of total unsaturated fatty acids, expressed as g/100 g of cheese **(A)** and g/100 g of total lipids **(B)**, measured in the control (CC) and probiotic cheeses (C1, C2, C3) at different ripening times; (n = 6). ^{a,b,c} Samples with different superscript letters, within the same ripening time, are significantly different at $P < 0.05$ (One-way ANOVA).

ratings of descriptive attributes such as odor, aroma, basic tastes, texture and overall acceptability are documented in **Table 4** and **Supplementary Figure 2** in Supplementary Material. Significantly higher scores for aroma intensity were recorded in C3 cheeses with respect to C1 and C2 types, while C2 was perceived as the most bitter and acid with respect to the other groups. Furthermore, the textural parameters elasticity and solubility were significantly higher in CC and C1 cheeses. In general, the sensory profile of C1 cheese, found to be the most similar to CC cheese, and the highest scores for aroma obtained from C3 cheese may explain their slightly higher values for overall acceptability. Our results are in agreement with other studies investigating the sensory properties of Caciotta cheese produced with the addition of autochthonous LAB (Fusco et al., 2019; Bancalari et al., 2020).

Fatty Acid Profile of Caciotta Cheese During Ripening

Quali-quantitative information on the main saturated (SFA) and unsaturated (UFA) FA that compose the lipid classes of Caciotta cheeses was obtained by HPLC analyses with ELSD detection. HPLC-ELSD is an alternative technology to UV analysis and

TABLE 4 | Sensory descriptors scores obtained for the control and probiotic Caciotta cheeses. Attributes were scored on a 7-point scale.

Cheese type	Basic taste							Texture attributes				
	Appearance	Odor	Aroma	Acid	Sweetness	Bitter	Saltiness	Elasticity	Firmness	Solubility	Granularity	Overall acceptability
CC	5.42 ± 0.38^{a}	5.08 ± 0.49^{a}	5.75 ± 0.27^{ab}	2.75 ± 0.68^{a}	4.42 ± 0.80^{a}	2.33 ± 0.75^{a}	4.00 ± 0.63^{a}	4.50 ± 0.32^{a}	2.42 ± 0.58^{ab}	5.08 ± 0.49^{a}	2.58 ± 0.58^{ab}	4.42 ± 0.66^{a}
C1	5.00 ± 0.45^{a}	5.00 ± 0.77^{a}	4.83 ± 0.60^{b}	3.17 ± 0.68^{ac}	3.25 ± 0.52^{ac}	2.25 ± 0.27^{a}	3.75 ± 0.70^{a}	4.50 ± 0.45^{a}	3.33 ± 0.52^{a}	5.08 ± 0.38^{a}	1.92 ± 0.66^{a}	5.17 ± 0.41^{a}
C2	3.42 ± 0.38^{b}	4.08 ± 0.58^{a}	4.75 ± 0.52^{b}	4.33 ± 0.70^{bc}	2.17 ± 0.26^{bc}	4.08 ± 0.49^{b}	4.33 ± 0.45^{a}	3.33 ± 0.26^{b}	2.67 ± 0.52^{ab}	4.08 ± 0.20^{b}	3.08 ± 0.80^{b}	4.16 ± 0.81^{a}
C3	4.83 ± 0.26^{ab}	4.08 ± 0.49^{a}	6.25 ± 0.42^{a}	4.08 ± 0.50^{bc}	2.92 ± 0.38^{ac}	3.67 ± 0.41^{ab}	4.50 ± 0.45^{a}	3.42 ± 0.20^{b}	2.00 ± 0.45^{b}	4.08 ± 0.49^{b}	2.17 ± 0.26^{ab}	4.75 ± 0.70^{a}

The scores are expressed as mean values ± SD of two batches.
Different letters within a column indicate significant differences ($P < 0.05$).

is often employed when compounds lack sufficient absorptivity (like saturated FA). The ELSD detector quantifies any solute less volatile than the solvents and functional groups, FA chain-length, or saturation has little or no effect on the detector response (Aradottir and Olsson, 2005). However, a non-linear ELSD response for FA and a low repeatability of ELSD response factors have been reported (Cebolla et al., 1997; Aradottir and Olsson, 2005). Therefore, for the comparative assessment of the total FA profiles of Caciotta cheese samples, FA quantification was based on the internal normalization method (Amaral et al., 2004; Rosa et al., 2017), assuming that the detector response was the same for all FA. It was based on the received peak area, the results were normalized without correction factor and the FA contents were expressed as area percentages (% area). This HPLC–ELSD analysis was set up to analyze up to 16 FA (saturated + unsaturated FA) in one single analysis without any derivatization. The ELSD is suitable for the analysis of only C_{12}–C_{22} FA since those with shorter chain length are not detected being too volatile (Bravi et al., 2006). FA compositions (expressed as % of total fatty acids) in control (CC) and probiotic Caciotta cheeses (C1, C2, C3) at 1 day of production measured by HPLC-ELSD are reported in **Table 5**. Control Caciotta cheese showed a concentration of approximately 53.3% of SFA (mainly palmitic acid 16:0, and stearic acid 18:0, 30, and 13%, respectively), 41.6% of monounsaturated FA (MUFA, mainly oleic acid 18:1 n-9 and 18:1 *trans* isomers, 35.6 and 5.9%, respectively), and 4.6% of polyunsaturated FA (PUFA), mainly constituted by conjugated linoleic acid (CLA) isomers and linoleic acid 18:2 n-6, 2.5 and 1.3%, respectively. The probiotic cheeses C1-C3 showed a SFA and UFA profile similar to the that of control cheese, with values of SFA, MUFA and PUFA in the range 53–55%, 40–41% and 4.6–5%, respectively. As indicated in **Table 5**, the absolute content of the main UFA detected in control and probiotic Caciotta cheeses at 1 day of production by HPLC with DAD detection and values, expressed as g/100 g of Caciotta cheese on a wet basis, showed UFA amounts of experimental cheeses in line with those measured in the control. The average values for the main UFA of both control and probiotic cheeses were in the range: 3.5–4.2 g/100 g of cheese for 18:1 n-9, 1.3-1.5 g/100 g for 18:1 *trans* isomers, 0.4-0.5 g/100 g for 18:2 n-6, and 0.3-0.4 g/100 g for CLA isomers.

A similar trend and no significant differences were measured in the SFA, MUFA and PUFA % levels among the different batches of Caciotta cheeses at all ripening stages (**Supplementary Figure 3** in Supplementary Material).

Some differences were observed in the absolute amounts of UFA during ripening. **Figure 2A** shows the total UFA amounts (expressed as g/100 g of cheese on a wet basis) measured in the control and probiotic cheeses at different ripening times. The absolute values of UFA increased during ripening in all cheese types mostly due to a corresponding decrease in the moisture content, as indicated by the high negative correlation coefficients measured between UFA absolute values/moisture level during ripening for CC (r = −0.9736), C1 (r = −0.9661), C2

TABLE 5 | Fatty acid profile of control (CC) and probiotic Caciotta cheese (C1, C2, C3) at 1 d of production measured by HPLC analysis with ELSD and DAD detection.

	% Area (ELSD detection)				g/100 g of Caciotta (DAD detection)			
	CC	C1	C2	C3	CC	C1	C2	C3
12:0	0.10 ± 0.02	0.10 ± 0.05	0.13 ± 0.06	0.11 ± 0.07	–	–	–	–
14:0	9.93 ± 0.78	10.31 ± 0.53	10.83 ± 1.19	11.20 ± 0.68	–	–	–	–
14:1	–	–	–	–	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
16:0	30.50 ± 1.36	31.89 ± 2.15	30.23 ± 2.57	30.14 ± 2.30	–	–	–	–
16:1 n-7	0.15 ± 0.02	0.17 ± 0.03	0.18 ± 0.03	0.19 ± 0.01	0.14 ± 0.01	0.12 ± 0.02	0.13 ± 0.01	0.12 ± 0.01
18:0	12.80 ± 0.89	12.44 ± 1.14	12.04 ± 0.96	12.52 ± 1.23	–	–	–	–
18:1 *trans* isomers	5.87 ± 0.65	5.71 ± 0.45	5.85 ± 0.59	5.91 ± 0.77	1.50 ± 0.22	1.36 ± 0.46	1.45 ± 0.27	1.26 ± 0.27
18:1 n-9	35.58 ± 2.49	34.30 ± 1.39	35.15 ± 2.45	34.43 ± 3.03	4.16 ± 0.26	3.69 ± 0.85	3.98 ± 0.37	3.52 ± 0.49
18:2 n-6	1.28 ± 0.15	1.23 ± 0.07	1.31 ± 0.15	1.30 ± 0.01	0.52 ± 0.03	0.45 ± 0.11	0.49 ± 0.05	0.43 ± 0.06
CLA isomers	2.50 ± 0.04	2.50 ± 0.22	2.76 ± 0.18	2.72 ± 0.33	0.41 ± 0.04	0.35 ± 0.04	0.38 ± 0.03	0.34 ± 0.01
18:3 n-3	0.56 ± 0.10	0.58 ± 0.07	0.62 ± 0.16	0.55 ± 0.04	0.27 ± 0.04	0.24 ± 0.08	0.26 ± 0.05	0.22 ± 0.04
18:3 n-6	–	–	–	–	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:4 n-3	–	–	–	–	trace	trace	trace	trace
20:4 n-6	0.30 ± 0.02	0.32 ± 0.02	0.34 ± 0.06	0.35 ± 0.02	0.06 ± 0.00	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.01
20:5 n-3	–	–	–	–	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
22:6 n-3	–	–	–	–	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
SFA	53.33 ± 2.43	54.74 ± 1.38	53.24 ± 2.68	53.98 ± 2.42	–	–	–	–
MUFA	41.60 ± 2.32	40.18 ± 1.37	41.18 ± 2.55	40.53 ± 2.62	5.83 ± 0.47	5.20 ± 1.33	5.59 ± 0.63	4.93 ± 0.77
PUFA	4.65 ± 0.22	4.63 ± 0.23	5.04 ± 0.37	4.92 ± 0.29	1.35 ± 0.05	1.18 ± 0.25	1.28 ± 0.10	1.25 ± 0.14
Others	0.43 ± 0.07	0.45 ± 0.13	0.55 ± 0.13	0.57 ± 0.09	–	–	–	–

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Data reported are the mean ± standard deviation (SD) of 6 values (n = 6).

(r = −0.8365), and C3 (r = −0.8480). Significant differences were detected in the UFA absolute values among cheese types from 15 days of ripening, with sample C2 and C3 showing the highest and the lowest UFA contents, respectively. These differences were ascribable to a different moisture level in Caciotta cheeses. In fact, no significant differences were observed between experimental and control group at the different ripening times when total UFA levels were measured as g per 100 g of lipids (**Figure 2B**), suggesting that the addition of probiotic bacteria did not affect the composition of FA that make up triglycerides of cheese fat. Similar results were reported by Perotti et al. (2014), who did not observe modifications in the FA composition (expressed as %, g FA/100 g of total FA) of Argentinean semi-hard ovine cheese made with a mix of probiotic microorganisms.

Isomers of conjugated linoleic acid (or CLAs) are of particular interest from a nutritional point of view, being *cis*-9, *trans* -11 CLA (rumenic acid) the most representative. Experimental evidence has suggested that CLA may have antiatherosclerotic, anticarcinogenic, antidiabetic and immunomodulanting effects (Mele et al., 2011; Prandini et al., 2011). Since CLA is naturally present in milk from ruminants, cheese represents a good source of this FA, its content in cheese being usually related to the CLA level of the unprocessed milk (Perotti et al., 2014). Sheep cheeses reported a higher level of CLA with respect to cow and goat cheeses (Prandini et al., 2011). Additional CLA quantities could be produced during manufacturing and ripening of cheeses, as some cheese-related microorganisms including probiotic bacteria have shown the ability to produce CLA (Laskaridis et al., 2013; Perotti et al., 2014). **Figure 3** shows the total values of CLA isomers, expressed as g/100 g cheese (**Figure 3A**) and g/100 g lipids (**Figure 3B**), measured in the control and probiotic cheeses C1-C3 at different ripening times. In our study, the absolute values of CLA isomers, like all the others FA, increased per 100/g of cheese in control and probiotic cheeses with ripening time, and the highest values were measured at 60 days ripening in the cheese samples characterized by a lower rate of moisture (CC and C2). Similar CLA contents were measured per 100 g of lipids, among Caciotta cheese batches at all ripening stages indicating that the probiotic cultures did not affect the CLA content of cheeses with respect to the control. Similar results were reported by other authors for ovine, caprine and bovine cheeses (Gursoy et al., 2012; Perotti et al., 2014).

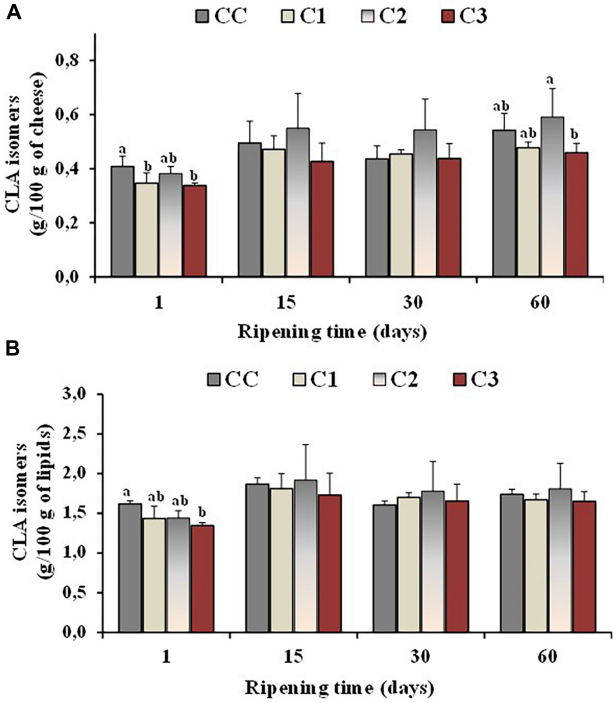

FIGURE 3 | Total values of CLA isomers, expressed as g/100 g of cheese **(A)** and g/100 g of total lipids **(B)**, measured in the control (CC) and probiotic cheeses (C1, C2, C3) at different ripening times; (n = 6). ^{a,b,c} Samples with different superscript letters, within the same ripening time, are significantly different at $P < 0.05$ (One-way ANOVA).

Total Cholesterol and α-Tocopherol Levels of Caciotta Cheese During Ripening

Dairy products play a major role in supplying dietary cholesterol, since cholesterol is the predominant sterol of milk (> 95 g/100 g of total sterol) (Collins et al., 2003). The association between plasma cholesterol and atherosclerosis has emphasized the importance of the assessment of cholesterol in dairy products (Marrone et al., 2014). Several probiotic strains have been shown to possess *in vitro* cholesterol-lowering ability (Ooi and Liong, 2010). **Figure 4** shows the values of total cholesterol, expressed as mg/100 g cheese and mg/100 g lipids (**Figures 4A,B**, respectively), measured in the control and probiotic cheeses at different ripening times. As expected, cholesterol content (mg/100 g cheese) increased in all cheese types during ripening because of the loss of water. After 60 days of ripening, the total cholesterol content was in the range 84–117 mg/100 g of cheese, with lower values observed in the probiotic cheeses C1 and C3, with respect to control CC and C2 sample. No significant differences were found in the cholesterol content expressed as mg/100 g of fat between control and probiotic cheeses even though in-vitro cholesterol-lowering *Lactobacillus* strains were used in C1 e C3 cheeses. On the other hand, possible differences between the expression of functional properties *in vitro* and *in vivo* have been pointed out, as various factors related to the environmental conditions and/or interaction with microbial communities prevailing *in situ* could affect the efficiency of the strains. In the study by Albano et al. (2018) seven LAB strains showed a lower ability to reduce cholesterol in cheese than in broth. Our results highlight the importance to perform in situ studies to confirm the in vitro functional characteristics of probiotic strains in order to assess their health-promoting properties and their performance as novel probiotic.

The level of the antioxidant α-tocopherol was also determined in all cheeses during ripening. In general, cheese is considered a product stable to oxidation (Mele et al., 2011), characterized by the presence of antioxidants like vitamin E, vitamin A and β-carotene (Revilla et al., 2014). Vitamin E is known to act primarily as a liposoluble antioxidant, protecting PUFA and

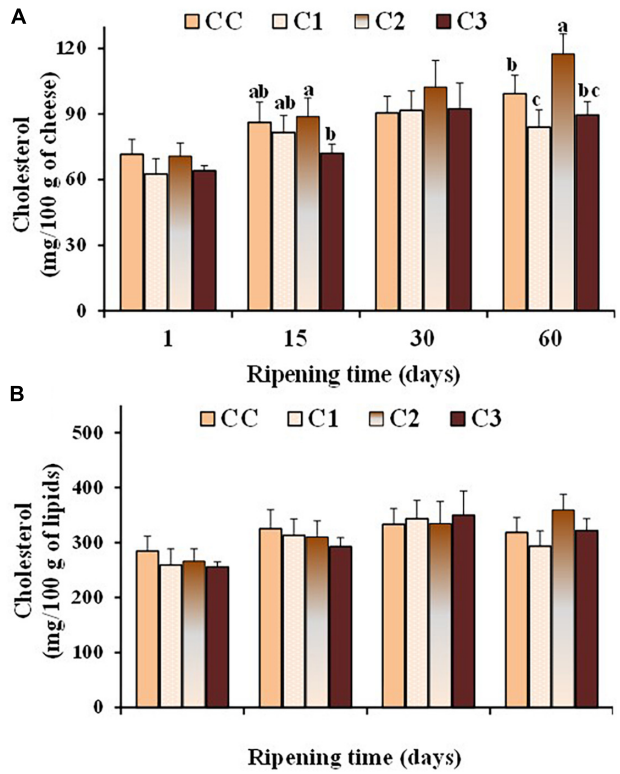

FIGURE 4 | Values of total cholesterol, expressed as mg/100 g of cheese **(A)** and mg/100 g of total lipids **(B)**, measured in the control (CC) and probiotic cheeses (C1, C2, C3) at different ripening times; (n = 6). ^{a,b,c,d} Samples with different superscript letters, within the same ripening time, are significantly different at $P < 0.05$ (One-way ANOVA).

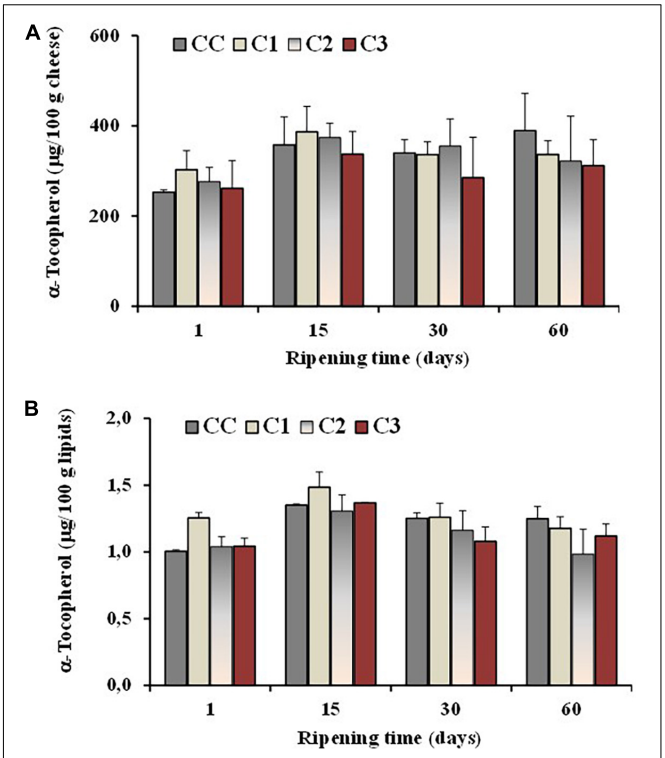

FIGURE 5 | Total values of α-tocopherol, expressed as µg/100 g of cheese **(A)** and µg/100 g of total lipids **(B)**, measured in the control (CC) and probiotic cheeses (C1, C2, C3) at different ripening times; (n = 6).

related substances from peroxidation, and α-tocopherol is the main component with vitamin E activity in sheep milk and cheese (Revilla et al., 2014). The effect of different probiotic organisms and ripening time on the α-tocopherol content of the control and probiotic cheeses is presented in **Figure 5**. A little increase in concentration, expressed as µg/100 g cheese, was observed in all cheese batches at 15 days of ripening, followed by similar or lower amount at 30 and 60 days of maturation (**Figure 5A**). No significant differences were observed among control and experimental cheeses and the average values for α-tocopherol of both control and probiotic cheeses were in the range 312–389 µg/100 g of cheese and 1–1.2 µg/100 g of lipids after 60 days of ripening (**Figure 5B**).

Metabolic Profile of Caciotta Cheeses During Maturation

Metabolomics analysis of cheese has been shown to be a valuable tool to investigate the association between the biochemical events occurring in this food and maturation process (Piras et al., 2013), microbial culture-dependent methods (Pisano et al., 2016), sensory properties (Ochi et al., 2012a), or cheese quality (Mazzei and Piccolo, 2012). In the present study, we have performed a NMR-based metabolomics analysis of the aqueous extract of Caciotta cheese to evaluate and compare the changes of the metabolic profile due to the ripening time and the added cultures.

Representative ^{1}H NMR spectra of the aqueous extracts of control Caciotta at different ripening days are reported in **Figure 6**, while those of C1, C2 and C3 cheeses are depicted in the Supplementary Material (**Supplementary Figures 4–7**). A variety of amino acids, carbohydrates, osmolytes, and organic acids were detected. In good agreement with the literature (Consonni and Cagliani, 2008; Rodrigues et al., 2011; Piras et al., 2013), it can be visually observed that ripening time has an impact on the NMR profile of all cheeses, as pointed out, in particular, by the increasing temporal intensities of many amino acid peaks and the decreasing of lactose signals. Among the various NMR peaks, those from lactic acid (La) were predominant in all Caciotta aqueous extracts at all ripening times (**Supplementary Figure 4** in the Supplementary Material). A high content of La is typical of the initial stage of cheese when the starter cultures cause a slight and rapid acidification of milk. The occurrence of high levels of La over the maturation time of Caciotta, indicated a sustained microbial activity during the ripening period. Compared to control, the levels of La in the experimental cheeses produced with autochthonous strains increased more markedly after 15 days of maturation, particularly in C1 and C3 (**Supplementary Figure 8** in Supplementary Material), then decreased up to 60 days of ripening, reaching the lowest values in C3, presumably as a consequence of

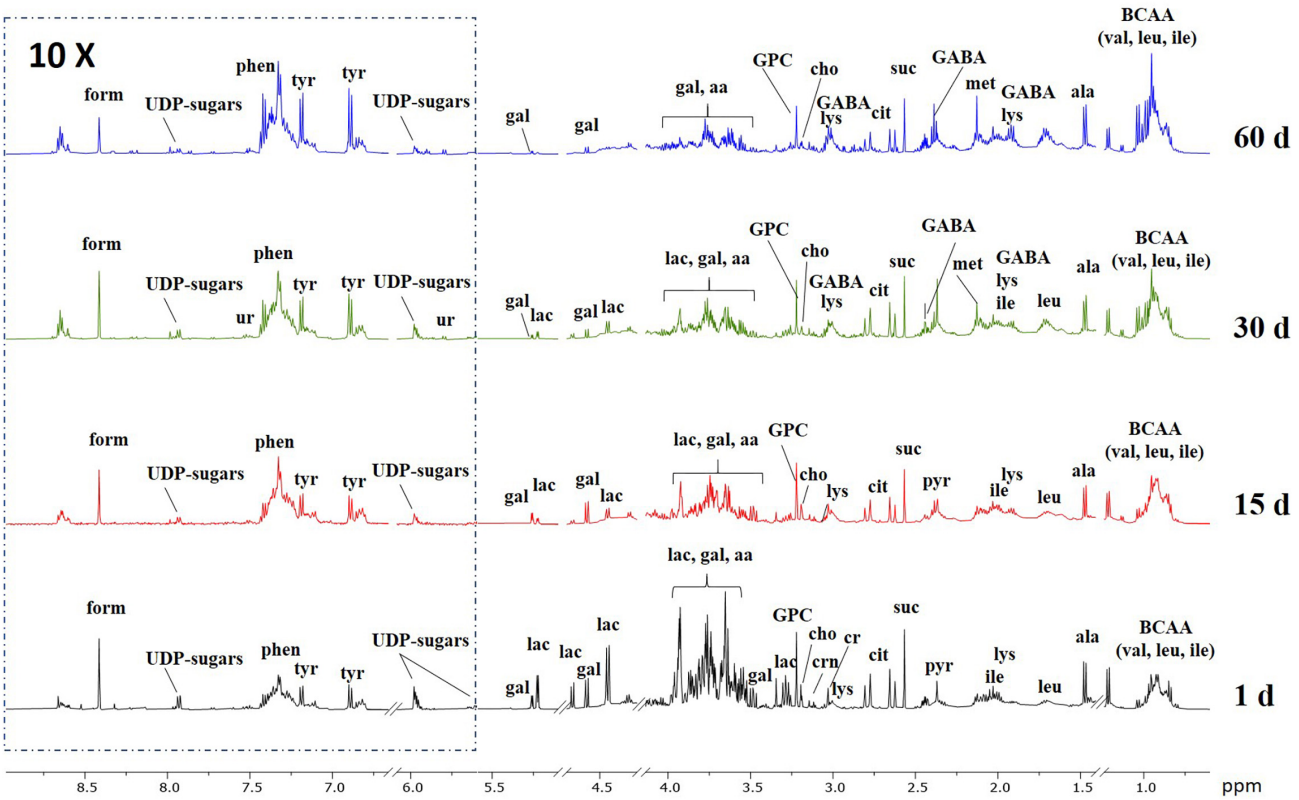

FIGURE 6 | Representative ^{1}H NMR spectra of the aqueous extracts of control Caciotta cheese at different ripening days. Abbreviations: aa, amino acids; ala, alanine; cit, citric acid; cho, choline; cr, creatine; crn, creatinine; form, formic acid; GABA, γ-aminobutyric acid; gal, galactose; GPC, glycero-phosphocholine; ile; isoleucine; lac, lactose; leu, leucine; lys, lysine; met, methionine; phen, phenylalanine; pyr, pyruvic acid; suc, succinic acid; tyr, tyrosine; UDP-sugars, uridine diphosphate-related sugars; ur, uracile; val, valine.

the lactate metabolism of the *K. lactis* strain included in the culture (Fadda et al., 2004). Since lactic acid is the most abundant organic acid in Caciotta, its content changes are reasonably the main source of the observed pH variations (**Table 2**).

A preliminary multivariate statistical analysis of the whole NMR data set was performed by PCA in order to explore the intrinsic variability. Due to the high intensity of lactic acid peaks, the corresponding bins were removed prior to analysis in order to monitor possible changes of the metabolic profiles due to the less abundant metabolites. **Figure 7** shows the PCA scores plot built with the first two principal components (PCs), PC1 and PC2 explaining 54.5 and 16.2% of the total variance, respectively. All samples in the plots were within the 95% Hotelling's T-squared ellipse, except those of C1 at 1 day of ripening, characterized by higher levels of lactose compared to the other samples. As it can be observed by the score distribution for each time point, we obtained a good reproducibility of NMR data in terms of replicates and trials. Furthermore, the score movement from the right to the left indicated a continuous metabolic change during cheese maturation. Differences in the scores distribution were visible also in terms of added cultures, in particular at the beginning of preparation. Overall, these findings suggested variations in the metabolome of cheeses in relation to both ripening time and added culture.

To focus on the metabolic changes mostly correlated with the ripening time, an OPLS model was built for each Caciotta cheese type (**Supplementary Figure 9** in Supplementary Material). All models were characterised by high statistical values, yielding a $Q^2 \geq 0.95$. Each model was further validated by permutation tests ($n = 400$), providing the Q^2 and R^2 values of the permutated models lower than the original ones (data not shown), and by CV-ANOVA ($p < 0.0001$) to ensure that there was no overfitting. The analysis of the S-line correlation coefficient plots evidenced for all cheeses a general increase of the content of free amino acids (AA) and organic acids during ripening, and a concomitant decrease of the carbohydrate levels. Among these metabolites, only a few exhibited a statistically significant correlation with cheese aging ($p(\text{cov}) \geq |0.05|$; $p(\text{cor}) \geq |0.5|$). In particular, branched chain amino acids (BCAA: valine, leucine, isoleucine) were positively correlated with time in all cheeses. Additionally, a positive correlation was observed for methionine, leucine, and 4-aminobutyric (GABA) only in CC and C3, for succinic acid only in C1 and C2, for alanine only in C3 and for acetic acid only in C2. Finally, for all cheeses, the most significant negative correlation with time was observed for lactose.

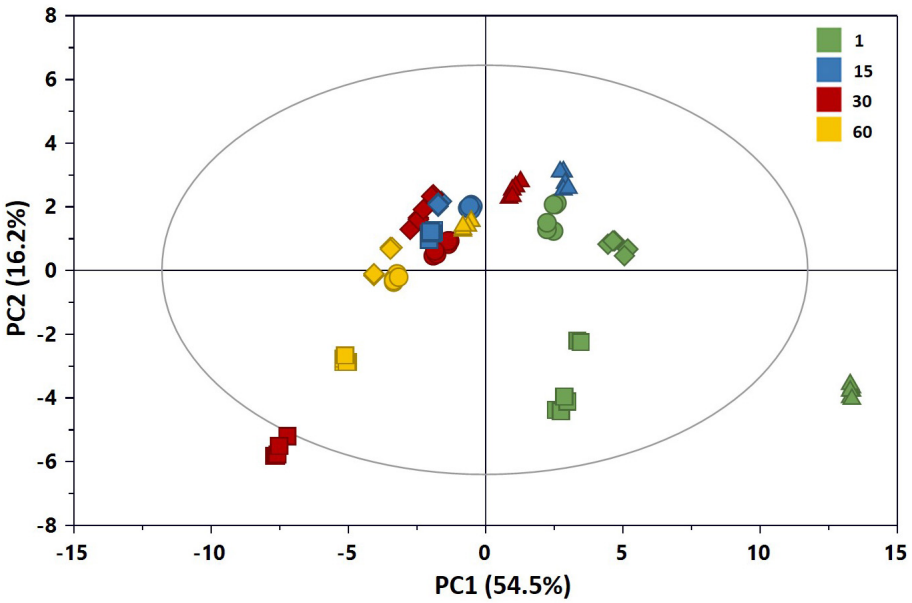

FIGURE 7 | PC1 vs. PC2 scores plot of the PCA model built with the ^{1}H NMR spectra of the aqueous extracts of Caciotta (R^2X = 0.707; Q^2 = 0.698): CC (●), C1 (▲), C2 (♦), C3 (■). The color of scores denotes the ripening days. *The reader is remanded to the color version of the figure.*

In order to examine the differences among the NMR profiles of cheeses in terms of the type of added culture, OPLS-DA models were built for pairwise comparisons among samples at 60 days of ripening. All models were found robust following permutation test (n = 400) and CV-ANOVA ($p < 0.0001$). The score plots of the models are shown in Supplementary Material (**Supplementary Figure 10**). Looking for candidate metabolites with an important impact on the group separation (**Figure 8**), we could note that C3 cheese had the highest content of BCAA followed in decreasing order by CC, C1, and C2. C3 exhibited also the highest levels of methionine and GABA, the latter being present only in the spectra of C3 and CC. As to the organic acids, CC was characterized by the highest content of citric acid, while high levels of formic, acetic and succinic acids were significantly associated with the use of autochthonous cultures. Acetic acid was more abundant in C2, while succinic acid in C1. In C1, of interest is the presence of erythritol. This polyol has been reported to occur in different type of cheeses by osmotolerant yeasts in response to salt stress (Breuer and Harms, 2006; Tomaszewska et al., 2014), and its presence may be indicative of metabolic activity by the *D. hansenii* strain added to C1, even though it was not detected either in culture or by DGGE analysis. Significantly higher levels of branched-chained fatty acids characterized C2 compared to the other cheeses.

Overall, the above-mentioned compositional changes observed in the metabolic profile of Caciotta relative to ripening time are in line with the occurrence of cheese fermentation (Mcsween, 2004). Lactose is the most abundant carbohydrate in milk. It is mostly consumed at the beginning of the fermentation as the main energy source for the growth of microorganisms. As fermentation progressed, the bacteria degraded also the milk proteins and released peptides and free AA, contributing to the general flavor and sensory quality of cheese. In particular, BCAA and methionine are some of the major precursors of cheese aroma compounds. These metabolites were particularly abundant in C3 and CC that, according to our sensorial analysis, were also the cheeses with the most intense aroma. GABA is the decarboxylation product of L-glutamate which is naturally present in caseins. It possesses several physiological activities, such as neurotransmission, antianxiety, and improves brain function and long-term memory (Wu and Sun, 2015). During fermentation, several LAB and NSLAB have been shown to produce GABA in response to stress due to acidic conditions (Dhakal et al., 2012; Valenzuela et al., 2019). This activity appears to vary widely among LAB strains and to depend on fermentation parameters such as pH and temperature. GABA has been also associated with a sour flavor note (Ochi et al., 2012b). Its high levels in C3 may contribute to the distinctive flavor profile of this cheese, besides giving also an added value compared to the other Caciotta due to its potentially beneficial health-promoting effects. Also organic acids have an important role on the sensory properties of cheese (Singh et al., 2003), besides acting as natural preservatives. They may arise from the hydrolysis of fatty acids, bacterial growth, or the addition of acidulants during cheesemaking. Succinic acid, in particular, is known to be produced by citrate-fermenting strains of LAB (Kaneuchi et al., 1988), while acetic and formic acids are derived mainly from lactose by the hetero-fermentative metabolism of NSLAB (McSweeney and Fox, 2004). The relatively lower levels of succinic and acetic acid and higher abundance of citric acid in the commercial Caciotta compared to C1, C2, and C3 suggests the presence of a higher proportion of non-citrate-fermenting strains in the control cheese.

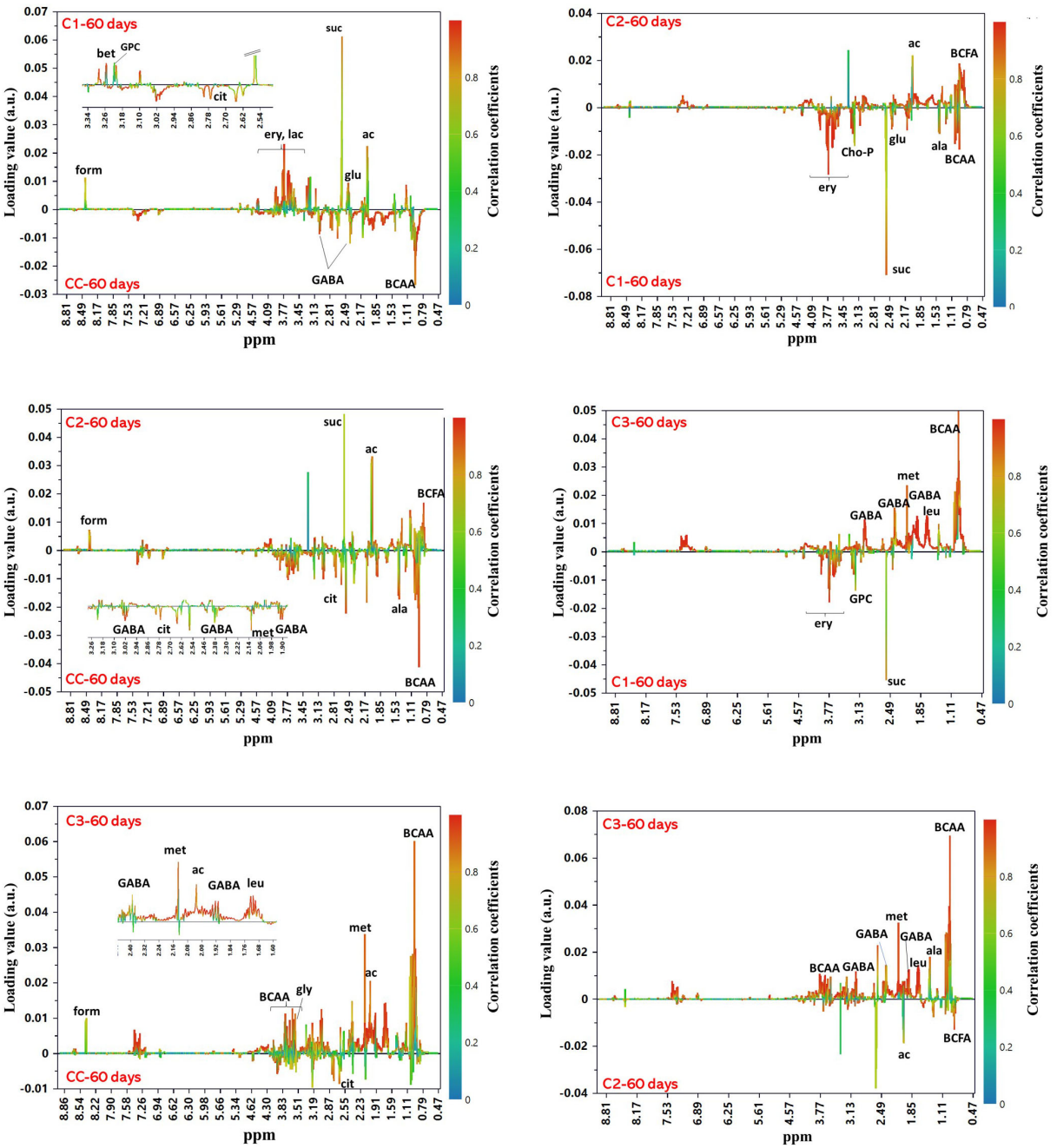

FIGURE 8 | OPLS-DA S-line correlation coefficient plots for the pairwise comparisons between groups of cheeses at 60 days of ripening. For all models $Q^2Y > 0.95$ and CV-ANOVA *p*-value < 0.0001. Only the signals from the major contributing metabolites toward the class separation are labeled (cutoff values: *p*(cov) ≥ |0.05| and *p*(cor) ≥ |0.5|). Abbreviations: ac, acetic acid; ala, alanine; BCAA, branched-chain amino acids; BCFA, branched-chain fatty acids; cho-P, phospho-choline; cit, citric acid; ery, erythritol; form, formic acid; GABA, γ-aminobutyric acid; gly, glycine; glu, glutamate; GPC, glycero-phosphocholine; lac, lactose; leu, leucine; met, methionine; suc, succinic acid.

CONCLUSION

Cheese has been demonstrated to be an optimal carrier product to deliver living probiotic bacteria, and autochthonous potential probiotic strains would be the best choice for use as adjunct cultures since they should be well-adapted to this food environment (Castro et al., 2015; Fusco et al., 2019; Bancalari et al., 2020). The data obtained in this study have indicated the applicative potential of autochthonous LAB cultures, containing putative probiotic *Lactobacillus* and *Kluyveromyces* strains, for the production of ovine Caciotta cheese. The use of combined inoculums with autochthonous cultures and probiotic strains

did not statistically affect the gross composition and lipid profile of experimental Caciotta with respect to the control, while improving the sensory characteristics in case of C1 and C3 cheeses. The NMR-based metabolomics approach used in our study highlighted differences in the cheese metabolome as a function of both ripening time and added autochthonous cultures. Both the *Kluyveromyces* and *Lactobacillus* probiotic strains survived manufacturing process and retained their viability till the end of ripening, the latter with a concentration much higher than 10^6 cfu/g, the amount of probiotic bacteria suggested at the time of consumption in order to exert a positive effect on human health. Further studies will aim to assess the survival of the probiotic strains in the human intestinal tract of volunteers fed with probiotic Caciotta cheese and to investigate the influence of the probiotic cheese intake on human metabolomics profile.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The panel test participants provided written informed consent to participate in this study. Sardinian Region ethic committee did not require the study to be reviewed or approved by an ethic committee.

AUTHOR CONTRIBUTIONS

AR and MP contributed to the experimental design of the study, interpretation of results, and wrote the manuscript. DP, VM, and SV carried out the experiments. FC performed NMR measurements and analyzed the data. MF carried out the DGGE experiments and supervised the study. SC contributed to the interpretation of the results and the critical revision of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.583745/full#supplementary-material

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Microbiota and Metabolite Profiling Combined With Integrative Analysis for Differentiating Cheeses of Varying Ripening Ages

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Cheese maturation and flavor development results from complex interactions between milk substrates, cheese microbiota and their metabolites. In this study, bacterial 16S rRNA-gene sequencing, untargeted metabolomics (gas chromatography-mass spectrometry) and data integration analyses were used to characterize and differentiate commercial Cheddar cheeses of varying maturity made by the same and different manufacturers. Microbiota and metabolite compositions varied between cheeses of different ages and brands, and could be used to distinguish the cheeses. Individual amino acids and carboxylic acids were positively correlated with the ripening age for some brands. Integration and Random Forest analyses revealed numerous associations between specific bacteria and metabolites including a previously undescribed positive correlation between *Thermus* and phenylalanine and a negative correlation between Streptococcus and cholesterol. Together these results suggest that multi-omics analyses has the potential to be used for better understanding the relationships between cheese microbiota and metabolites during ripening and for discovering biomarkers for validating cheese age and brand authenticity.

Keywords: cheese, 16S rRNA-based microbiota analysis, GC-MS untargeted metabolomics, cheese maturity, integrative analysis

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INTRODUCTION

In large-scale cheddar cheese manufacture, starter bacteria (normally selected strains of *Lactococcus lactis*) together with adjunct bacteria (these may or may not be added and typically comprise strains of *Lactobacillus* spp. and/or other bacteria) are inoculated into the milk (Fox et al., 2017). As these bacteria grow, they produce lactic acid and break down milk proteins (caseins) to release peptides and amino acids and also produce many diverse secondary metabolites that determine the final quality and flavor of cheese (Fox et al., 2017). Cheese ripening is a highly complex and time-dependent process that is necessary for full flavor development. Ripening involves successional changes in microbial communities and in their associated enzymatic and biochemical reactions that underpin the release of hundreds or thousands of flavorsome compounds (Blaya et al., 2018). While it has long been established that balanced ripening is pivotal for optimum quality and flavor development (Ochi et al., 2013), being able to consistently predict and control cheese

maturation processes between batches during cheesemaking remains a challenge, even for large-scale commercial operations. Following production, cheeses are graded for quality and those of lower quality are commonly diverted into processed cheese manufacture, but at a reduced price. In recent years, DNA sequencing has revealed that in addition to lactic acid bacteria (LAB), other adventitious species may also be present during ripening with their associated role in cheese ripening less well-understood. These adventitious species or taxa include *Arthrobacter* spp., *Prevotella*, *Faecalibacterium* (Quigley et al., 2012), marine-associated γ-Proteobacteria (Wolfe et al., 2014), coagulase-negative staphylococci, members of the *Enterobacteriaceae* and other unclassified genera (Yeluri Jonnala et al., 2018).

The application of high-throughput DNA sequencing and metabolomics approaches combined with new computational algorithms and data-analysis platforms (multi-omics) are now providing significant advances in understanding of the complex microbial and metabolic interactions involved in cheese ripening (Walsh et al., 2020; Afshari et al., 2018; Wolfe et al., 2014). Together, they have the potential to provide important new understanding of cheese production and maturation processes and the potential for identification and application of biomarkers that could be used to predict, optimize and control cheese ripening outcomes (Afshari et al., 2018). This is important as the global consumption of cheese is projected to increase by ∼13% between 2016 and 2025 (OECD/FAO, 2016). Consumers are increasingly demanding high-quality products with excellent sensory properties at a reasonable cost (Braghieri et al., 2014). In response, large cheese manufacturers typically map consumer preferences in different geographical and demographic markets, aiming for optimally targeted products within competitive sales environments, for example as has been explored for Cheddar cheeses of different maturities (Young et al., 2004). The provision of improved tools to discriminate between cheese of varying quality that could be incorporated into cheese manufacturing processes during cheese ripening would aid manufacturers so that their final products can increasingly and more consistently closely match the preferences of consumers.

This research has applied a multi-omics approach combining 16S rRNA-based microbiota and untargeted metabolomics [gas chromatography-mass spectrometry (GC-MS)] analyses in combination with data integration analysis to investigate the interrelationships between cheese microbiota and metabolomes in Cheddar cheeses from different manufacturers and of varying maturity (ripening age). The aims of this research were to identify key microbiota and/or metabolites that are characteristic of these cheeses and to determine interrelationships between these microbiota and metabolites.

MATERIALS AND METHODS

Sampling

Cheddar cheeses produced by three Australian commercial manufacturers were purchased from local supermarkets (these three brands of cheddar were designated in this study as A, B, and C). For each brand, cheeses of three or four different maturities were available from each manufacturer. For brand A, four types of cheese were purchased: "mild," "tasty," "extra tasty," and "vintage." However, no specific ripening times were stated on the packs. For Brand B, cheese was labeled as "tasty" (ripened up to 12 months), "extra-tasty (ripened for up to 18 months)" or "epicure" (ripened for up to 32 months). For brand C, each cheese was labeled as "sharp" (ripened for up to 12 months), "extra-sharp" (ripened for up to 20 months) or "special reserve" (ripened for up to 32 months). For simplicity, in this study we have labeled cheeses from all three brands using the same terminology (in increasing order from minimum to maximum ripening level). These definitions are "mild" (up to 6 months), "tasty" (up to 12 months), 'extra-tasty' (up to 18 months) and 'vintage' (up to 32 months). We have also assumed that the ripening times for the four brand A cheeses are approximately similar to those of the corresponding cheeses for brands B and C. For each brand and level of ripening, four 250 g (approximate) commercially packaged shrink-wrapped blocks were purchased and sampled aseptically. Each individual sample was divided into two sub-samples; one of these was frozen at −80 °C until subsequent DNA-sequencing analysis, while the second was immediately homogenized using a mortar and pestle with liquid nitrogen and then freeze-dried for subsequent GC-MS metabolomics analysis.

16S rRNA-Based Cheese Microbiota Analysis

Total DNA was extracted from 200 mg of each cheese sample using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) following the manufacturer's instructions. DNA purity and concentration were determined and PCR amplification and DNA sequencing of bacterial 16S rRNA genes were performed as described previously (Afshari et al., 2020). Briefly, the V4 region of DNA was amplified using primers 515F and 806R. PCR conditions consisted of 95°C for 3 min, followed by 25 cycles of: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. 16S rRNA amplicons were purified and indexed using the Nextera XT DNA library prep kit as according to the 16S Metagenomic Sequencing Library Preparation instructions (Illumina, San Diego, CA, United States). Indexed PCR amplicons were pooled in equal concentrations and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, United States).

Raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using GHAP v2.1 (Greenfield Hybrid Amplicon Pipeline, developed by Paul Greenfield at CSIRO, Canberra, ACT, Australia) as described previously (Afshari et al., 2020). The relative abundance of each taxon in each sample was determined using the vegan package Rv.3.4.3. Beta diversity was calculated based on a Bray-Curtis dissimilarity matrix using Primer v7 (Primer-E, Plymouth, United Kingdom). A non-metric-multi-dimensional scaling (nMDS) plot was generated from the resulting distance matrix. Permutational multivariate analysis of variance (PERMENOVA) with 999 permutations was used to test the significant differences in phylogenetic diversity between cheeses of different ages within and between each

manufacturer based on a Bray-Curtis matrix. Since the number of unique permutations was less than 50, the marginal *p*-value which was significant in PERMANOVA was further tested by Monte Carlo analysis.

Gas Chromatography-Mass Spectrometry Metabolomics

Freeze-dried cheese (60 mg) was extracted as described by Afshari et al. (2020). Briefly, 60 mg of cheese was extracted in 500 μL of $MeOH/H_2O/CHCl_3$ (2.5:1:1, *v*:*v*:*v*). Internal standards (100 μL of $^{13}C_6$-sorbitol/$^{13}C_5{}^{15}N$-valine in water, 0.2 mg mL^{-1}) were then added to this mixture. The extract was homogenized using a MP homogeniser (FastPrep)® for 1 min at 4.5 m/s, then incubated at 37°C for 15 min in a thermomixer at 850 rpm, centrifuged at 15700 *g* for 15 min and the supernatant was then decanted into a new tube. The remaining pellet was mixed with 500 μL of $MeOH/H_2O/CHCl_3$, centrifuged at 13000 rpm for 15 min and the resulting supernatant was then combined with the previous extract. Following extraction, 40 μL aliquots were transferred into glass vial inserts and dried *in vacuo* for subsequent trimethylsilyl (TMS) polar metabolite derivatization. Online, chemical derivatization and acquisition were performed as previously described by Afshari et al. (2020). This method specifically enables the extraction of non-volatile compounds.

Resulting GC-MS data were analyzed using the Agilent Mass Hunter Workstation software, Quantitative analysis, Version B.07.01/Build 7.1.524.0 (Agilent Technology, Inc). Mass spectra of eluted compounds were identified using the commercial mass spectra library NIST 08,[1] the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany,[2] an *in-house* mass spectral library at RMIT University and also by comparing their retention time to authentic standards. Relative response ratios (area of analyte divided by area of the internal $^{13}C_6$-sorbitol standard and sample dry weight) were calculated for each analyzed metabolite and used for multivariate analysis. Principal component analysis (PCA) was used to analyze the GC-MS data of cheeses between and within each manufacturer. Principal component analysis and PCA biplots were performed in SIMCA 15.0.1 (Umetrics AB, Umea, Sweden).

Data Integration Analysis

Multifactorial analyses (MFA) were performed in R using the FactoMineR package[3] to assess variation with respect to cheese maturity based on the microbiota and metabolite compositions of cheeses and to find canonical correlation between metabolite and microbiota profiles. Multifactorial analysis is a generalization of PCA in which sample similarity is determined by multiple different sets of variables (Escofier and Pages, 1994), in this case, microbiota and metabolite profiles.

Random forest (RF) is a non-parametric machine learning technique, where multiple regression or classification trees are constructed using RF subsets of the data (Breiman, 2001). While a linear regression would fit only a linear relationship between the predictors and the outcome, RFs allow for any type of relationship, including complex interactions. Random forest analysis was used to predict associations between taxa and metabolites (regression model) for each manufacturer. Over 500 trees were constructed using the RF package and a 10-fold cross-validation was used to evaluate these RFs. Based on the mean decrease in Gini-coefficient, the most 'important' parameters were selected (Louppe et al., 2013). The variable with the highest mean decrease in Gini index is considered the most important variable in the optimized model. Random forest analysis does not provide a regression coefficient; therefore, partial plots were used to show the adjusted relationship between the taxa and metabolites as other metabolites are held constant at their mean observed value (Friedman, 2001). The PartialPlot function in R was used to generate partial dependence plots for the five most important variables.

RESULTS

Bacterial Community Structure in Cheddar Cheeses of Different Brands and Age

Sequencing of PCR-amplified 16S rRNA gene amplicons was applied to investigate variation in the bacterial communities in cheddar cheeses of different brands and ages. Across all samples, a total of 115 operational taxonomic units (OTU) were identified at 97% identity. At the phylum level, Firmicutes comprised the highest proportion of detected OTUs (84% of all OTUs) and more than 99% of all sequence reads (**Figure 1**). Twelve genera dominated across the cheeses. For brand A, *Lactococcus* and then *Lactobacillus* were most dominant (**Figure 1A**). For brand B, *Lactococcus* and *Streptococcus* were most abundant in tasty (up to 12-month ripened) and extra-tasty (up to 24-month ripened) cheeses, while *Lactococcus* (only) was dominant in vintage cheeses (up to 32-month ripened) (**Figure 1B**). For brand C, the bacterial community was dominated by *Lactococcus* and *Lactobacillus* in varying proportions in tasty and extra tasty cheeses with *Lactobacillus* present in higher proportions (over 90%) in vintage cheeses (**Figure 1C**). Within these cheeses *Streptococcus* (presumably mostly *Streptococcus thermophilus*) and *Macrococcus* (0.1% of total reads) were also present at low abundance, except for brand B where *Streptococcus* constituted up to more than 50% of the communities in the tasty and extra tasty cheese (**Figure 1B**). *Thermus* was present at low abundance in all brand A cheeses with a maximum of 2.5% of total reads in Brand A vintage cheeses (**Figure 1A**).

The bacterial community composition in the cheeses differed more between brands than within brands (**Figure 2A**), with permutational multivariate analysis of variance confirming that this variation between brands was statistically significant ($P < 0.001$). Within each brand, the bacterial communities within cheeses of different maturities all varied significantly from each other ($P < 0.05$; **Figures 2B–D**) but with the exception of the bacterial communities in tasty and extra tasty cheeses from

[1] http://www.nist.gov

[2] http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html

[3] http://factominer.free.fr/

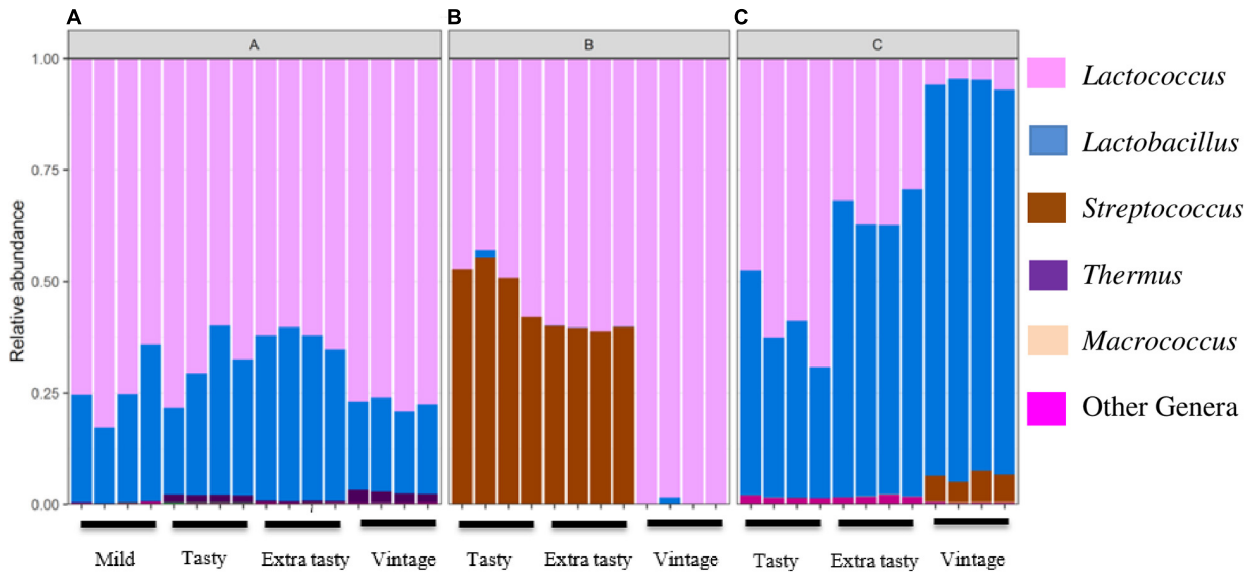

FIGURE 1 | Variation in the relative abundance of bacterial genera in cheeses between brands and between cheeses of different maturity. Brands **(A–C)** are as indicated. Only genera detected at >0.1% relative abundance are shown.

brand B in which there was no significant variation between these communities ($P = 0.12$; **Figure 2C**).

Variation in Metabolome Profiles in Cheddar Cheeses of Different Brands and Ripening Age

Gas chromatography-mass spectrometry untargeted metabolomics profiling revealed a total of 46 primary metabolites across all cheese brands and maturities. These metabolites comprised of 22 amino acids and amines, 11 carboxylic acids, seven free fatty acids and steroids and six-sugar and -sugar derivatives (**Supplementary Table 1**). Principal component analysis showed that the variance between cheese samples based on their metabolite profiles (**Figure 3**). The first two principal components accounted for over 59% of the total variance (32.5 and 26.8% for PC1 and PC2, respectively) in the metabolome data. PCA showed that the metabolite profiles of brand C cheeses clustered separately from those of brands A and B (**Figure 3A**). Within brands, metabolite profiles also varied between cheeses of different maturities. Within brand A, metabolite profiles of mild cheeses (shown as triangles) were distinct from those of the more mature cheeses. Conversely, within brand B and C cheeses, metabolite profiles of vintage cheeses (circles) were distinct from those in tasty and extra-tasty cheeses. For all three brands, PC1 was the component which explained the largest proportion of the variance and best characterized the level of cheese maturity. To further investigate the relationships between individual metabolites and cheese maturity, PCA biplots were generated (**Figures 3B–D**).

The PCA biplot of brand A cheeses (**Figure 3B**) showed that mild cheeses contained a higher relative abundance of glycerol, lactose and mannose, whereas the mature cheeses (extra tasty and vintage) had higher relative abundances of numerous amino acids and carboxylic (such as citrate, malate, oxalate, succinate, and hydroxy-glutaric acid), free fatty acids (such as pentadecanoic acid, myristic acid, lauric acid, and palmitic acid) and also one amine (piperidine). This might be expected as the number of such metabolites would increase as ripening progresses. In contrast and perhaps surprisingly, in brand B cheeses (**Figure 3C**), these metabolites were present in higher relative abundance in the tasty cheeses when compared to the more mature extra tasty and vintage cheeses. More specifically, a total of 16 amino acids,and four carboxylic acids (citrate, oxalate, orotic, and uric acids) were more strongly associated with the brand B tasty cheeses. In contrast, only seven metabolites (GABA, glutamine, succinic acid, glycerate, octadecanoic acid, inositol, and galactose) were found to be present in higher abundance in the extra tasty cheeses while vintage cheeses were highly associated with just two metabolites, urea and lactose (**Figure 3C**). For brand C (**Figure 3D**), the relative abundance of 19 amino acids and one amine (piperidine) were strongly associated with the vintage cheeses, whereas (in contrast) leucine was more strongly associated with the extra tasty cheeses and glutamine with both tasty and extra tasty cheeses (**Figure 3D**). The relative abundance of carboxylic acids and fatty acids were also higher in the brand C vintage cheeses with the exceptions of succinic acid which was more strongly associated with the extra tasty cheese and of stearic acid which was present in similar proportions in all brand C cheeses.

Overall, GC-MS untargeted metabolomics profiling showed that there was an increase in the relative abundance of amino acids and amines, carboxylic acids and free fatty acids (especially malic acid, hydroxy-glutaric acid, citric acid, lauric acid, myristic acid pentadecanoic acid and palmitic acid) for brands A and C which correlated positively with increasing cheese age (PC1). For

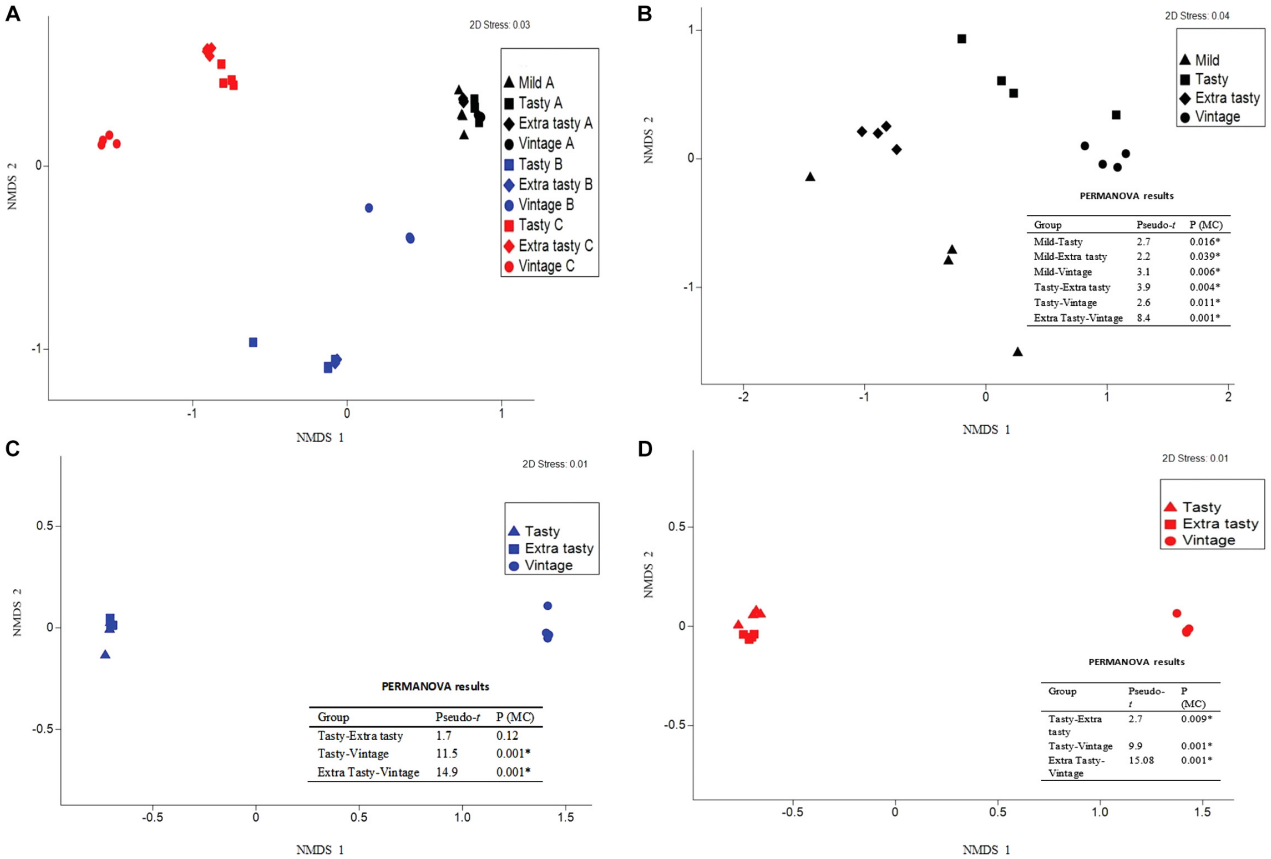

FIGURE 2 | Variation in bacterial community composition in cheese between brands and between cheeses of different maturity. Bray-Curtis non-metric multi-dimensional scaling (nMDS) plots and PERMANOVA results are shown comparing **(A)** variation between cheeses from different manufacturers and **(B–D)** variation between cheeses of different maturity for each manufacturer: brand A **(B)**, brand B **(C)**, and brand C **(D)**. Different colors and shapes show different cheese manufactures and different ages, as indicated. PERMANOVA results are shown; Pseudo-*t* = the Pseudo-*t* statistic, whereby higher values indicate greater differences between the community structure of cheeses of the two groups compared; P (MC) = Monte Carlo *P-value,* with significant values ($P < 0.05$) indicated by an asterisk.

brand B this increase unexpectedly ceased after the cheese had aged beyond 12 months (i.e., tasty).

Determining Relationships Between Cheese Microbial Composition, Metabolome Profiles, and Cheese Maturity

Multifactorial analysis was used to determine the similarity/dissimilarity between cheeses of different ages within each brand based on the combined microbiota and metabolites profiles (integrated multi-omics datasets). Multifactorial analysis was also used to investigate relationships between cheese microbiota, cheese metabolites and cheese age within each brand and to identify correlations between individual bacterial taxa and metabolites. The scatter plots visualized the cheeses into a two-dimension space using the first two dimensions (Dims) which captured 43.2, 60, and 88.3% of the total variability among cheeses within brand A, B, and C, respectively (**Figures 4A–C**). The scatter plots showed that cheeses of different ages made by the same manufacturer could be separated based on the combined microbiota (bacterial taxa) and metabolite profiles (**Figures 4A–C**). **Figures 4D–F** identifies correlations between variables (herein: microbiota, metabolites, and age) and dimensions of MFA scatter plots for each brand. For brand A, the coordinate of metabolites on Dim 1 is higher than for microbiota, indicating the greater contribution of metabolites compared to the microbiota to the separation of mild cheeses from the other more matured cheeses (**Figure 4D**). For brand B, the contribution of microbiota and metabolites to Dim 1 is almost identical. However, on Dim 2 for which tasty and extra tasty cheeses were separated from each other, the contribution of microbiota was higher (**Figure 4E**). For brand C cheeses, both microbiota and metabolites had identical contributions to Dim 1 and very similar contributions for Dim 2 (**Figure 4F**). This could reflect the higher correlation that was obtained between these two datasets (microbiota and metabolites) (RV = 0.73) for cheeses of brand C when compared to other two brands (for brand A, RV = 0.33; for brand B, RV = 0.57).

Numerous significant correlations were observed between specific bacterial taxa and metabolites for brands B and C (**Figures 5B,C**). For brand A cheeses, however, this correlation

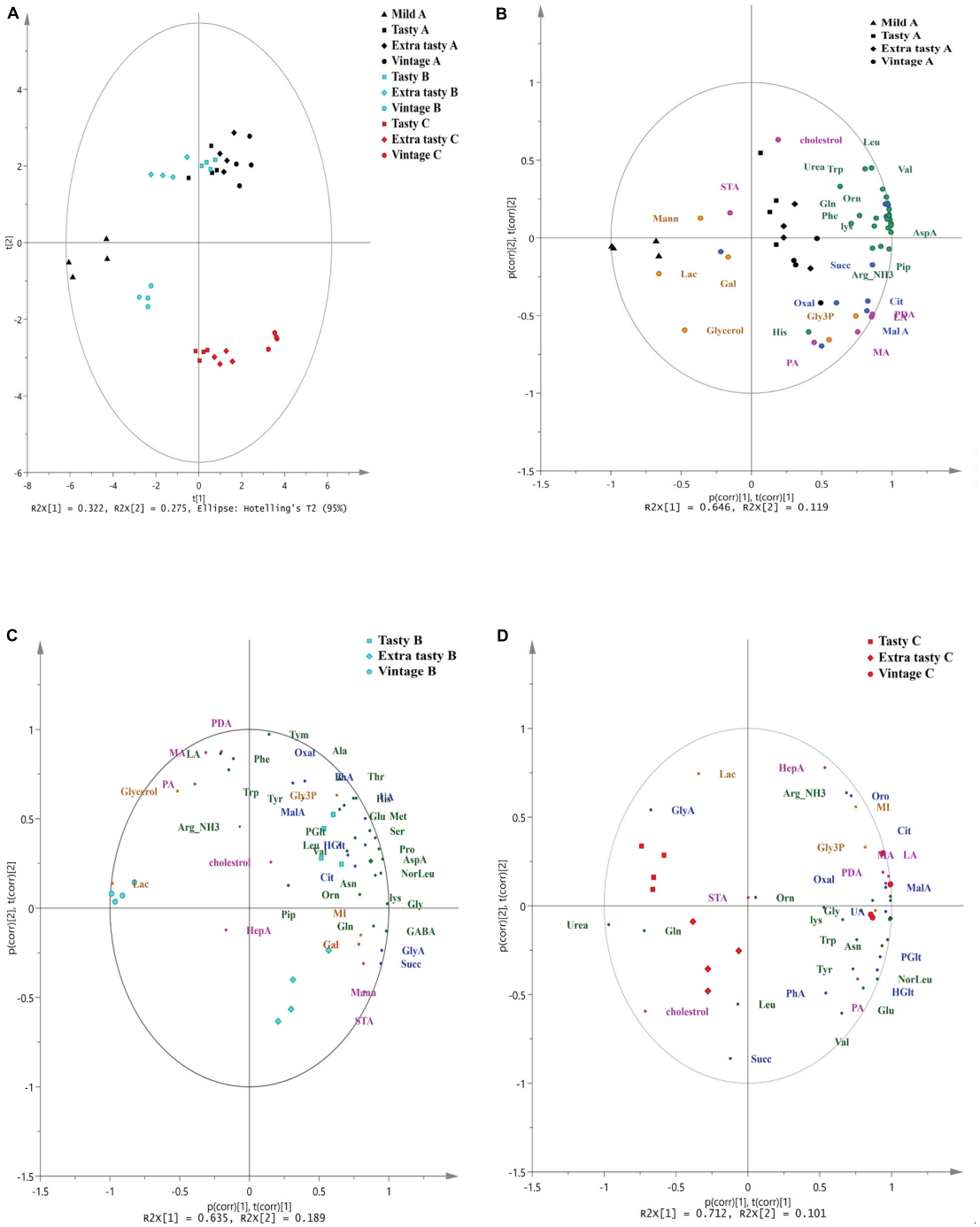

FIGURE 3 | Principal Component Analysis (PCA) of untargeted GC/MS metabolomics of cheeses made by different manufacturers. **(A)** PCA of untargeted GC/MS metabolomics of cheeses made by different manufacturers. PC1 and PC2 account for 32.2% and 27.5% of the variance, respectively. **(B–D)** The biplot superimposed on the scores and loadings of PCA analysis based on a correlation scaling method for cheeses for brand A **(B)**, brand B **(C)**, and brand C **(D)** Brands and maturity of cheeses are as indicated. p(corr), t(corr) is a combined vector, p(corr) represents loading p scaled as correlation coefficient between X and t; t(corr) represents score t scaled as correlation coefficient resulting in all points falling inside the circle with radius 1. Different colors represent different brands and different classes of metabolites: black; brand A, cyan; brand B, red; brand C, green; amino acids and amines, blue; carboxylic acids, pink; fatty acids and sterols, orange; sugar and sugar phosphates. Orn, ornithine; Tyr, tyrosine; GABA, gamma amino butyric acid; Lys, lysine; Gly, glycine; Val, valine; Ser: serine; Leu, leucine; Noreleu, noreleucine; Thr, threonine; Pro, proline; Pip, piperedine; Asn, asparagine; AspA, aspartic acid; Glu, glutamic acid; Met, methionine; Arg, arginine; PDA, pentadecanoic acid; HepA, heptadecanoic acid; LA, lauric acid (dodecanoic acid); PA, palmitic acid (hexadecanoic acid); STA, stearic acid (octadecanoic acid); MA, myristic acid (tetradecanoic acid); Oxal, oxalic acid; Succ, succinic acid; GlyA, glyceric acid; Glt, glutaric acid; MalA, malonic acid; HGlt, hydroxy-glutaric acid; Citric, citric acid; GalA, galactonic acid, Pglu, pyroglutamic acid; Oro, orotic acid; UA, uric acid; PhA, phosphoric acid; Mann, mannose; Gal, galactose; MI, inositol myo; Lac, lactose; Gly3p, glycerol-3-phosphate.

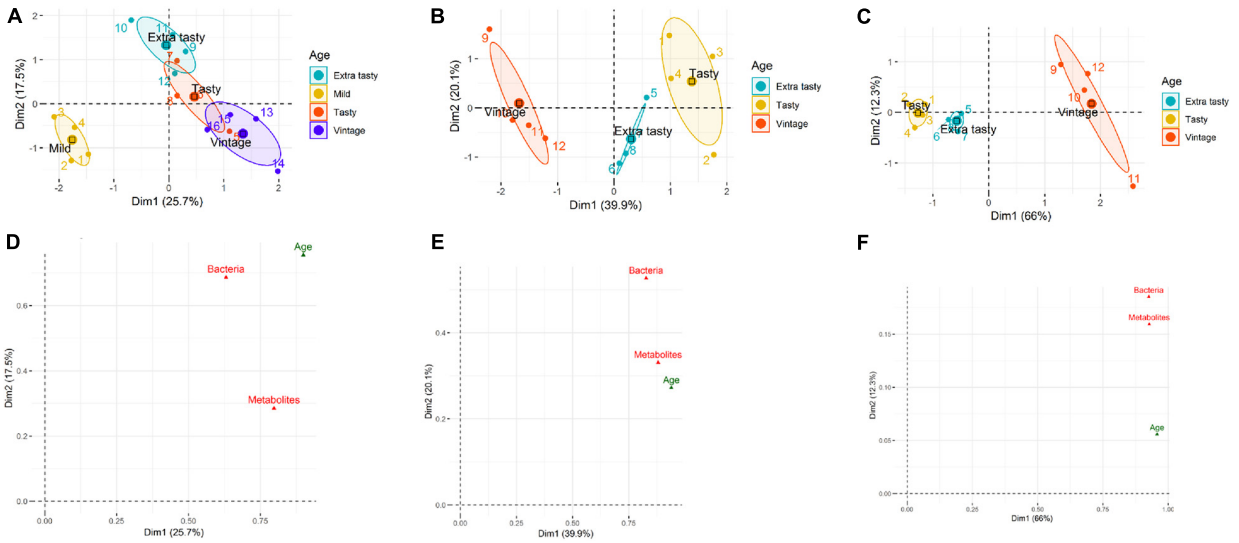

FIGURE 4 | Multifactorial analysis (MFA) of cheese microbiota and cheese metabolite profiles for cheeses of different maturities from different manufacturers (A, B, and C). **(A–C)** MFA scatter plots for cheeses from brands A, B, and C, respectively. Ellipses representing the barycentre of the sample groups with 95% confidence. Maturity (age) of chesses is as indicated. **(D–F)** MFA group representations to illustrate the relationships between variables (bacterial genus composition, metabolite profiles, maturity of cheeses) and Dim 1and Dim 2 for brands A, B, and C, respectively.

was less pronounced (RV coefficient = 0.33) (**Figure 5A**). The only notable exception was the positive correlation between *Thermus* and relative abundance of phenylalanine (**Figure 5A**). The relationship between age and metabolites for brand A (RV coefficient = 0.65) was also stronger than the relationship between age and microbiota profiles (RV coefficient = 0.57). Similarly, for brands B and C cheeses, the relationships between age and metabolites (RV coefficients = 0.81 and 0.77, respectively) were stronger than the relationships between age and microbiota (RV = 0.50 and 0.58, respectively). The MFA correlation circle for brand B and specifically for brand C revealed several positive microbiota-metabolite relationships. These included, for brand B, relationships between both *Lactococcus* and *Acinetobacter* (present at low abundance) and medium chain fatty acids including lauric acid, myristic acid, pentadecanoic acid and palmitic acid; and between *Streptococcus* and the relative abundance of amino acids (**Figure 5B**). For brand C, relationships were found between both *Streptococcus* and *Pediococcus* (but not *Lactococcus*) and increased relative abundances of amino acids which have sensory properties, including pyroglutamate, tyrosine, and proline (**Figure 5C**). In addition, *Streptococcus* was found to be associated with decreased abundance of both cholesterol and urea in brand C.

Random Forest Analysis to Predict Associations Between Cheese Metabolites and Bacterial Genera

Random forest regression analysis was used to predict the association between the dominant bacterial genera and the cheese metabolites within each brand. For brand A cheeses, the optimized model showed that the relative abundance of *Thermus* was highly positively associated with phenylalanine (pseudo R^2 = 0.85; **Table 1** and **Supplementary Figure 1A**), supporting the results of MFA. The RF model did not identify any significant association between the microbiota and other metabolites for brand A. For brand B, the optimized model showed that the relative abundance of *Streptococcus* was negatively associated with the levels of urea and lactose (pseudo R^2 = 0.93; **Table 1** and **Supplementary Figure 1B**) and conversely that *Lactococcus* (presumably *Lc. lactis* added as the starter culture) was positively associated with heptadecanoic acid (**Table 1** and **Supplementary Figure 1C**). For brand C (**Table 1** and **Supplementary Figures 1D–F**), *Streptococcus* was associated with decreased cholesterol and urea, whilst *Lactococcus* was associated with decreased pyroglutamic acid and piperidine levels, and increased urea (pseudo R^2 = 0.96). *Lactobacillus* in brand C cheeses was associated with decreased ornithine (not present in casein) and glutamine levels and increased tyrosine.

DISCUSSION

This research demonstrates that similar commercial cheddar cheeses of different maturity levels (ripening ages) made by independent manufacturers can be differentiated by the application of multi-omics-microbiota and metabolomics analyses combined with data integration analysis. Notably, GC-MS untargeted metabolomics identified metabolites that were specific to cheeses of particular ages (maturity) and have the potential for use as markers for monitoring cheese ripening progression, validating cheese age, improving the quality and efficiency of cheese ripening outcomes. The identification of metabolites as diagnostic biomarkers further offers the potential for their inclusion in the cheese grading process. Our research

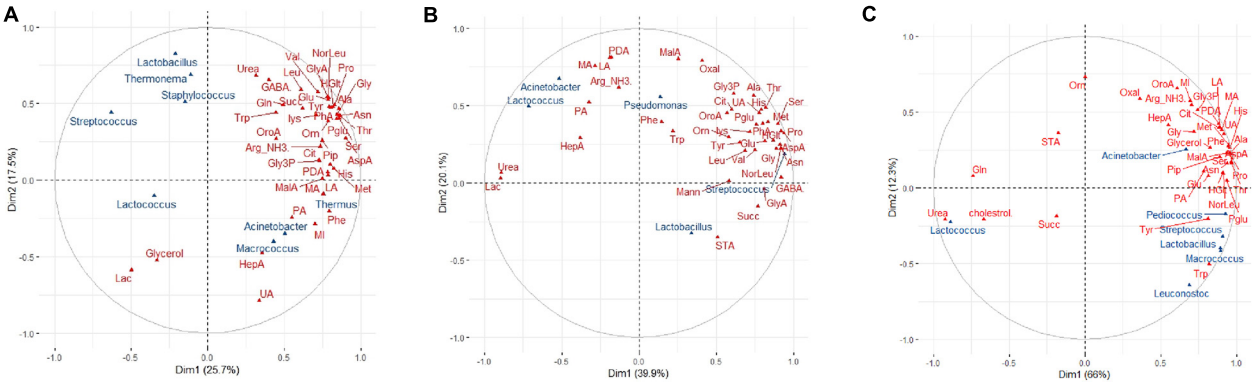

FIGURE 5 | Multifactorial analysis (MFA) showing correlations between bacterial genera and metabolites in cheese. The Correlation circle depicts correlations in normalized abundance between cheese, bacterial genera (blue) and cheese metabolites (red) along the MFA axes for **(A)** brand A, **(B)** brand B, and **(C)** brand C cheeses. Those metabolites and genera which are depicted together in the same direction along an axis indicate positive correlations; those which are depicted together in the opposite direction indicate negative correlations; metabolites and genera depicted in different directions indicate no correlations. The strength of the correlation is shown by the increasing distance from the center of the plot. To improve the readability of the plots, only the greatest correlations for metabolites in each dimension are shown.

TABLE 1 | Adjusted associations between the bacterial genera and selected metabolites in the Random forest model to predict associations between genera and metabolites.

Brand	Genus	Selected metabolites	Adjusted association	Pseudo-R^2
A	*Thermus*	Phe	Positive	0.85
B	*Streptococcus*	Lac	Negative	0.93
		Urea	Negative	
	Lactococcus	HepA	Positive	0.98
C	*Streptococcus*	Urea	Negative	0.98
		Cholesterol	Negative	
		Pglu	Negative	
	Lactococcus	Urea	Positive	0.96
		Pip	Negative	
		Orn	Negative	0.83
	Lactobacillus	Gln	Negative	
		Tyr	Positive	

Partial plots were used to determine the direction of the associations between the genera and the metabolites (plots are not shown). See ***Figure 3*** *for metabolite abbreviations.*

has demonstrated that strong and significant associations exist between cheese microbiota and metabolites. To our knowledge, some of these associations have not been previously described, such as the positive association between the levels of phenylalanine and the presence of *Thermus,* while others were consistent with the known biochemical characteristics of bacterial species, such as the association between urease-positive *S. thermophilus* and decreased levels of urea (Afshari et al., 2020).

Lactococcus was the dominant genus in cheeses from brands A and B, presumably reflecting the growth of *Lc. lactis* derived from the cheese starter culture (16S rRNA gene sequence data from amplicons were most closely related to sequences from *Lc. lactis*; data not shown), while *Lactobacillus* was present at ∼16–38% in brand A cheeses and <1% in brand B cheeses. Brand C cheeses were dominated by *Lactobacillus* spp. and *Lactococcus* spp., with the relative abundance of *Lactobacillus* increasing in cheeses of increasing maturity (**Figure 2C**). This is consistent with the standard cheese ripening model, which predicts that numbers of starter lactococci, which are very high at the start of ripening then decrease, accompanied by growth of *Lactobacillus* spp. derived from added flavor adjunct cultures or other adventitious microflora, or both (Fitzsimons et al., 2001; Stefanovic et al., 2018). In contrast to brands A and C, cheeses from brand B contained very low proportions of *Lactobacillus*, potentially suggesting that *Lactobacillus* were not used as adjunct cultures in the manufacture of brand B cheeses.

Streptococcus was present at high relative abundances (up to ∼50%) in both tasty and extra tasty cheeses from brand B, but not in the vintage cheddar. This might be explained if *S. thermophilus* was present in addition to *Lc. lactis* in the starter culture. While not traditionally present in mesophilic (or "O"-type) DVS cheese cultures (Blaya et al., 2018; Høier et al., 2010), some more recent "O"-type cultures do contain added *S. thermophilus* in order to enhance acid production at the cheddar cook stage (see for example Christian Hansen DVS cataloge, 2014, pp. 11–13[4]). Alternatively, though less likely given such high levels in the cheese itself, *S. thermophilus* may have been present as biofilms in the downstream cooling side of the pasteurizer, some of which may have sloughed off into the cheese milk during vat filling (Bouman et al., 1982). Either way, *S. thermophilus* inoculated into the cheese milk during vat filling would increase in numbers even after the cook stage, but would then die off quickly once ripening commenced. In contrast, communities in vintage cheeses from brand B were dominated by *Lactococcus* (**Figure 2B**); whether a different starter culture that did not contain *S. thermophilus* was used to produce the vintage cheeses (brand B) is not known. This comparison of cheeses from different brands and of differing

[4]Available at https://hjemmeriet.com/da/ChrHansen/Brochures/GlobalCheeseCultureCatalogue_EN-2014.pdf.

maturities highlights that both intended or unintended variation in cheese microbiota composition during dairy manufacturing is a variable that needs to be considered and that is likely to influence cheese quality and sensory characteristics, even within cheeses of the same type.

In brand A cheeses, *Thermus* spp. were present at a relative abundance of up to 2.5% but were absent from other brands and their presence may have originated from hot water sources in the factory (Quigley et al., 2016). Based on microbiome DNA sequencing studies, Quigley et al. (2016) suggested that *Thermus* may be the causative agent of pink discoloration in cheese, a problem that has affected the dairy industry over many years (Daly et al., 2012) and yet remains without a definitive explanation. In our study, the presence of this genus was associated with high levels of phenylalanine (**Table 1**), although none of the cheeses in our study exhibited a pink discoloration defect. This is consistent with the observations of Quigley et al. (2016) who found no association between the presence of free amino acids and the development of pink discoloration. Rather, these authors have suggested that the defect may be due to a microbially produced carotenoid when *Thermus* is present at a higher relative abundance (up to 6% of the total 16S rRNA reads).

Cheese is known to contain many thousands of metabolites present in varying abundance (Afshari et al., 2020). Although the impact of metabolites found in low relative abundances remains largely unexplored, there is evidence that some may have significant impacts on cheese flavor and quality. For example, esters present in very low amounts in cheese can be detected in taste testing (Holland et al., 2005). GC-MS untargeted profiling revealed differences in metabolite profiles between the three different brands of cheddar as well as between cheeses of different maturity and additionally, identified cheese metabolites that were correlated with aging. These results are in agreement with other research that suggests metabolome fingerprinting may be a useful indicator of cheese maturity (Ochi et al., 2013; Gan et al., 2016). As cheddar cheeses mature, proteolysis and lipolysis results in release of peptides, amino acids, and free fatty acids, all contributing to flavor (McSweeney, 2004). Our research has shown progressive increases in the production of seven carboxylic acids together with 16 amino acids and one heterocyclic amine (piperidine) during cheese ripening in cheeses from brand A and C cheeses. This is consistent with previous studies that reported an increase in amino acids, especially lysine, proline, glycine and pyroglutamic acid with ripening time (Ardo et al., 2002; Zheng et al., 2018). The association of threonine with aged cheddars (30 months ripening) and of isoleucine and leucine with relatively younger (24 months ripening) hard cheeses has also been reported (Consonni and Cagliani, 2008). Mucchetti et al. (2000) also reported a linear correlation between the concentration of pyroglutamic acid and ripening age with the age of extensively ripened Italian Grana Padano cheese. Furthermore, in contrast to brands A and C, the less mature tasty cheeses from brand B were characterized by a higher relative abundance of amino acids than in the more mature extra tasty and vintage cheeses (**Figure 3C**). Such differences in amino acid abundance between brands could be due to differences in processing methods such as salt content, geography and the microbial compositions of different cheeses made by different manufacturers (Yvon and Rijnen, 2001; Masotti et al., 2010; Moser et al., 2018).

Cheese is a complex ecosystem where many metabolites can be re-metabolized or catabolized by multiple microbial species (Irlinger and Mounier, 2009). In addition, enzymes released into the curd even after cell death may continue to catalyze reactions. This means that relationships between cheese microbiota and metabolites are unlikely to be linear. A model which allows many different types of relationships, including complex interactions, is expected to be more accurate and versatile in predicting associations between microbiota and metabolites. Hence, in this study, the RF regression model (Breiman, 2001) was used to predict such associations between cheese microbiota and cheese metabolites. Similarly, MFA integrative analysis showed that the overall (global) associations between microbiota and metabolite composition in cheeses varied between brands. The higher associations seen between bacterial taxa and metabolites for brand C (RV = 0.73) as revealed by MFA analysis, may be due to its different microbial community structure and/or varying succession, since different microbes will possess different enzymatic capacities affecting metabolite production. It is to be noted that DNA sequencing of PCR amplified 16S rRNA genes (as has been performed in this study) will detect both live and dead cells (see Emerson et al., 2017) and not specifically identify those cells that are active. There is a complex interplay between growth of specific genera and subsequent death and lysis of cells, and the subsequent production of metabolites in cheese, since many enzymes such as peptidases released by bacterial autolysis remain active after cell death. Indeed, some enzymes appear to be more stable in the cheese environment than they are in intact stressed cells (Crow et al., 1995). This makes validation of biomarkers using more diverse and larger sample sets extremely important. Furthermore, the RF regression modeling for brand C cheeses showed that both cholesterol and urea were negatively associated with the abundance of *Streptococcus* (*S. thermophilus*). The ability of several strains of LAB, including *Streptococcus thermophilus* (and also *Lactobacillus*) species to reduce cholesterol levels in vitro and in cheese matrix has previously been demonstrated (Albano et al., 2018; Belviso et al., 2009; Ziarno et al., 2007). Understanding of this association between certain LAB and reduced cholesterol levels offers opportunities to improve human health in relation to cheese consumption.

In terms of other functional relationships between microbiota and metabolites, the presence and abundance of *Lactococcus* was associated with decreased levels of amino acids in brand C cheeses (as shown by MFA and RF) and in particular, with pyroglutamic acid and piperidine (a heterocyclic amine) both of which have previously been reported in mature cheeses (Golovnya et al., 1969). It has long been established that *Lactococcus lactis* contributes to cheese flavor by metabolizing amino acids and converting them into flavorsome compounds, for example, by deamination to α-ketoacids and subsequent conversion to amino acids to aldehydes, esters, alcohols and carboxylic acids (Kieronczyk et al., 2003). However, the biochemistry which underlies the negative association between piperidine and *Lactococcus* warrants further investigation. The contribution of

this amine to cheese flavor is unknown. The RF optimized model also showed that the relative abundance of *Lactobacillus* was inversely correlated with ornithine and glutamine in brand C cheeses. Ornithine, produced by the decarboxylation activity of LAB through the arginine deiminase (ADI) pathway, has been shown to be physiologically active (Kurata et al., 2011; Zúñiga et al., 2002). The ability of *Lactobacillus paracasei* to convert a wide range of amino acids including glutamine and ornithine, but not pyroglutamic acid *in vitro* has been shown previously (Tammam et al., 2000). Similarly, for brand B cheeses we determined the overall correlation of 57% (RV = 0.57) between microbiota and metabolites composition by MFA. Some of these correlations between microbiota and metabolites were of interest; for example, a positive association between a low -abundant taxa, *Acinetobacter* (a common spoilage organism) (<0.1% of total reads) and medium chain fatty acids. This association may be due to the ability of this genus to produce lipase in the milk and/or cheese (Pratuangdejkul and Dharmsthiti, 2000). However, the effects of lipolysis on milk quality cannot be discounted (Hickey et al., 2007).

While our findings cannot prove causation, they demonstrate that the metabolome profiles of cheeses (and of individual metabolites therein) which influence cheese quality and flavor also may be a useful predictor of the microbial composition of cheeses (Gallegos et al., 2017). This improved understanding could additionally be applied to informing decision-making on choice of "desirable" starter or adjunct cultures to optimize cheese quality and flavor. Future targeted and controlled studies involving more diverse and larger sample sets and whole genome sequencing for differentiation of species and strains, together with detailed profiling of volatile and non-volatile metabolites and sensory analysis, are needed to validate the associations between cheese microbiota and metabolomes and identify potential biomarkers for monitoring cheese quality and authenticity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here in NCBI, under accession PRJNA673975.

AUTHOR CONTRIBUTIONS

RA designed and performed the experiments, analyzed the data, and drafted the manuscript. Other authors listed made a substantial, direct and intellectual contribution to the work, and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.592060/full#supplementary-material

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Heat Resistance of *Listeria monocytogenes* in Dairy Matrices Involved in Mozzarella di Bufala Campana PDO Cheese

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The presence of *Listeria monocytogenes* in Mozzarella di Bufala Campana Protected Designation of Origin cheeses may depend on curd stretching conditions and post contaminations before packaging. To avoid cross-contamination, thermal treatment of water, brines and covering liquid may become necessary. The present study aimed to improve knowledge about *L. monocytogenes* thermal resistance focusing on the influence of some cheese making operations, namely curd stretching and heat treatment of fluids in contact with cheese after molding, in order to improve the safety of the cheese, optimize efficacy and sustainability of the processes. Moreover, the role that cheese curd stretching plays in *L. monocytogenes* inactivation was discussed. The 12 tested strains showed a very heterogeneous heat resistance that ranged from 7 to less than 1 Log_{10} Cfu/mL reduction after 8 min at 60°C. *D*-values (decimal reduction times) and *z*-values (thermal resistance constant) calculated for the most heat resistant strain among 60 and 70°C were highly affected by the matrix and, in particular, heat resistance noticeably increased in drained cheese curd. As cheese curd stretching is not an isothermal process, to simulate the overall lethal effect of an industrial process a secondary model was built. The lethal effect of the process was estimated around 4 Log_{10} reductions. The data provided may be useful for fresh pasta filata cheese producers in determining appropriate processing durations and temperatures for producing safe cheeses.

Keywords: food safety, *L. monocytogenes*, thermal resistance, dairy matrices, stretching, *D*-value, *z*-value

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INTRODUCTION

Fresh pasta filata cheeses have been usually considered among the microbiologically safe cheeses because of the supplementary effect of the stretching of the acid cheese curd with hot or boiling water. Nonetheless, Mozzarella di Bufala Campana (MBC) Protected Designation of Origin (PDO) cheese is characterized by pH (>5.1) and water activity (a_w > 0.975) values that do not allow to automatically consider it as food not able to support *Listeria monocytogenes* growth, according to Commission Regulation (EC) No. 2073/2005 (2020). From 1998 to 2017 out of more than

370 cases of cheese microbial contamination, only three cases of Mozzarella withdrawn from the market because of the presence of *L. monocytogenes* have been reported by European Rapid Alert System for Food and Feed (RASFF Portal, 2020). Suddenly, in 2018 and 2019 three recalls of MBC occurred in France (RASFF 2018/1198 and 2018/2459) and in Canada (Canadian Food Inspection Agency (CFIA), 2019), because of the presence of *L. monocytogenes*. This unusual contamination has urgently raised the need to investigate on its possible reasons, since the risk profile of MBC may be considered lower than the more widely consumed cow milk Mozzarella, because of the higher acidity of the cheese curd (pH 4.8 vs pH 5.1) and the higher temperature of the stretched curd at the end of the process (about 68–72°C vs 58–63°C; Mucchetti and Neviani, 2006). The stretching process of buffalo cheese curd is associated to significant but extremely variable decimal reductions of *L. monocytogenes* count, ranging from 1 to 8, as resulted by some studies performed with laboratory scale cheese making trials (Villani et al., 1996; Raimundo et al., 2013; Serraino et al., 2013; Murru et al., 2018). The survival probability depends on the cheese curd contamination, the effective conditions of stretching applied during the experiments, often not fully described, and the heat resistance of biotypes. Up to present, at the best of our knowledge, no *L. monocytogenes* contamination has been reported in MBC cheese curds. Its presence at the moment of stretching is believed to be occasional and lower than the counts measured during challenge tests, where artificial curd contamination ranged from 10^3 to 10^7 CFU/g. In these challenge tests, *L. monocytogens* was added to milk, and during cheese curd maturing a decrease (Murru et al., 2018) or even a small but significant growth (Serraino et al., 2013) have been observed. The degree of *L. monocytogenes* survival after stretching may be associated to the stretching conditions, as the overall amount of thermal and mechanical energy applied, that determine the temperature and time of permanence of the cheese curd at lethal temperature, is inversely related to the degree of cheese curd maturity and demineralization. Indeed, to obtain a cheese with the standard and typical MBC structure, the cheesemaker lowers the stretching temperature when the cheese curd has a too low pH and, as a consequence, an excessive extent of casein demineralization (Mucchetti and Neviani, 2006; Mucchetti et al., 2016).

Moreover, further aspects should be considered to explain the variable effects of curd stretching on *L. monocytogenes* survival. First, heterogeneity (genetic or phenotypic diversity) within a population, can promote adaptation and survival when the population experiences sudden environmental changes (Ryall et al., 2012; Davis and Isberg, 2016). These phenomena may explain the ability of a fraction of a bacterial population to withstand stresses that kill the majority of the population itself (Booth, 2002), and could be associated both to *Listeria* spp. biodiversity (Bernini et al., 2013) and to the presence of isogenic variants, able to influence its heat tolerance, e.g., because of a different ability to repair cell membrane after sub lethal heat injury (Somolinos et al., 2010). Heterogeneity can further contribute to explain the limits of the widely used primary model of Bigelow (Bigelow, 1921), when deviations from linear relationship between time of heat treatment and survival are observed (Cebrian et al., 2017). The heat resistance range of *L. monocytogenes* biotypes is quite variable, as reported by Doyle et al. (2001) and Aryani et al. (2015b), with *D*-values at 60°C ranging from few seconds to more than 4 min, and decreases at low pH, high a_w and low NaCl content (Jørgensen et al., 1995; Mazzotta, 2001; Bucur et al., 2018). Heat resistance of *L. monocytogenes* is also influenced by the growth phase, with the cells in exponential phase being more heat sensitive than those in stationary phase (Lou and Yousef, 1996; Jørgensen et al., 1999; Aryani et al., 2015b), and by stress adaptation phenomena, e.g., acid and or osmotic stress (Jørgensen et al., 1995; De Jesús and Whiting, 2003). Moreover, it may be interesting to note that some *D*- and *z*-values measured in some complex food matrices (meat, eggs) were significantly higher than those determined in culture media as Tryptone Soy Broth (TSB; Quintavalla and Campanini, 1991; Doyle et al., 2001), confirming the role of the interaction of microorganisms with the matrix in determining its stress tolerance.

Furthermore, the presence of *L. monocytogenes* in MBC can also be the result of post-contamination during the processing steps after stretching and before packaging. After stretching, MBC cheese is hardened and cooled by dipping into flowing tap water, salted by immersion in a brine and finally packed by dipping into a covering liquid, composed by salt and organic acids (e.g., lactic acid; Mucchetti and Neviani, 2006). Potentially, the contamination of these fluids can be transferred to the cheese, but at present time no data demonstrated the presence of *L. monocytogenes* in tap water and/or brines and/or covering liquids used for MBC cheese making. However, even if the presence of *L. monocytogenes* in tap water is not largely documented (Lyautey et al., 2007), its ability to survive (Budzińska et al., 2012), and to create biofilm (Gião and Keevil, 2014) has been determined. Otherwise, the presence of *L. monocytogenes* in brines of different cheeses has been detected using analytical tools as quantitative polymerase chain reaction (PCR) (Alessandria et al., 2010; Barancelli et al., 2014), and its ability to survive was inversely related to the acidity and NaCl content of the brine itself (Schirmer et al., 2014). The presence of this pathogen on cheese surface and/or on equipment may result in water and brine contamination, causing the beginning of a non-controlled cross-contamination. Also, the more frequent presence of *Listeria* spp. on non-food contact surfaces (floor, drains, walls, and platforms) may become a potential route of cheese contamination (Barancelli et al., 2014). To avoid cheese cross-contamination, the treatment of water, brines and covering liquid may become necessary. Beside heat processes, filtration, and/or UV-C treatment (Gayan et al., 2015) can be effective in reducing the count of *L. monocytogenes*. However, heat treatments can lead to more reproducible results.

Knowledge of decimal reduction time (*D*-value) and thermal resistance constant (*z*-value) in each out of these matrices can allow to build primary and secondary models able to foresee the lethal effect of heat treatments, contributing to improve the efficacy and the sustainability of the processes, avoiding to waste energy and/or time, mainly when batch treatments are applied as occur in many small sized MBC dairies. The *D*-value is the time

required to obtain a ten-fold reduction of a microbial population at a constant lethal temperature and it varies according to the microorganism and to the medium where heat is applied. The *z*-value is the temperature variation required for the *D*-value to change by a factor of ten. Using these parameters, it is possible to foresee the effect of a thermal treatment, accepting that the death rate follows a first order reaction kinetics. Despite the death rate of many microorganisms does not always or completely follow this order of kinetics, suggesting that other models (e.g., Weibull frequency distribution models or non-log-linear Geeraerd's model) could be applied (Peleg and Cole, 1998; Mafart et al., 2002; Geeraerd et al., 2005; Cebrian et al., 2017), the linear model (Bigelow, 1921) continues to offer a relevant contribution to predict the effect of a heat treatment. However, in the presence of a non-isothermal temperature profile, as in the case of curd stretching operation, it is necessary to develop a secondary model able to simulate the overall lethal effect of the treatment.

The aims of this study were (i) the selection of the most heat resistant *L. monocytogenes* among a pool of dairy origin strains, (ii) the estimation of *D* and *z* values of this strain using as matrices MBC cheese curd and the fluids coming into contact with MBC cheese after cheese molding, such as hardening water, brine and fresh covering liquid; (iii) the proposal of a secondary model able to estimate the cumulative effect of the stretching process on *L. monocytogenes* survival. Following this approach, we aimed to improve the knowledge about the influence of the matrix on heat resistance and to better understand the role that cheese curd stretching plays in *L. monocytogenes* inactivation.

MATERIALS AND METHODS

Culture Preparation

Twelve *L. monocytogenes* strains were overall considered in this study. Among these, ten strains (Lm1, Lm2, Lm3, Lm4, Lm5, Lm8, Lm15, Lm16, Lm21, and Lm28) isolated from Gorgonzola cheese (Bernini et al., 2013) and belonging to the collection of Food and Drug Department of the University of Parma, and two reference strains (Lmg 21263 corresponding to Atcc 13932, isolated from a clinical patient affected by meningitis, and Lmg 13305 corresponding to Dsm 15675, isolated from soft cheese) purchased from Bccm (Belgian Co-ordinated Collections of Microorganisms) of Ghent University, Belgium were considered. The stock cultures were kept frozen at −80°C in Tsb (Vwr, Milano, Italy) added with 12.5% glycerol (v/v). They were recovered in TSB (VWR) enriched with 0.6% yeast extract (Oxoid, Basingstoke, United Kingdom) and incubated at 37°C overnight. The procedure was replicated three times. The counts after overnight growth were determined in Agar Listeria according to Ottaviani and Agosti (ALOA; VWR, Milano, Italy) incubated at 37°C for 48 h. Before being used for heat resistance experiments, microbial cells were separated by centrifugation (Centrifuge 5810 R, Eppendorf) at 12,857 *g* for 5 min at 25°C, washed twice in Ringer solution (Oxoid, Milan, Italy) and finally resuspended in Ringer solution to reach the proper cell concentration.

Thermal Resistance Screening of *L. monocytogenes* Strains *in vitro* Conditions

The selection of the strain with the highest heat resistance was carried out as follows. Small volumes of Tsb (Vwr; 2.970 mL) were pre-heated in conical plastic tubes (50 mL capacity) in a water bath (Type M900-Ti Basic, Instruments s.r.l, Italy) until they reached the temperature of 60°C. Then 0.03 mL of each strain was inoculated in different pre-heated Tsb aliquots in order to obtain a final concentration ranged from 6 to 7 Log_{10} Cfu/mL. The inoculated solutions were rapidly mixed and kept for 8 min at 60°C. In order to obtain a rapid temperature decrease and stop the heat treatment the suspensions were chilled by immersion into a water bath with melting ice to fulfill the condition of iso-temperature, monitoring the temperature with type-K thermocouples. The initial and the residual microbial concentration were checked on ALOA (Vwr) at 37°C for 48 h. The detection limit was 1 Log_{10} Cfu/mL and the tests were performed in duplicate.

Determination of *L. monocytogenes* Heat Resistance in TSB, Hardening Water, Brine, Covering Liquid, and Cheese Curd

The strain that showed the highest heat resistance was selected and further tested to estimate *D* and *z* values in the range from 60 to 70°C in TSB and in different matrices of MBC cheese production chain: hardening water, brine, covering liquid, and cheese curd. Matrices were provided by Consorzio Tutela MBC DOP, with these main characteristics:

(i) hardening water represents a sample of pasteurized tap water that was used to chill MBC in a continuous equipment without recycle of the water;
(ii) brine is a freshly prepared light brine (NaCl concentration 4 *g*/100 *g*, titratable acidity 6°Soxhlet-Henkel Sh/50 mL) used to salt MBC according to the rules of MBC production standard;
(iii) covering liquid is a pasteurized solution of 0.05 *g*/100 mL of lactic acid (titratable acidity 6°Sh/50 mL; pH 2.9);
(iv) cheese curd is mature MBC cheese curd (about 45% of moisture content, a_w 0.99) sampled before the stretching step and immediately frozen at −18°C.

A small volume (0.985 mL) of each fluid matrix was warmed at the selected temperature immerging conical plastic tubes (50 mL capacity) into a water bath (Type M900-Ti Basic, Instruments s.r.l, Italy) to reach the target equilibrium temperature. Then 0.015 mL of *L.* monocytogenes washed cell suspension was added; the inoculated fluid matrix was then rapidly mixed and kept for the scheduled time at the set temperature (that ranged from 60 to 70°C). The suspensions (1 mL) were chilled by dilution into 9 mL of Ringer solution (Oxoid) at 4°C. In this way, as checked by temperature monitoring with type-K thermocouples, the conditions of iso-temperature were fulfilled. The initial and

residual counts were made on Aloa (Vwr) at 37°C for 48 h. The detection limit was 1 Log_{10} Cfu/mL.

The test in MBC cheese curd was performed introducing a step of preparation of the matrix, as its warming determines a whey syneresis. So, 10 *g* of cheese curd were poured into a conical plastic tube (50 mL capacity) and kept into a water bath at the set temperature (62 to 70°C) up to 30 min for completing the whey separation. Total solids and a_w of the drained cheese curd were measured according to Idf standard 4A (1982) and by AquaLab Water Activity Meter Series 3TE with internal temperature control (Decagon Devices, Inc., Pullman, WA, United States), respectively. Then 1.97 *g* of drained cheese curd was poured into a new conical plastic tube and, when equilibrated at the target temperature of treatment, 0.03 mL of *L. monocytogenes* washed cell suspension was inoculated directly into the hot mass and kept for the scheduled times. After treatment, instantaneous chilling to non-lethal temperature was obtained by the addition of 18 mL of Ringer solution (Oxoid) at 4°C. All the trials were performed in duplicate.

Estimation of *D*- and *z*-Values for the Primary Model

The *D*-value, that is the absolute value of the inverse slope of the linear regression line between the Log_{10} of surviving cells number and time (s), was calculated considering at least three out of four or more measures performed at different times for each experiment and falling into the linear portion of the regression line. The absolute value of the inverse slope of the linear regression line between the average Log_{10} *D*-values and temperature was the *z*-value.

Simulation of the Effect of Cheese Curd Stretching on *L. monocytogenes* Survival

In order to benchmark the results of *L. monocytogenes* thermal inactivation obtained in cheese curd, an exemplificative Mbc curd stretching process was considered according with temperature data obtained from observations made in Mbc dairies (**Table 3**). The process was divided into four key steps: melting caused by curd mixing with hot water, kneading of melted curd, cheese molding, and cooling (**Table 3**). To estimate the effect of cheese curd stretching step on *L. monocytogenes* survival, a secondary model was built applying the following procedure:

(i) Estimation of *D*-values at all the lethal temperatures (Tx) involved in the process on the basis of Eq. 1, using a primary linear model, with T_{REF} = 70,0°C;

$$D_{T_X} = D_{T_{\mathrm{REF}}} 10^{-\frac{T_X - T_{\mathrm{REF}}}{z}} \quad (1)$$

(ii) calculation of the estimated time of permanence of the coldest spot of the cheese curd at lethal temperature (e.g., from 60 to 70°C) both during the stretching operation and the initial step of cooling;

(iii) the time of permanence at each temperature was divided by the corresponding *D*-values to obtain the Log_{10} count reduction at each temperature;

(iv) finally, the sum of the lethal effects at each temperature represents the overall lethal effect given by the process.

RESULTS

Selection of the Most Heat Resistant *L. monocytogenes* Strain

In general, the heat resistance of the 12 strains was heterogeneous. Among all the tested strains, Lm15 was the most heat resistant showing a decrease in cell concentration of less than 1 Log_{10} CFU/mL after 8 min of heating at 60°C in TSB. *L. monocytogenes* LMG 21264, isolated from a patient affected by meningitis, was the most heat sensitive strain; *L. monocytogenes* Lm3, Lm8, Lm21, Lm28, and LMG 13305 were the most sensitive among the strains isolated from cheese (**Table 1**).

Estimation of *D*- and *z*-Values of *L. monocytogenes* Lm15 Strain in Dairy Matrices

D-values of Lm15 estimated at temperatures ranging from 60 to 70°C in fluid media and between 62 and 70°C in drained cheese curd have been strongly affected by the matrix (**Table 2**). Considering the fluid matrices, the high presence of organic matter in TSB (31.5 g/L) appeared to protect the cells from heat damage at all three temperatures considered. The acidic pH (2.9) of covering liquid that was due to the presence of lactic acid contributed to negatively affect the heat resistance of Lm15; on the contrary, this strain showed a higher thermal resistance in the hardening water and in the brine. Heat resistance of Lm15 increased noticeably at all the tested temperatures when measured in the drained cheese curd, characterized by a total solid content of 63.4% and a_w value of 0.989, because of

TABLE 1 | Log_{10} reduction of *L. monocytogenes* tested strains after 8 min at 60°C in *in vitro* conditions.

Strain	Log_{10} CFU/mL			
	Trial A	Trial B	Mean	SD
Lm1	1.86	1.56	1.71	0.21
Lm2	1.12	1.83	1.48	0.50
Lm3	5.28	5.31	5.29	0.03
Lm4	3.50	4.38	3.94	0.62
Lm5	3.21	2.76	2.98	0.32
Lm8	4.98	5.34	5.16	0.25
Lm15	0.52	0.72	0.62	0.14
Lm16	2.92	2.61	2.76	0.22
Lm21	4.55	6.20	5.37	1.17
Lm28	6.72	6.37	6.55	0.25
LMG 21264	7.08	7.04	7.06	0.03
LMG 13305	4.90	5.70	5.30	0.56

TABLE 2 | *D*-values and *z*-values of *L. monocytogenes* Lm15 in different matrices.

Matrix	pH	Temperature (°C)	*D*-value (s)	*z*-value (°C)
TSB	7.2	60.1 ± 0.3	284.4 ± 17.3	6.4
		67.3 ± 0.1	19.3 ± 2.3	
		70.2 ± 0.6	8.4 ± 0.3	
Hardening water	7.2	60.0 ± 0.2	90.7 ± 5.0	8.2
		67.4 ± 0.2	6.9 ± 0.1	
		70.2 ± 0.2	6.1 ± 0.4	
Brine	3.2	60.0 ± 0.1	84.4 ± 4.9	10.1
		67.5 ± 0.2	16.4 ± 0.4	
		70.4 ± 0.1	7.7 ± 0.1	
Covering liquid	2.9	60.1 ± 0.2	22.1 ± 0.7	9.8
		67.2 ± 0.1	4.3 ± 0.1	
		70.2 ± 0.2	2.1 ± 0.2	
Drained cheese curd	5.1	62.3 ± 0.4	423.5 ± 26.6	11.1
		66.4 ± 0.1	188.9 ± 19.4	
		70.0 ± 0.3	85.4 ± 7.2	

whey separation that was caused by the preliminary equilibration step to heating temperature. Even the *z*-values were affected by the different matrices, with values much higher for the dairy matrices (about 10°C) compared to the synthetic medium TSB (6.4°C).

Simulation of the Effect of Cheese Curd Stretching on *L. monocytogenes* Survival

As stretching is not a fully isothermal operation, its lethal effect on Lm15 should be foreseen based on the temperature profile of the curd. On the basis of the hypothesized curve temperature vs cumulative time (**Table 3**), four steps of the process contributing to the overall lethal effect can be identified. The cumulative lethal effect of the first three steps, where mixing and kneading contribute to a roughly homogeneous heating rate, can be estimated around 4 Log_{10} reductions (3.92 Log_{10} in the example) of Lm15 strain (**Table 3**). In the center of the MBC cheese ball, being the slowest point to be chilled, the lethal effect can increase to more than 6 Log_{10} count reduction. Conversely, in the external part of the cheese (which temperature decrease is not estimated in **Table 3**) because of the very fast cooling to sublethal temperature the count reduction does not overcome the one obtained during stretching (∼4 Log_{10} count reduction). To foresee the overall lethal effect of the thermal treatment, the zone where the effect is lower has to be considered. Furthermore, the outer zone of the cheese is the most subjected to post-contamination by contact with hardening water, brine, and covering liquid.

DISCUSSION

A large variability of heat resistance among the *L. monocytogenes* strains tested was observed in this study and it has to be taken into account in future studies on *L. monocytogenes* survival during heat processes of food products. Our results in TSB medium agree with previously reported data (Doyle et al., 2001; Aryani et al., 2015b). A similar high heat resistance was found by Quintavalla and Campanini (1991) for a strain isolated from meat and tested in the same matrix. Curiously, the most heat resistant strain among the 20 tested by Aryani et al. (2015b), with the majority of them of food origin, was isolated from milk. Otherwise, some strains showed a very low heat resistance; the most heat sensitive strain was a reference strain (LMG 21264, isolated from human meningitis), whose heat resistance was about 11 times lower than that of Lm15 and similar to Lm28 that was about 10 times less heat resistant than Lm15. De Jesús and Whiting (2003) found a narrower distribution from 1.98 to 5.31 min of the $D_{60°c}$ values among 21 *L. monocytogenes* strains isolated from humans and animals, while the range for the same acid-adapted strains was between 0.8 and 4.59 min.

The influence of the matrix on the heat resistance of the selected strain used in the present study was clear and it was probably linked to a combination of protective and inhibiting factors. The high amount of total solids of the drained cheese curd (63.4%) and of the synthetic TSB medium (31.5 g/L) may be related to the high *D*-values measured at all the tested temperatures. No literature data are available about the heat resistance of *L. monocytogenes* in MBC cheese curds, while $D_{60°c}$ values in TSB (4.74 min corresponding to 284.4 s in **Table 2**) resulted similar to those reported by Quintavalla and Campanini (1991) and Aryani et al. (2015b). A higher thermotolerance of *L. monocytogenes* in half cream, double cream or butter compared to TSB was reported by Casadei et al. (1998), but no relationship was found between the *D*-values and the fat amount of the product. The presence of undissociated lactic acid in the covering liquid may be responsible for the lowest *D*-values reported in the present study. As the fresh covering liquid contained 0.5 *g* of lactic acid/100 mL at pH 2.9, according to the Henderson-Hasselbalch equation.

The amount of undissociated acid is near to 90% corresponding to about 50 mmol/L, largely above the concentration of 6.35 mM indicated by Aryani et al. (2015a) and Wemmenhove et al. (2018) as inhibitory of *L. monocytogenes* growth. Considering the values obtained in hardening water and brine, the relatively low NaCl concentration (4 *g*/100 *g*) of the latter fluid did not markedly affect Lm15 heat resistance, probably because the protective effect of salt was counterbalanced by the low pH value of the brine. The direct relation between salt concentration and *L. monocytogenes* thermotolerance was observed by several authors (Jørgensen et al., 1995; Juneja et al., 2013; Li et al., 2017).

The calculation of *z*-values between 60 and 70°C in the different matrices gave results generally higher (from 8.2 to 11.1°C) than most of the data present in literature, with the exception of *z*-value related to TSB medium (6.4°C). However, similar or higher *z*-values were found for *L. monocytogenes* strains tested in some foods such as egg albumen or yolk, meat or flour (Palumbo et al., 1996; Michalski et al., 2000; Taylor et al., 2018). A higher *z*-value means that the strain is more tolerant to the changes in temperatures and this behavior should be taken into account in the design of heat processes.

As the knowledge of *D*- and *z*-values is pivotal to successfully design food processes, the accuracy of the simulation of the lethal

TABLE 3 | Estimation of the overall lethal effect of cheese curd stretching on Lm15 ($D_{70°C}$ = 85.36 s; z = 11.1°C).

	Estimated Lm15 D_T (s)	Temperature (°C)	Estimated time of curd residence at "constant" temperature (s)	Cumulative time of stretching operation (s)	Log_{10} count reduction	Log_{10} count reduction cumulative
Curd mixing with hot water	681	60	3	3	0.00	0.00
	553	61	4	7	0.01	0.01
	449	62	6	13	0.01	0.02
	365	63	8	21	0.02	0.05
	297	64	10	31	0.03	0.08
	241	65	15	46	0.06	0.14
	196	66	20	66	0.10	0.24
	159	67	25	91	0.16	0.40
	129	68	30	121	0.23	0.63
	105	69	45	166	0.43	1.06
Kneading of melted curd	105	69	200	366	1.90	2.97
Cheese molding	105	69	100	466	0.95	3.92
Cheese cooling and hardening	129	68	80	546	0.62	4.54
	159	67	80	626	0.50	5.04
	196	66	65	691	0.33	5.37
	241	65	60	751	0.25	5.62
	297	64	50	801	0.17	5.79
	365	63	40	841	0.11	5.90
	449	62	30	871	0.07	5.97
	553	61	30	901	0.05	6.02
	681	60	30	931	0.04	6.06

effect due to stretching and other heating operations depends both on the accuracy of the *D*- and *z*-value estimation, both on the accuracy of the determination of the heat treatment. While temperature changes during fluid heating by flowing through continuous heat exchangers can be predicted by computational fluid dynamics (Rinaldi et al., 2018) or direct measurements, the measurement of the change of temperature of a cheese curd during continuous cooking-stretching operation in industrial conditions, by means of the typical diving arms equipment used in the geographical area, is difficult to be monitored or estimated, and for this reason is lacking in literature. Cheese curd stretching is considered as an asymmetrical heat treatment (Villani et al., 1996; Murru et al., 2018) and just few known examples (Serraino et al., 2013) of description of the temperature evolution are referred to lab scale experiments or to industrial trials performed with batch operations (Mucchetti et al., 1997). The process involves solid/fluid mixing operations, rheological changes due to curd melting, and a flow in a channel with not constant section, responsible for a variable speed and residence time in the different sections of the equipment (tween screw conveyor, diving arms, and molding). The process can be divided in four steps: (i) a first step of fast rise of temperature of the curd mass, which homogeneity depends on the efficacy of cheese curd mixing with hot water into the horizontal counter rotating twin screw conveyor that transports the mixture to the kneading section; (ii) a second step where the melted cheese curd is kneaded by the movement of diving arms and temperature is quite constant and homogenous within the mass; (iii) a third step where the stretched cheese mass enters the molding equipment and it is pulled by a second screw conveyor toward the molding drum; (iv) a final step during which the molded cheese balls fall from the molding drum into flowing tap water where they start to harden and to decrease their temperature; the temperature decrease in the center of the cheese, that is slower than in its external part, is governed by the conductive heat transfer from the center to the surface of the cheese and it is dependent of the cheese ball size. The minimum overall sterilizing effect on *L. monocytogenes* is the result of the cumulative effect of non-isothermal (the steps of curd mixing with hot water and cheese chilling by dipping in tap water) and quasi-isothermal (the steps of curd kneading and cheese molding, performed without water addition) contributions. The accuracy of lethal effect prediction depends on the correct measurement of the temperature profile of the coldest spot in the non-isothermal steps of the process. This discussion shows the complexity of the phenomenon of heat exchange during the curd stretching operation and the difficulties to foresee the rate of inactivation of microorganisms during cheese curd stretching and cheese hardening. At the same time, beside the strain biodiversity (Murru et al., 2018), the potential process variability can contribute to explain the heterogeneous results obtained by

challenge tests, where the inactivation of *L. monocytogenes* ranged from 1 to more than 8 decimal reductions (Villani et al., 1996; Kim et al., 1998; Raimundo et al., 2013; Serraino et al., 2013; Murru et al., 2018). The technological importance of bacterial heterogeneity is also connected to the ability of a small fraction of any population to survive exposure to stresses that kill the majority of the population. The knowledge of heat inactivation kinetics parameters for all the other fluids involved in chilling, salting and storing of MBC cheese is critical to more effectively and efficiently manage the treatment of these fluids to prevent MBC cheese surface post contamination.

CONCLUSION

The data supplied in this *in vitro* study may prove to be useful for MBC and other Mozzarella cheese producers in determining appropriate durations and temperatures for producing fresh pasta filata cheeses avoiding the presence of *L.* monocytogenes. To do so, a further model should be studied to foresee the amount of hot water required to heat the MBC cheese curd mass, considering both the cases of batch or in continuous stretching processes. Finally, the knowledge of the kinetic parameters of heat inactivation together with a deeper knowledge of the temperature vs time evolution of the cheese mass can be a tool to foresee the lethal effect of the process and to better manage the process itself also With the aim to improve the microbial safety of the cheese.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AR: investigation and methodology. MA: methodology and data analysis. FM: investigation and methodology. VB: project administration, supervision, and writing -review and editing. AG: resources. GP: resources. EN: supervision, writing -review and editing. GM: conceptualization, supervision, and writing – original draft. All authors contributed to the article and approved the submitted version.

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Development of a High-Throughput Microfluidic qPCR System for the Quantitative Determination of Quality-Relevant Bacteria in Cheese

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The composition of the cheese microbiome has an important impact on the sensorial quality and safety of cheese. Therefore, much effort has been made to investigate the microbial community composition of cheese. Quantitative real-time polymerase chain reaction (qPCR) is a well-established method for detecting and quantifying bacteria. High-throughput qPCR (HT-qPCR) using microfluidics brings further advantages by providing fast results and by decreasing the cost per sample. We have developed a HT-qPCR approach for the rapid and cost-efficient quantification of microbial species in cheese by designing qPCR assays targeting 24 species/subspecies commonly found in cheese. Primer pairs were evaluated on the Biomark (Fluidigm) microfluidic HT-qPCR system using DNA from single strains and from artificial mock communities. The qPCR assays worked efficiently under identical PCR conditions, and the validation showed satisfying inclusivity, exclusivity, and amplification efficiencies. Preliminary results obtained from the HT-qPCR analysis of DNA samples of model cheeses made with the addition of adjunct cultures confirmed the potential of the microfluidic HT-qPCR system to screen for selected bacterial species in the cheese microbiome. HT-qPCR data of DNA samples of two downgraded commercial cheeses showed that this approach provides valuable information that can help to identify the microbial origin of quality defects. This newly developed HT-qPCR system is a promising approach that will allow simultaneous monitoring of quality-relevant species in fermented foods with high bacterial diversity, thereby opening up new perspectives for the control and assurance of high product quality.

Keywords: real-time qPCR, microbial community composition, microfluidic, cheese quality, cheese microbiome, fermented food, food microbiology, Fluidigm

Abbreviations: BLAST, basic local alignment tool; Cq, quantification cycle; DNA, deoxyribonucleic acid; *E.*, *Enterococcus*; HT-qPCR, high-throughput qPCR; *L.*, *Lactobacillus*; LAB, lactic acid bacteria; NSLAB, non-starter lactic acid bacteria; *Pd.*, *Pediococcus*; *Pr.*, *Propionibacterium*; qPCR, quantitative real-time polymerase chain reaction; *S.*, *Streptococcus*; Tm, melting temperature.

INTRODUCTION

Cheese can be considered a complex ecosystem that is characterized by multiple interactions between its diverse microbial community and environmental conditions. The cheese rind exhibits a high microbial diversity, whereas the composition of the microbiome within the cheese body is less complex (Wolfe et al., 2014; Dugat-Bony et al., 2016). Although the microbiota of raw milk is diverse, several factors, such as pretreatment of the milk, the use of starters, and the thermal conditions applied during cheese making, strongly influence the initial composition of the cheese microbiome. Moreover, the harsh environmental conditions occurring during ripening favor the development of a characteristic ripening microbiota that is especially adapted to an environment characterized by limited levels of fermentable carbohydrates, acidic pH, elevated salt concentrations, and low temperatures (De Filippis et al., 2014; Gobbetti et al., 2018).

The study of the bacterial community composition and of the bacterial population dynamics in cheese has been greatly improved with the advent of culture-independent molecular techniques. Methods such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE), single strand conformational polymorphism (SSCP), length heterogeneity PCR (LH-PCR), and terminal restriction fragment length polymorphism (T-RFLP), were commonly used in the past decades to study the microbial composition of raw milk and cheese, as well as the rind microbiota (Quigley et al., 2011). However, the recent development of next-generation sequencing (NGS) techniques has enabled an even more detailed study of complex microbiomes, and these are now the most widely used approaches in food microbial ecology (De Filippis et al., 2017). The commonest NGS technique used in the analysis of food microbiomes is 16S rRNA gene amplicon-based sequencing, which provides an extensive overview of the food microbiota (Cao et al., 2017). However, identification beyond the genus level is often not possible with this method (Claesson et al., 2010). In the case of cheese and dairy products, species level classification has been achieved by optimization of primer pairs for variable 16S rRNA gene regions, by improved data analysis procedures, and by the establishment of high-quality databases, such as the manually curated DAIRYdb (Meola et al., 2019).

Even though NGS is now routinely used by academic researchers, its use in the food industry is rare. An inherent limitation of the 16S rRNA gene sequencing method is that it only provides the relative abundances of the individual members of the community [operational taxonomic units (OTUs), amplicon sequence variants (ASVs), and taxa]. Complementary approaches, such as quantitative real-time PCR (qPCR) or flow cytometry, are then required to assess the quantitative aspects of the communities (Props et al., 2017). This quantitative analysis is particularly important for fermented foods, as off-flavors may arise due to the abundance of certain microbial populations (Giraffa, 2004). The composition of the cheese microbiome has an important impact on the sensory quality and safety of the final cheese product (Fox et al., 2017). The sensorial quality depends on the microbial biodiversity as well as on the bacterial counts of each individual species (Giraffa, 2004). The metabolic activity of desired and undesired bacterial species is usually sensorially perceivable at counts of $>10^5$ colony-forming units per gram (CFU/g); however, easily noticeable flavor characteristics and off-flavors are typically associated with bacterial counts of 10^6–10^9 CFU/g (Fox et al., 2017).

Quantitative real-time PCR is a well-established method for the detection and quantification of bacteria, such as in pathogen detection in clinical and veterinary diagnostics and in food safety (Curran et al., 2007; Ramirez et al., 2009; Cremonesi et al., 2014; Sartori et al., 2017; Garrido-Maestu et al., 2018). The major limitation of standard qPCR methods is their low throughput, but this has been overcome in recent years with the development of high-throughput qPCR (HT-qPCR) platforms (Ishii et al., 2013; Waseem et al., 2019). HT-qPCR has now been validated and applied to investigate synthetic bacterial soil communities (Kleyer et al., 2017), to determine functional genes in soils (Crane et al., 2018), to quantify pathogens in spiked fecal and environmental water samples (Ishii et al., 2013), to study the gut microbial diversity in piglets (Hermann-Bank et al., 2013), and to quantify dairy *Lactococcus* (*Lc.*) *lactis* and *Leuconostoc* species bacteriophages (Muhammed et al., 2017). However, to our knowledge, HT-qPCR has not yet been used to quantify bacteria in fermented foods, such as cheese. Particularly in the case of raw milk cheeses, microbially induced quality defects, such as off-flavors caused by faulty secondary fermentation or the formation of high quantities of biogenic amines, can frequently lead to a downgrading of cheeses, with significant financial losses. A cost-effective monitoring of desirable and undesirable microorganisms could therefore improve the surveillance of product quality and enable the identification of the causes of microbial cheese defects at an early stage of ripening.

The present study describes the design, validation, and application of a novel microfluidic HT-qPCR system for the simultaneous quantification of multiple bacterial species that are frequently present in raw milk cheeses. We evaluated 24 qPCR assays targeting 23 different bacterial species, including two *Lactococcus lactis* subspecies. The selected target bacteria included lactic acid bacteria (LAB) often used as starters for cheese production, non-starter lactic acid bacteria (NSLAB), and selected species associated with undesired secondary fermentation. A workflow was also developed to facilitate the experimental setup, data filtering, and analysis of the HT-qPCR results. The developed HT-qPCR system was tested under practical conditions by inoculating experimental cheeses with different target species and by including two downgraded commercial cheeses with quality defects in the analysis.

MATERIALS AND METHODS

Selection of Target Species and Primer Design

Twenty-four target species were selected based on a review of the literature and our own preliminary results from 16S

rRNA gene amplicon-based sequencing of Gruyere and Raclette cheeses (unpublished data). The selection criteria were the abundance and frequency of detection, as well as known impacts on cheese quality (**Table 1**). The primer pairs used in this study are listed in **Supplementary Table S1**. New primer pairs were designed for 20 species according to the workflow described in a previous study (Dreier et al., 2020). Briefly, genome assemblies of the target species were downloaded from the National Center for Biotechnology Information (NCBI) and a pan-genome analysis was performed, single copy core genes were selected for primer design and species-specific primer pairs were identified. Three primer pairs were previously published (Dreier et al., 2020). LbhelvF1 and a modified version of LbhelvR1, described elsewhere (Moser et al., 2017), were selected as the primer pair for *Lactobacillus helveticus*. All primers were validated *in silico* by BLAST and Primer-BLAST searches (Johnson et al., 2008; Ye et al., 2012).

Bacterial Strains

For each species, the type strain was selected; for additional strains, isolates from food were preferred. Strains (**Supplementary Data Sheet 1**, target and off-target strain sheets) were obtained from the Agroscope Culture Collection stored at −80°C in sterile reconstituted skim milk powder (10% w/v) and were reactivated and cultivated according to the conditions specified in **Supplementary Data Sheet 1** (cultivation conditions sheet).

DNA Extraction

DNA was extracted from bacterial single strains and from cheese samples, as follows. Bacterial pellets from single strains were harvested from 1 ml overnight cultures by centrifugation (10,000 × g, 5 min, room temperature). Bacterial pellets from cheese were obtained by adding 10 g of cheese to 90 ml modified peptone water (10 g/l peptone from casein, 5 g/l sodium chloride, 20 g/l trisodium citrate dihydrate, pH 7.0) and incubating for 10 min at 40°C. The sample was then homogenized for 3 min in a Stomacher (Masticator, IUL Instruments, Königswinter, Germany). A 50 µl volume of 10% (w/v) SDS was then added to 10 ml of the homogenate, which was then thoroughly mixed and centrifuged (4,000 × g, room temperature, 30 min). The bacterial pellets from the single strains and from the cheese samples were then subjected to a pre-lysis treatment, as described previously (Dreier et al., 2020). Briefly, the pre-lysis treatment included a 15 min incubation in 50 mM sodium hydroxide, followed by an incubation with 2.5 mg/ml lysozyme for 1 h at 37°C. Cell lysis and genomic DNA extraction was performed using the EZ1 DNA Tissue kit and a BioRobot® EZ1 workstation (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was eluted in a volume of 100 µl and the concentration was measured using a NanoDrop® ND-1000 spectrophotometer

TABLE 1 | Selected species/subspecies and their impact on cheese quality.

Species	Group	Associated defect	Incidence level
Clostridium tyrobutyricum	Raw milk contaminant	Butyric acid fermentation	Species
Enterococcus durans	NSLAB	Biogenic amines (T)	Species
Enterococcus faecalis	NSLAB	Biogenic amines (T)	Species
Enterococcus faecium	NSLAB	Biogenic amines (T)	Species
Levilactobacillus brevis	NSLAB	Biogenic amines (T)	Strain
Lacticaseibacillus casei	NSLAB	–	–
Loigolactobacillus coryniformis	NSLAB	Biogenic amines (H)	Strain
Latilactobacillus curvatus	NSLAB	Biogenic amines (T, P)	Strain
Lactobacillus delbrueckii	Starter	–	–
Limosilactobacillus fermentum	NSLAB/(Whey starter)	(Excess gas formation)	Species
Lactobacillus helveticus	Starter/Adjunct	–	–
Lentilactobacillus parabuchneri	NSLAB	Biogenic amines (H)	Strain
Lacticaseibacillus paracasei	NSLAB/Adjunct	–	–
Lactiplantibacillus paraplantarum	NSLAB	–	–
Lactiplantibacillus plantarum	NSLAB	–	–
Lacticaseibacillus rhamnosus	NSLAB/Adjunct	–	–
Latilactobacillus sakei	NSLAB	–	–
Lactococcus lactis subsp. cremoris	Starter	–	–
Lactococcus lactis subsp. lactis	Starter	–	–
Leuconostoc mesenteroides	Starter/Adjunct	–	–
Pediococcus acidilactici	NSLAB	–	–
Pediococcus pentosaceus	NSLAB	–	–
Propionibacterium freudenreichii	Adjunct/Raw milk	Propionic acid fermentation	Species
Streptococcus thermophilus	Starter	–	–

T, Tyramine; P, Putrescine; H, Histamine.

(NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA, United States).

Reagents and Conditions for Standard qPCR

The inclusivity of the primer pairs was assessed by performing qPCR with 2 ng DNA of 2–34 strains of the target species in technical duplicates (**Supplementary Data Sheet 1**, target strains). The qPCR assays were performed in a total reaction mix volume of 12 µl, containing 6 µl 2× SsoFast™ EvaGreen® Supermix with low ROX (Biorad, Cressier, Switzerland), 500 nM of forward and reverse primers, and 2 µl of DNA. The qPCR cycling conditions consisted in an initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 5 s and 60°C for 1 min. The melting curve analysis was performed using a gradient from 60 to 95°C, with 1°C steps per 3 s. All qPCR assays were run on a Corbett Rotor-Gene 3000 (Qiagen). Rotor-Gene 6000 Software 1.7 was used for analysis, with dynamic tube normalization and a threshold of 0.05 for quantification cycle (Cq) value calculation; the five first cycles were ignored for the determination of the Cq values. The peak calling threshold for the melt curve analysis was set to −2 dF/dT, and the temperature threshold was set at 2°C lower than the positive control peak.

Preamplification of DNA Samples

An assay mix was prepared by pooling 1 µl of each primer (100 µM) in a total volume of 200 µl DNA suspension buffer [10 mM tris(hydroxymethyl)aminomethane, 0.1 mM ethylenediaminetetraacetic acid, pH 8]. A volume of 1.25 µl DNA sample was mixed with 3.75 µl preamplification pre-mix consisting of 2.5 µl 2× TaqMan PreAmp Master Mix (Thermo Fisher Scientific, Waltham, MA, United States), 0.5 µl of pooled assay mix, and 0.75 µl DNase-free water. Preamplification was performed using a Labcycler (SensoQuest, Göttingen, Germany) thermal cycler using the following conditions: an initial denaturation step at 95°C for 10 min, followed by 14 cycles at 95°C for 15 s and 60°C for 4 min. The preamplification primers were eliminated from the reactions by treating the samples with 2 µl diluted Exonuclease I (4 U/µl, Thermo Fisher Scientific, Waltham, MA, United States) at 37°C for 30 min, followed by enzyme inactivation at 80°C for 15 min. The final reactions were diluted 10-fold with DNA suspension buffer and stored at -20°C.

Microfluidic HT-qPCR

HT-qPCR was performed using a 192.24 Dynamic Array integrated fluidic circuit (IFC; Fluidigm Corporation, San Francisco, CA, United States). DNA samples from pure bacterial cultures were diluted to 3 ng/µl prior to qPCR measurement. The assay mix consisted of 3 µl 2× Assay Loading Reagent (Fluidigm Corp.) added to 3 µl primer mix (forward and reverse, 10 µM). A sample pre-mix was prepared by combining 3 µl 2× SsoFast™ EvaGreen® Supermix with low ROX (Biorad, Cressier, Switzerland) and 0.3 µl 192.24 Delta Gene Sample Reagent (Fluidigm Corp.). Finally, 2.7 µl of each sample were added to 3.3 µl sample pre-mix. The IFC was loaded according to the manufacturer's instructions (Fluidigm, 2015). Briefly, 3 µl of each assay and 3 µl of each sample were distributed to the respective inlet, and the IFC was loaded using the Juno Load Mix 192.24 GE script. The loaded IFC was transferred to the Biomark instrument and run with the GE 192x24 PCR+Melt v2 program, as follows: hot start 95°C for 1 min, followed by 30 cycles of denaturation at 96°C for 5 s and annealing and elongation at 60°C for 20 s. A melting curve analysis was performed with a temperature increase of 1°C per 3 s from 60 to 95°C.

HT-qPCR Standards

The standards for quantification in the HT-qPCR system were produced using standard calibration curves of gBlock™ Gene Fragments (Integrated DNA Technologies, LubioScience, Switzerland), consisting of 24 double stranded target species sequences separated by thymine spacers five base pairs in length. A map representation of the HT-qPCR standard is shown in **Figure 1**, and the sequence is available in **Supplementary Data Sheet 2**. The dried gBlock gene fragment pellet (Molecular weight: 1440635.7 u) was resuspended with DNA suspension buffer [10 mM tris(hydroxymethyl)aminomethane, 0.1 mM ethylenediaminetetraacetic acid, pH 8] at a concentration of 10 ng/µl. Copy numbers were calculated using the following equation:

$$10\frac{ng}{l} \times 0.69\frac{fmol}{ng} \times 1 \times 10^{-15}\frac{mol}{fmol} \times 6.022 \times 10^{23}\frac{copies}{mol}$$

$$= 4.16 \times 10^{9}\ copies/l$$

HT-qPCR Standard Calibration Curves

Copy numbers for quantification were calculated using duplicate standard calibration curves ranging from 10^8 to 10^3 copies/µl (**Supplementary Figure S1** in **Supplementary Data Sheet 3**).

HT-qPCR Samples

We assessed the specificity of the primer pairs using DNA from pure cultures of 84 strains (**Supplementary Data Sheet 1**, off-target strains sheet). For each strain, the cultivation from stock culture and the DNA extraction were performed twice independently. With the exception of *Leuconostoc mesenteroides* (four strains) and *Lacticaseibacillus casei* (two strains), three strains of each target species were selected. In addition, we also selected DNA of 12 type strains of species often occurring in dairy products or closely related to one of the target species (*Leuconostoc carnosum* and *Streptococcus salivarius*). The HT-qPCR was performed with DNA samples diluted to 3 ng/µl.

A mock community consisting of the type strains of the 24 target species/subspecies at concentrations of about 1×10^6 copies/µl was also prepared (**Supplementary Data Sheet 1**, Mock community sheet). The DNA concentration for the corresponding number of genome copies was estimated by taking the genome size of the type strain, if available. Otherwise, we used the average genome size[1] and an average weight of 1.096×10^{-21} g per base pair. A 10-fold dilution series

[1]https://www.ncbi.nlm.nih.gov/genome

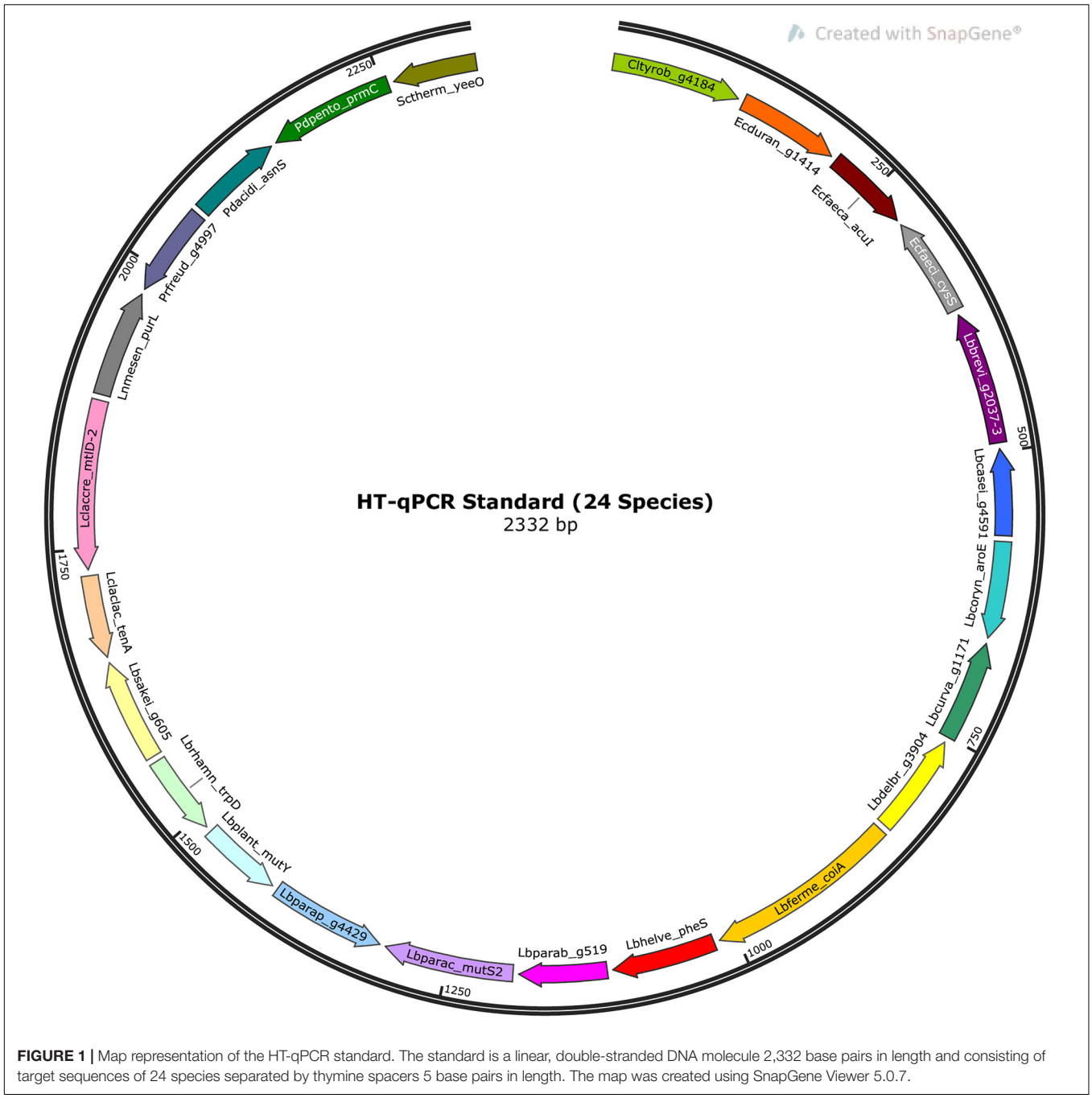

FIGURE 1 | Map representation of the HT-qPCR standard. The standard is a linear, double-stranded DNA molecule 2,332 base pairs in length and consisting of target sequences of 24 species separated by thymine spacers 5 base pairs in length. The map was created using SnapGene Viewer 5.0.7.

of the mock community was prepared and subjected to preamplification to enrich the target sequences in the mock community dilutions (10^4–10 copies/µl). Mock community dilutions without preamplification (10^5–10^2 copies/µl) were also measured.

192.24 Dynamic Array IFC Setup

The validation was performed on multiple 192.24 Dynamic Array IFCs. All samples (pure bacterial culture DNAs, no template controls, mock community, and HT-qPCR standard dilution series) were included, and eight primer pairs were measured in triplicate in each run.

Production of Model Cheeses With Adjunct Cultures

Fifteen model cheeses with adjunct cultures of selected target species [*Levilactobacillus brevis, L. casei, Loigolactobacillus coryniformis, Latilactobacillus curvatus, Limosilactobacillus fermentum, L. helveticus, Lentilactobacillus parabuchneri, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum,*

Lacticaseibacillus rhamnosus, *Latilactobacillus sakei*, *Leuconostoc mesenteroides*, *Pediococcus* (*Pd.*) *acidilactici*, *Pd. pentosaceus*, and *Propionibacterium* (*Pr.*) *freudenreichii*] and 4 control cheeses (without adjunct cultures) were produced in the experimental cheese dairy at Agroscope (Bern, Switzerland) on four different days. The experimental design for the production of the 19 model cheeses and the conditions used for the preparation of the 15 adjunct cultures are listed in **Supplementary Table S2**. The pasteurized vat milk was inoculated by centrifuging 50 ml of each adjunct culture (4,000 × g, room temperature, 10 min) and resuspending in 50 ml sterile reconstituted skim milk powder (10% w/v) before addition to the milk. The estimated concentration of adjunct culture in the milk vat was 10^4–10^5 CFU/ml.

The Raclette-type semi-hard model cheeses were produced from 50 l of pasteurized milk, using a combination of the mesophilic starter RSW 901 (*Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *diacetylactis*) and the mixed mesophilic/thermophilic starter MK 401 (*Lc. lactis* ssp. *lactis*, *Lactobacillus delbrueckii* subsp. *lactis* and *S. thermophilus*) (Liebefeld Kulturen AG, Switzerland). The milk was pre-ripened at 28–32°C for 30 min, followed by rennet addition and coagulation for 25 min at 32°C, and then cutting and stirring at 32°C for 25 min. The temperature was then increased to 36°C for 10 min and the milk was stirred for a further 35 min. The whey-curd mixture was filled into molds and pressed for 4 h at 34°C, 4 h at 32°C, and finally 8 h at 28°C. The cheeses (30 cm in diameter, about 6 kg) were immersed in a 20% (w/w) saline solution (11–13°C, 14 h), and smear-ripened in a maturing cellar (10–11°C, 90–96% relative humidity) for up to 120 days. The samples were collected after 111, 113, 118, and 120 days of ripening for the cheeses manufactured on days 1, 2, 3, and 4, respectively.

HT-qPCR Application on Cheese Samples

We calculated the copy numbers for quantification using standard calibration curves ranging from 10^7 to 10^3 copies/µl (**Supplementary Figure S2** in **Supplementary Data Sheet 3**). The measurement was performed on a single 192.24 Dynamic Array IFC. All samples (HT-qPCR standard dilutions, no template controls, and cheese samples) were measured in technical triplicates.

Data Analysis

Results from the 192.24 Dynamic Array IFCs for the validation runs were combined for the analysis with the Fluidigm Real-Time PCR Analysis Software version 4.5.2 (Fluidigm Corp.). The quality threshold was set to 0.5, the quantification cycle (Cq) threshold for all reactions was set to 0.05, and the baseline correction was set to constant. The settings used for the melting curve analysis were: a peak sensitivity of 3 and a peak ratio threshold of 0.8, the qPCR assay-specific peak detection ranges are available in **Supplementary Table S3**. The melting curve peak threshold was set to 0.05 −dRn/dT for the validation runs and to 0.025 −dRn/dT for the run with the cheese samples, based on visual inspection of the baseline fluorescence. The Real-Time PCR Analysis Software flags all reactions that do not conform to the selected thresholds (i.e., low quality score, multiple or no melting curve peaks, or reactions where the normalized fluorescence is below the threshold). The data were then exported to a csv file. A python script (biomarkdataparser.py) was used to filter the data and to calculate the number of copies/µl in the samples based on the calibration curves. All reactions flagged by the Real-Time PCR Analysis Software were interpreted as negative results. The copies/µl of the specific targets were calculated for each reaction using the standard calibration curves, and all reactions below an 800 copies/µl cut-off were interpreted as negative, as recommended by the manufacturer (Fluidigm, 2018). Average copies/µl were only calculated if at least two of three reactions were positive; otherwise, the results were interpreted as negative. The raw data (csv export) from the Real-Time PCR Analysis Software, the biomarkdataparser.py script and the jupyter-notebooks used to make the figures are available in **Supplementary Data Sheet 4** and on GitHub[2].

Analysis of Volatile Carboxylic Acids and Biogenic Amines

Volatile carboxylic acids in cheese were esterified with ethanol, and analyzed by gas chromatography as described by Fröhlich-Wyder et al. (2013) using a Hewlett Packard HP 6890 gas chromatograph (Agilent Technologies, Basel, Switzerland) equipped with a Hewlett Packard Ultra 2 cross linked phenyl methyl silicone fused silica capillary column (50 m, 0.32 mm, 0.52 mm) and a flame ionization detector (FID). Biogenic amines in cheese were analyzed as described by Ascone et al. (2017) using a UPLC system (UltiMate 3000 RS; Thermo Fisher Scientific, Reinach, Switzerland) equipped with a C18 column (Accucore C18: 2.6 mm, 150 × 4.6 mm; Thermo Fisher Scientific, Reinach, Switzerland). All measurements were carried out in duplicate.

RESULTS

Specificity of the qPCR Assays

The inclusivity of the qPCR assays was assessed by performing standard qPCR with DNA from single strains of each target species (**Supplementary Data Sheet 5**). The inclusivity was 100% for all the tested qPCR assays (**Table 2**). The qPCR assay for *L. casei* was only tested with two *L. casei* strains, due to the limited availability of these strains in public strain collections. The *in silico* validation of the primer pair showed that all available genomes of *L. casei* and *L. zeae* contain a perfectly matching target sequence, in contrast to genomes of any other species of the *Lactobacillaceae* family (NCBI:txid33958) available in the NCBI Microbial Genomes BLAST database, including complete and draft genomes (as of July 2020; data not shown).

The specificity of the qPCR assays was assessed by performing HT-qPCR with DNA from single strains of two to four strains of the target and selected type strains of off-target species. The raw Cq data showed high quantification cycles for several off-target reactions, mainly for the qPCR assays for the detection

[2]https://github.com/biologger/htqpcr_validation_data

TABLE 2 | Standard qPCR results of inclusivity assessment.

Species	Mean Cq	SD	Mean T_m	SD	Inclusivity
Clostridium tyrobutyricum	14.32	1.05	80.02	0.14	25/25
Enterococcus durans	14.5	1.27	81.3	0.22	25/25
Enterococcus faecalis	14.55	0.71	75.5	0.0	22/22
Enterococcus faecium	11.95	0.53	80.8	0.11	25/25
Levilactobacillus brevis	14.65	2.21	82.55	0.11	18/18
Lacticaseibacillus casei	14.36	0.41	83.4	0.12	2/2
Loigolactobacillus coryniformis	12.95	0.4	83.19	0.16	19/19
Latilactobacillus curvatus	13.4	0.58	82.21	0.09	25/25
Lactobacillus delbrueckii	14.62	1.15	83.52	0.09	34/34
Limosilactobacillus fermentum	14.19	1.29	87.12	0.1	24/24
Lactobacillus helveticus	14.42	1.32	78.42	0.13	24/24
Lentilactobacillus parabuchneri	13.98	1.45	81.56	0.29	25/25
Lacticaseibacillus paracasei	13.88	1.06	84.34	0.23	21/21
Lactiplantibacillus paraplantarum	14.04	1.02	84.21	0.23	14/14
Lactiplantibacillus plantarum	13.86	0.67	76.94	0.1	24/24
Lacticaseibacillus rhamnosus	13.93	1.42	81.92	0.48	24/24
Latilactobacillus sakei	11.98	0.45	83.0	0.06	24/24
Lactococcus lactis subsp. cremoris	14.28	1.18	81.23	0.17	25/25
Lactococcus lactis subsp. lactis	14.53	0.87	80.02	0.2	25/25
Leuconostoc mesenteroides	13.68	1.14	82.28	0.32	23/23
Pediococcus acidilactici	11.61	0.46	78.89	0.22	20/20
Pediococcus pentosaceus	12.64	0.96	78.48	0.1	25/25
Propionibacterium freudenreichii	15.1	0.85	85.5	0.03	25/25
Streptococcus thermophilus	13.73	0.82	78.89	0.24	25/25

Mean values and standard deviation (SD) of quantification cycle (Cq) and melting temperature (T_m) of inclusivity assessment by standard qPCR for strains of the target species. Raw data is available in ***Supplementary Data Sheet 5****.*

of *L. delbrueckii*, *Lc. lactis subsp. lactis,* and *S. thermophilus* (**Supplementary Figure S3** in **Supplementary Data Sheet 3**). Background noise was reduced by applying two filter criteria to the data: all reactions flagged by the analysis software and all reactions with fewer than 800 copies/µl were interpreted as negative reactions, as recommended by the manufacturer (**Supplementary Figure S4** in **Supplementary Data Sheet 3**). All target species strains were detected by HT-qPCR, and only one cross-reaction was detected with the filtered average Cq values (**Figure 2**). The cross-reaction of the *Lactiplantibacillus paraplantarum* assay with the off-target strain *L. coryniformis* (DSM 20004) was only detected in one of two different DNA extracts, and the Cq value was about eight cycles higher than that for the *L. paraplantarum* DNA samples. In summary, the *L. paraplantarum* assay had a specificity of 0.9939, while all other tested assays were specific.

Sensitivity and Dynamic Range of the qPCR Assays

The qPCR assay performance was assessed with a 10-fold dilution series of the qPCR standard consisting of all 24 target sequences in a range from 10^8 to 10^3 copies/µl. The calculated efficiency of the qPCR assays ranged between 87 and 97%. The linear regression equations ($Cq\ slope * log$[copies] intercept) had slopes between −3.39 and −3.68 and correlation coefficients between 0.992 and 0.998. The sensitivity of the assays without preamplification is given by the cut-off Cq value corresponding to 800 copies/µl (Cq 23.8–26.4), as calculated using the linear equations of the standard calibration curves (**Supplementary Figure S1** in **Supplementary Data Sheet 3**).

We validated the quantification of the targets in mixtures by HT-qPCR analysis of samples of a 10-fold dilution series of a mock community consisting of DNA from 24 type strains in a range between 10^5 and 10^2 copies/µl. All targets were detected in the diluted mock community sample containing 10^4 copies/µl, and 14 of 24 assays detected the target a dilution of 10^3 copies/µl (**Figure 3**). The concentrations of target DNA in the mock community were calculated based on the initial DNA concentration of the single strain sample and the genome size of the target species (**Supplementary Data Sheet 1**, Mock community sheet). The predicted concentrations were compared to the measured copies/µl (**Supplementary Figure S5** in **Supplementary Data Sheet 3**). The assays for the detection of *L. brevis, L. sakei*, *L. paracasei*, *Pr. freudenreichii,* and *S. thermophilus* had lower initial concentrations of the target sequence than predicted. By contrast, the assays for *Enterococcus* (*E.*) *durans*, *E. faecalis*, *Lc. lactis subsp. lactis*, *Pd. acidilactici,* and *Pd. pentosaceus* showed similar values for the predicted and measured number of copies/µl, though the assays did not detect the target in the diluted sample containing 10^3 copies/µl, whereas the 14 other assays did.

Preamplification Efficiency

The increase in sensitivity due to preamplification reactions was assessed by preamplification of a 10-fold dilution series of a mock community consisting of DNA from 24 type strains in a range between 10^4 and 10 copies/µl and subsequent HT-qPCR analysis. All species were detected down to a dilution of 10^2 copies/µl in the pre-amplified mock community sample, whereas in the samples with the highest dilution of 10 copies/µl, 14 of 24 targets were detected (**Figure 3**). The efficiency of the preamplification reaction for the qPCR assays was assessed by comparing the Cq values obtained for a diluted mock community sample (10^4 copies/µl) with preamplification to Cq values without preamplification (**Table 3**). The Cq values for the sample with preamplification decreased, on average, by 7.43 cycles (range

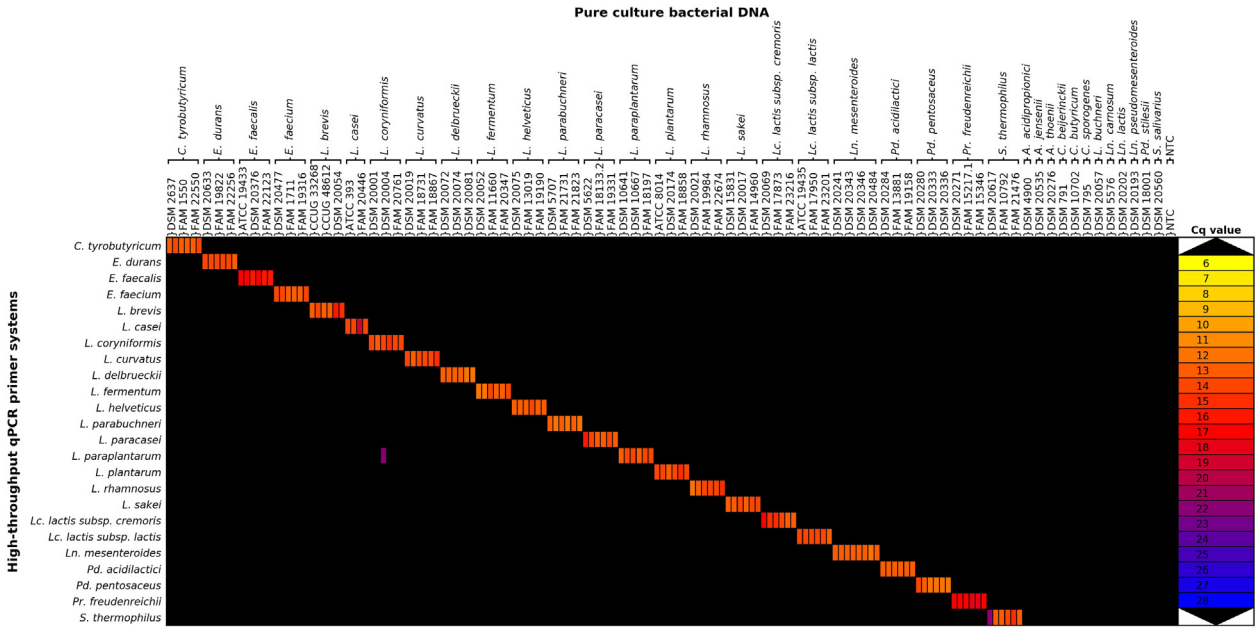

FIGURE 2 | Heatmap of filtered average quantification cycle data of the exclusivity assessment. Average Cq values of the filtered Cq data are represented, but only data points where at least two of the three technical replicates were positive are depicted. Raw Cq data and filtered Cq data heatmaps are available in **Supplementary Figures S3, S4** in **Supplementary Data Sheet 3**. *A.*, *Acidipropionibacterium*; *C.*, *Clostridium*; *E.*, *Enterococcus*; *L.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *Pd.*, *Pediococcus*; *Pr.*, *Propionibacterium*; *S.*, *Streptococcus*; NTC, no template control.

6.49–9.85) compared to the Cq values for the sample without preamplification.

Application of HT-qPCR to Raclette-Type Model Cheese

The ability of the HT-qPCR system to quantify the target species in real cheese DNA samples was verified by manufacturing 19 Raclette-type model cheeses with the target species adjuncts. The DNA extracts from 19 Raclette-type model cheeses were analyzed by HT-qPCR (**Figure 4**). The volatile carboxylic acids and biogenic amines of the cheeses were also analyzed, as these metabolites are often elevated in defective cheeses and serve as indicators of the presence of undesirable microorganisms.

The four starter LAB species (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *L. delbrueckii,* and *S. thermophilus*) were detected in all cheese DNA samples. All 15 adjunct culture species were detected in the corresponding cheese DNA sample, except for sample S07, where no *L. helveticus* was detected. Low concentrations of *L. helveticus* were detected in sample S06, indicating that the adjunct culture with *L. helveticus* had mistakenly been added to the wrong cheese vat. In several samples, cross-contaminations of target species from cheeses that were produced on the same production day were detected at distinct lower concentrations. The concentration of propionic acid was elevated in cheese samples S13 (14.93 mmol/kg) and S18 (37.83 mmol/kg) and, to a lesser extent, in cheese S17 (4.53 mmol/kg) and S19 (2.5 mmol/kg). Increased amounts of tyramine were measured in cheese samples S2 (171.78 mg/kg), S3 (284.67 mg/kg), S5 (482.33 mg/kg), and S19 (155.72 mg/kg), while in samples S5 and S8, the concentration of putrescine (292.8 mg/kg) and histamine (320.35 mg/kg) were increased, respectively.

Application of HT-qPCR to Downgraded Commercial Cheeses With Quality Defects

The potential of the HT-qPCR system to identify the microbial causes of cheese defects was demonstrated by HT-qPCR analysis of DNA extracts from two commercial cheese samples with quality defects (the C1 alpine cheese and C2 Raclette cheese, **Figure 5**).

The alpine cheese sample (C1) had increased concentrations of propionic acid (36.5 mmol/kg) and biogenic amines, mainly histamine (733 mg/kg) and to a lesser extent tyramine (398 mg/kg) and putrescine (417 mg/kg). The cheese contained high concentrations of the typical thermophilic starter species *S. thermophilus,* whereas *L. delbrueckii. L. helveticus,* and *L. parabuchneri* were present at concentrations over 10^5 copies/µl and *L. coryniformis*, *L. curvatus*, *L. paracasei,* and *Pd. pentosaceus* had concentrations between 10^4 and 10^5 copies/µl. Low concentrations ($<10^4$ copies/µl) of *E. faecalis* and *L. paraplantarum* were detected.

The Raclette cheese sample (C2) had elevated levels of biogenic amines, mainly tyramine (545 mg/kg), but also histamine (185 mg/kg). Both subspecies of *Lc. lactis* and *Leuconostoc mesenteroides* used in mesophilic starters were detected, with *Lc. lactis subsp. lactis* as the predominant species at more than 10^6 copies/µl. *L. helveticus* and *L. parabuchneri*

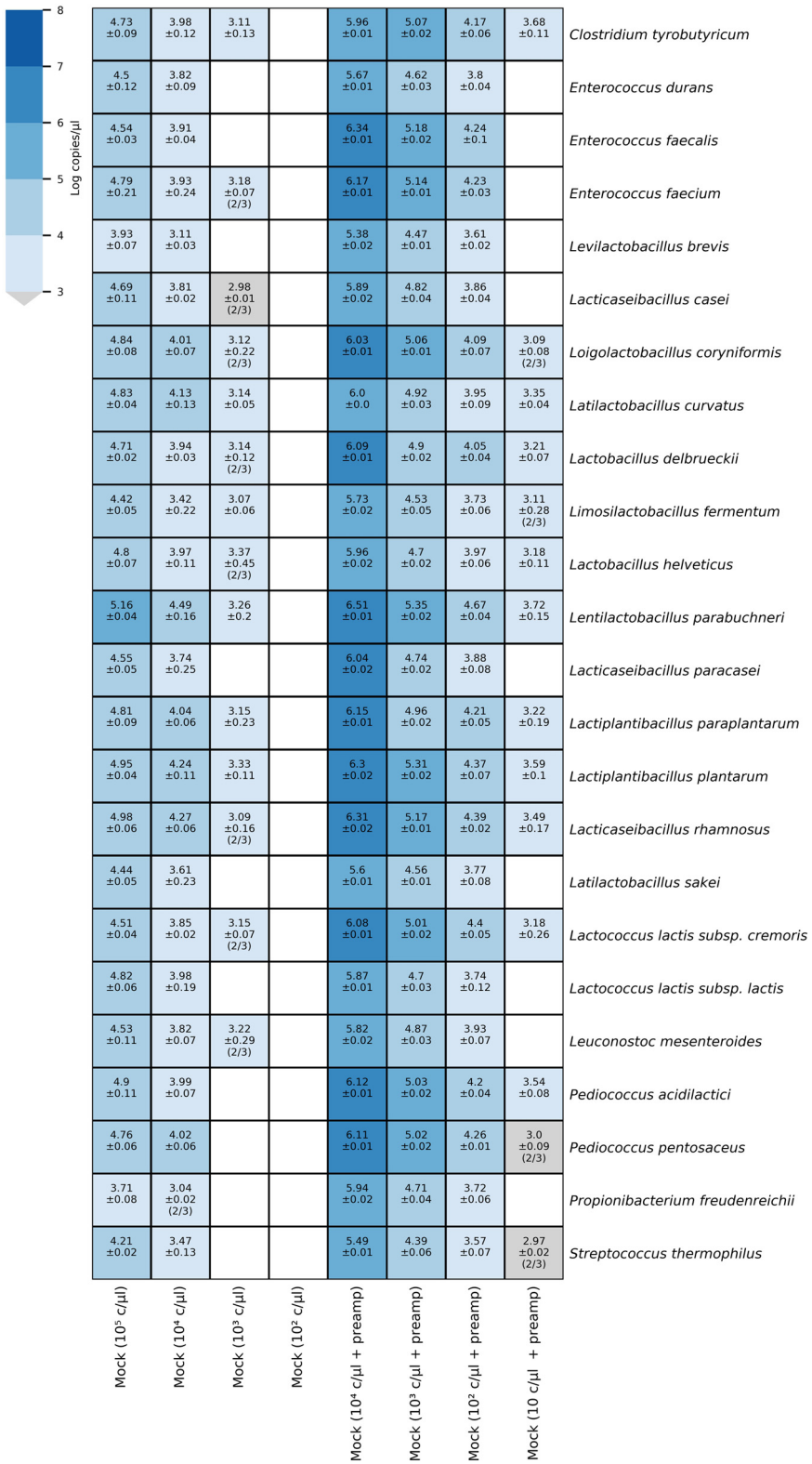

FIGURE 3 | Heatmap of microfluidic qPCR results for mock community dilutions. The heatmap annotation depicts the average logarithmic copies/µl and the standard deviation. When not all samples were positive, the number of positive samples out of the total number of samples (triplicates) is given in brackets. On the left side, the results correspond to the diluted mock community DNA samples quantified without preamplification whereas, the diluted mock community DNA samples depicted on the right side were pre-amplified.

TABLE 3 | Quantification cycle values of a mock community sample with and without preamplification.

Species	Cq before preamplification	Cq after preamplification	ΔCq
Clostridium tyrobutyricum	20.71	13.67	7.05
Enterococcus durans	21.51	15.02	6.49
Enterococcus faecalis	22.89	14.43	8.45
Enterococcus faecium	21.10	13.32	7.78
Levilactobacillus brevis	23.42	15.51	7.90
Lacticaseibacillus casei	21.70	14.40	7.30
Loigolactobacillus coryniformis	20.65	13.56	7.09
Latilactobacillus curvatus	20.68	14.20	6.49
Lactobacillus delbrueckii	20.53	13.13	7.40
Limosilactobacillus fermentum	22.51	14.28	8.23
Lactobacillus helveticus	21.07	14.08	6.99
Lentilactobacillus parabuchneri	19.35	12.22	7.12
Lacticaseibacillus paracasei	21.21	13.24	7.97
Lactiplantibacillus paraplantarum	20.22	12.86	7.36
Lactiplantibacillus plantarum	20.96	13.60	7.36
Lacticaseibacillus rhamnosus	20.15	12.96	7.19
Latilactobacillus sakei	21.86	14.99	6.87
Lactococcus lactis subsp. cremoris	22.42	14.20	8.22
Lactococcus lactis subsp. lactis	20.85	14.32	6.53
Leuconostoc mesenteroides	21.00	13.96	7.04
Pediococcus acidilactici	20.55	13.15	7.40
Pediococcus pentosaceus	20.05	12.76	7.29
Propionibacterium freudenreichii	23.31	13.46	9.85
Streptococcus thermophilus	22.04	15.20	6.84

were found at concentrations of about 10^5 copies/μl, whereas *L. paracasei*, *L. rhamnosus,* and *Pr. freudenreichii* were present at concentrations below 10^4 copies/μl.

DISCUSSION

Validation of qPCR Assays and the Microfluidic HT-qPCR System

The qPCR assays validated in this study were highly specific. However, the qPCR assay for *L. casei* is not able to differentiate between *L. casei* and *L. zeae* species, two similar species for which a reclassification was recently proposed (Huang et al., 2020). The false positive cross-reaction of the *L. paraplantarum* assay in one *L. coryniformis* DNA sample was most likely due to a cross-contamination. A similar melting curve peak and the negative result of an independent DNA extraction from the same pure cultured strain support this assumption.

Background fluorescence signals in the raw data were mainly caused by three qPCR assays, specifically the assays for *L. delbrueckii*, *Lc. lactis* subsp. *lactis*, and *S. thermophilus*. Background signals may occur due to weak amplification of primer dimers in samples without the target sequence. The qPCR assay-specific cut-off values (equivalent to 800 copies/μl) were calculated from standard calibration curves and used to reduce background signals. Measures to increase the signal to the background ratio have also been reported in other microfluidic HT-qPCR studies. For example, a previous study (Ishii et al., 2013) reported that some (TaqMan) probes had to be redesigned because the probes failed to obtain sufficiently strong signals to separate them from background signals. Another study (Hermann-Bank et al., 2013) developed the Gut Microbiotassay on a 48 × 48 Access Array (Fluidigm Corp.) and excluded Cq values exceeding primer-specific cut-off values during data analysis.

The sensitivity of the tested qPCR assays was limited by the nanoliter-scale reactions used in the microfluidic qPCR system. This limitation for microfluidic HT-qPCR can be addressed by adding a preamplification step as a part of the experimental workflow. Preamplification increased the sensitivity of all assays in the mock communities. However, the delta Cq values calculated from target sequences and pre-amplified target sequences differed considerably for the 24 qPCR assays; consequently, the Cq data from samples with pre-amplification do not allow a reliable quantitative analysis and can therefore, only be used for qualitative detection of targets.

Application of Microfluidic HT-qPCR to Cheese Samples

Given the technical limit of 800 copies/μl for qPCR reactions, the theoretical limit of detection of the assays was calculated as 8×10^4 genome equivalents/g cheese. However, it should be noted that for culture-independent quantitative methods, the DNA extraction method can have a significant impact on the results obtained. It is known that residues from the food matrix such as fats, proteins and calcium in DNA samples can inhibit subsequent PCR reactions (Wilson, 1997). DNA extraction can also have an influence on the recovery rates of different bacteria, e.g., due to the different composition of cell walls and the resulting differences in the efficiency of cell lysis, such as between Gram-positive and Gram-negative bacteria (Quigley et al., 2012). Starter LAB grow very fast during cheese production, typically reaching counts of $>10^8$ CFU/g within the first 24 h. By contrast, the growth of NSLAB is significantly slower and occurs mainly during the first weeks of ripening, reaching bacterial counts of 10^6–10^8 CFU/g, depending on the species (Fox et al., 2017). Quantitative studies have shown that the population density of species relevant for the organoleptic quality of cheese typically ranges from 10^6 to 10^{10} genome equivalents/g cheese (Falentin et al., 2010, 2012; Turgay et al., 2011; Desfosses-Foucault et al., 2012; Moser et al., 2018). At lower population densities, the formation of metabolites is too low to be reliably perceived by

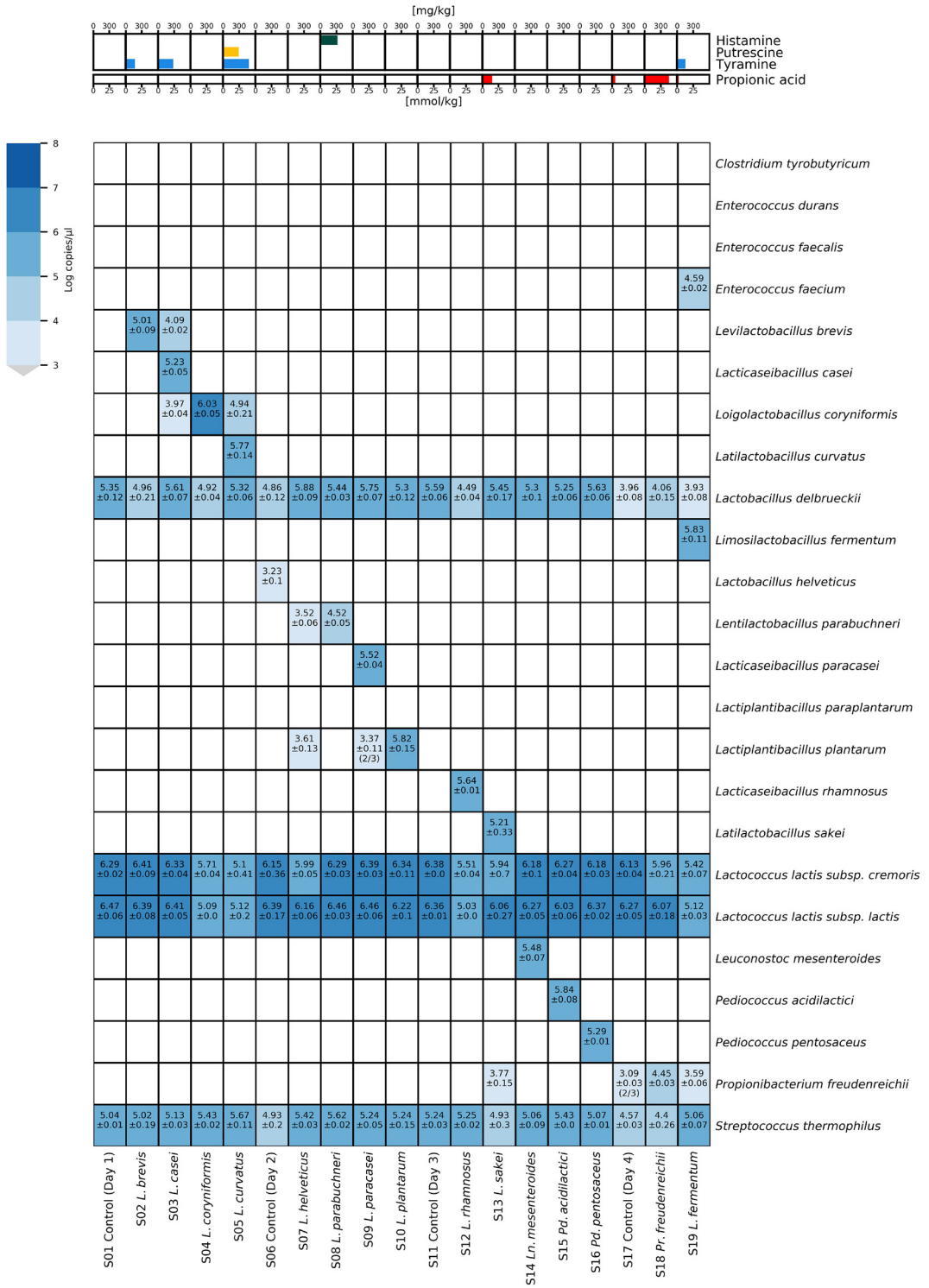

FIGURE 4 | Heatmap of microfluidic HT-qPCR results of Raclette-type model cheese samples produced with adjunct cultures. The results of the chemical analysis of the model cheese are shown in the upper panel, while the results of the HT-qPCR analysis (without preamplification) are shown in a heatmap in the lower panel.

sensory perception. The results obtained from the HT-qPCR analysis of the mock community dilutions indicate that all assays are able to quantify a minimal population density of 10^6 genome equivalents/g cheese. Despite this rather high detection limit, HT-qPCR would still be a valuable tool for cost-effective monitoring of species relevant for the sensory quality of cheese. In addition,

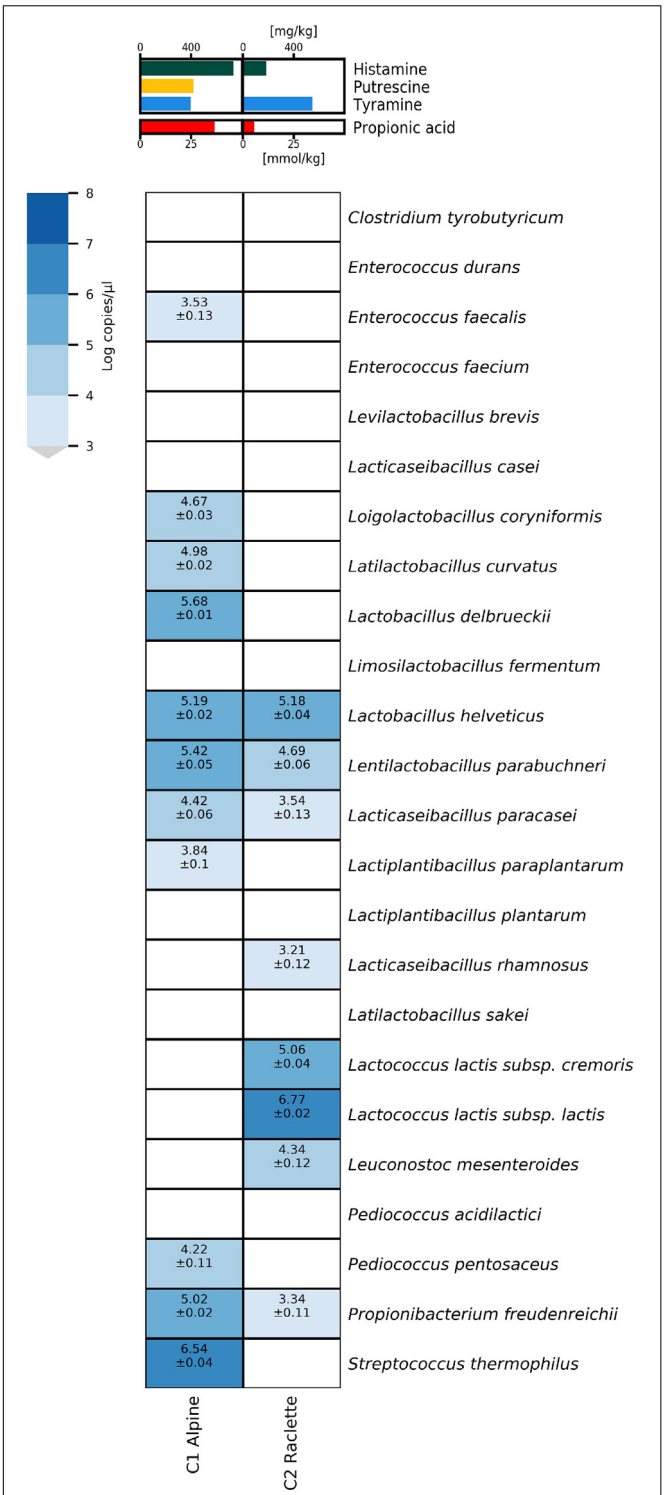

FIGURE 5 | Heatmap of microfluidic qPCR results (without preamplification) for commercial cheese samples with defects.

detection of species with lower abundancies (e.g., NSLAB species) in early stages of ripening could optionally be achieved using a preamplification step.

The application of the HT-qPCR system to model and commercial cheese samples was used to show the potential of the new method to detect a broad range of quality-relevant species in cheese samples, including starter LAB, NSLAB, and raw milk-associated contaminants that may cause severe cheese defects during ripening. The application of the microfluidic qPCR assays on model cheeses with adjunct cultures of selected target species confirmed the successful detection and quantification of these target species in cheese DNA samples. In addition, we observed the presence of bacteria that had not been deliberately added with the adjunct cultures in several cheese samples. These cross-contaminations most likely originated from equipment used in parallel during the simultaneous production of the experimental cheeses on the same day (e.g., cheese harps used for cutting the curd and the system used for filling the curd/whey mixture into the cheese molds). However, the unexpected presence of *Pr. freudenreichii* in sample S13 remains unexplained, as no adjunct culture with *Pr. freudenreichii* was used on that production day. The growth of *Pr. freudenreichii* in the cheese in sample S13 resulted in a similarly increased concentration of propionic acid (14.9 mmol/kg) as in other cheeses (S17, S18, and S19) in which *Pr. freudenreichii* was detected (**Figure 4**). Studies examining the environment and production facilities of cheese dairies show that bacteria present in raw milk and cheese are quite abundant and can persist on surfaces, despite frequent cleaning (Somers et al., 2001; Bokulich and Mills, 2013; Stellato et al., 2015). The source of the *E. faecium* contamination in S19 was identified as a contaminated stock culture of one of the used *L. fermentum* strains, as confirmed by partial 16S rRNA gene sequencing of single-colony DNA (**Supplementary Data Sheet 6**).

The selection of qPCR assays designed for the HT-qPCR system included species of undesirable bacteria found in raw milk. Various microbiologically induced quality defects in cheese are related to contamination of the processed milk with undesirable bacteria. The most common microbial causes of cheese defects are faulty fermentations, such as butyric acid fermentation (typically caused by *Clostridium tyrobutyricum*) and propionic acid fermentation (typically caused by *Pr. freudenreichii*), and the formation of biogenic amines (Bachmann et al., 2011). Tyramine, histamine, cadaverine, putrescine, and β-phenylethylamine (PEA) are the most abundant biogenic amines in cheese (Linares et al., 2011). Various NSLAB species play an important role in the excessive formation of biogenic amines in cheese (Barbieri et al., 2019). The formation of biogenic amines is a strain-specific characteristic of various NSLAB species. For example, strains of *L. parabuchneri* have been repeatedly isolated from cheeses heavily contaminated with histamine, whereas aminogenic strains of *E. faecium* are often present in cheeses with elevated tyramine content. Similarly, strains of *L. curvatus* have been shown to be potent producers of tyramine and putrescine (Benkerroum, 2016; Diaz et al., 2016; Wüthrich et al., 2017). The determination of metabolites like volatile carboxylic acids and biogenic amines often provides helpful information that clarifies the microbial origin of faulty fermentations and other cheese defects. However, the simultaneous quantitative determination of undesirable bacterial species using HT-qPCR opens up new perspectives for an efficient and cost-effective

diagnosis of the causes of microbially induced cheese defects. Notably, the early and reliable detection of the microbial causes of cheese defects is an important precondition for tracing the sources of contamination and taking corrective actions.

In the model cheese experiments, samples with elevated tyramine content contained either *L. brevis*, *L. curvatus,* or *E. faecium*; all three species are known tyramine producers (Coton and Coton, 2009; Bunkova et al., 2010; Ladero et al., 2012). Sample S05 containing *L. curvatus* also showed elevated levels of putrescine, while sample S08 containing *L. parabuchneri* had elevated levels of histamine.

In the alpine cheese (sample C1), the histamine concentration was strongly increased (733 mg/kg), and an increased population density (5.42 log copies/µl) of *L. parabuchneri* was detected. The additional presence of *E. faecalis* and *L. curvatus* likely explains the formation of tyramine and putrescine. Moreover, the increased concentration of propionic acid correlates with the increased numbers of *Pr. freudenreichii* detected in this sample.

Similarly, the detection of *L. parabuchneri* most likely explains the increased concentration of histamine in the defective commercial Raclette cheese (sample C2). However, the results of the HT-qPCR analysis did not allow identification of a species that could account for the elevated tyramine content. In all likelihood, a species not covered by our qPCR assays was responsible for the high concentrations of tyramine. Strains of several *Lactobacillus* species other than the NSLAB species targeted here have been reported to produce tyramine (Bunkova et al., 2010; Benkerroum, 2016).

The setup of the method described here allows the exchange or extension of the qPCR assays for the detection of additional species or functional genes (e.g., the *hdc* gene, important in histamine production). Furthermore, the outlined workflow allows an efficient validation of new primer pairs for integration into the HT-qPCR system. We demonstrated here the potential of the HT-qPCR system to quantify simultaneously multiple bacterial species in cheese DNA samples. However, this approach could also be of interest for the investigation of other fermented foods such as kimchi, sauerkraut or sausages that also contain complex microbial compositions which include to some extent the same LAB species as present in cheese (Plengvidhya et al., 2007; Cocolin et al., 2009; Jung et al., 2011).

The HT-qPCR approach presented in this study offers a fast and affordable simultaneous quantitative screening of 24 species/subspecies relevant for the quality of cheese. A single 192.24 Dynamic Array IFC chip enables the screening of 56 cheese DNA samples in technical triplicates from 24 species/subspecies in several hours. Moreover, the developed script for data cleaning and visualization then allows immediate visualization and interpretation of the data exported from the Fluidigm Real-Time PCR Analysis software, thereby facilitating the rapid interpretation of the data. The high sample capacity of the microfluidic high-throughput system and the high specificity of the qPCR assays are key factors required for fast, accurate, and cost-efficient monitoring of desired and undesired microorganisms affecting sensory cheese quality.

Another advantage is that the system can easily be expanded with additional assays to cover further product-specific species or to adapt the system to other fermented products. Preliminary results from model cheeses and downgraded commercial cheeses showed that the application of HT-qPCR to complex fermented products such as cheese could be of interest for identification of the microbiological causes of sensorially perceivable quality defects. Particularly in the production of raw milk cheese, the application of HT-qPCR could be very useful for monitoring the composition of the ripening microbiota, thereby ensuring a constant product quality.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MD, HB, NS, DW, and PJ conceived and designed the experiments, authored or reviewed drafts of the manuscript, and approved the final draft. MD performed the experiments, analyzed the data, and prepared figures and tables. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.619166/full#supplementary-material

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Population Genomic Analysis of *Listeria monocytogenes* From Food Reveals Substrate-Specific Genome Variation

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Listeria monocytogenes is the major causative agent of the foodborne illness listeriosis. Listeriosis presents as flu-like symptoms in healthy individuals, and can be fatal for children, elderly, pregnant women, and immunocompromised individuals. Estimates suggest that *L. monocytogenes* results in ∼1,600 illnesses and ∼260 deaths annually in the United States. *L. monocytogenes* can survive and persist in a variety of harsh environments, including conditions encountered in production of fermented dairy products such as cheese. For instance, microbial growth is often limited in soft cheese fermentation because of harsh pH, water content, and salt concentrations. However, *L. monocytogenes* has caused a number of deadly listeriosis outbreaks through the contamination of cheese. The purpose of this study was to understand if genetically distinct populations of *L. monocytogenes* are associated with particular foods, including cheese and dairy. To address this goal, we analyzed the population genetic structure of 504 *L. monocytogenes* strains isolated from food with publicly available genome assemblies. We identified 10 genetically distinct populations spanning *L. monocytogenes* lineages 1, II, and III and serotypes 1/2a, 1/2b, 1/2c, 4b, and 4c. We observed an overrepresentation of isolates from specific populations with cheese (population 2), fruit/vegetable (population 2), seafood (populations 5, 8 and 9) and meat (population 10). We used the Large Scale Blast Score Ratio pipeline and Roary to identify genes unique to population 1 and population 2 in comparison with all other populations, and screened for the presence of antimicrobial resistance genes and virulence genes across all isolates. We identified > 40 genes that were present at high frequency in population 1 and population 2 and absent in most other isolates. Many of these genes encoded for transcription factors, and cell surface anchored proteins. Additionally, we found that the virulence genes *aut* and *ami* were entirely or partially deleted in population 2. These results indicate that some *L. monocytogenes* populations may exhibit associations with particular foods, including cheese, and that gene content may contribute to this pattern.

Keywords: *Listeria*, dairy, microbial genomics, pathogen, food safety

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INTRODUCTION

Listeria monocytogenes is a gram positive, foodborne pathogen that causes the severe infection listeriosis (Buchanan et al., 2017). Listeriosis infects an estimated 1,600 people and causes about 260 deaths annually in the United States[1]. Although listeriosis is relatively rare, it is regarded as a serious public health concern due to its high mortality rate (20–30%) (Choi et al., 2018). Additionally, infants, the elderly, and immunocompromised individuals are at an increased risk of severe cases of listeriosis, which can result in symptoms such as meningitis and septicemia (Jacquet et al., 2004). *L. monocytogenes* infections can also threaten pregnant women because the pathogen possesses the unique ability to permeate the placental wall and infect the fetus which can lead to miscarriage or stillbirth (Cotter et al., 2008; Hilliard et al., 2018).

L. monocytogenes is ubiquitous in the environment as a saprophyte, and is abundant in soil and water (Smith et al., 2018). The wide distribution of *L. monocytogenes* in the environment is due to its ability to persist under extreme conditions including cold temperatures, dry conditions, low pH, and high salt environments (Magalhães et al., 2016; Hingston et al., 2017). The ecological range of this species in addition to its ability to form biofilms enables *L. monocytogenes* to contaminate and persist on a variety of substrates. These characteristics result in *L. monocytogenes* frequent contamination of different foods including dairy, meat, seafood, and fresh produce (Jalali and Abedi, 2008; Shamloo et al., 2019). A recent outbreak linked to a mushroom supplier in Korea resulted in 47 illnesses and 4 deaths over the last 3 years across five countries (Centers for Disease Control and Prevention, 2020; Food Safety News, 2020). Another outbreak occurred between February and June of 2020, when six illnesses and two deaths were reported in the Netherlands from contaminated smoked trout filets (Food Safety News, 2020).

There are 13 known serotypes of *L. monocytogenes* that fall into four major phylogenetic lineages (Orsi et al., 2011). Lineage I and II are commonly found in food, human outbreak cases, and in the environment, while lineages III and IV are primarily isolated from ruminant animals (Orsi et al., 2011). Lineage I has been associated with epidemic listeriosis cases while lineage II is has been associated with isolates sourced from food and food environments (Nightingale et al., 2005, 2006, 2008; Orsi et al., 2011). This trend suggests that genetic variation between the two lineages results in lineage I's ability to infect humans more efficiently, and lineage II's ability to colonize food substrates. Additionally, lineage II isolates have reduced virulence compared to lineage I (Jacquet et al., 2004; Nightingale et al., 2005). This reduction in virulence may, in part, be due to the observation that over 30% of lineage II isolates contain a premature stop codon in *inlA*, a gene encoding Internalin A, a key virulence factor required for the invasion of epithelial cells (Nightingale et al., 2005; Bonazzi et al., 2009).

L. monocytogenes often occurs in ready-to-eat foods that require minimal heating or cooking, such as cheeses, and other fermented dairy products. There are several possible routes *L. monocytogenes* can enter and contaminate dairy, including transmission from infected ruminants to milk, and through improper pasteurization or the usage of contaminated equipment during post-processing (Melo et al., 2015). The production process and physical attributes of cheese make it a suitable substrate for *L. monocytogenes* growth. The pH range, water content, and salt concentrations of soft cheeses are often inhibitory to other microorganisms, but suitable for *L. monocytogenes* (Melo et al., 2015; Hingston et al., 2017). A survey of 374 European red-smear cheeses revealed 6.4% of cheeses were contaminated with *L. monocytogenes* (Rudolf and Scherer, 2001). In the United States since 2011, a number of multistate foodborne *L. monocytogenes* outbreaks were the result of contaminated cheese, raw milk, and ice cream[2].

The objective of this study was to investigate the association between population structure and the food source from which *L. monocytogenes* strains were isolated. We analyzed the genomes of hundreds of *L. monocytogenes* strains from food, and identified 10 major populations. Specific populations showed associations with cheese, fruit/vegetables, seafood and meat. Using comparative and population genomic analysis, we identified genes that were present at greater frequency in the population associated with cheese and a closely related population. Our results shed light on potential candidate genes involved in the specialization to particular food substrates.

MATERIALS AND METHODS

Whole-Genome Data

We were specifically interested in addressing whether *L. monocytogenes* serotypes display associations with food types, with particular interest in strains isolated from dairy and cheese. To compile a diverse collection of genomes from meat, seafood, dairy, and fruit/vegetables, we used the NCBI Pathogen Detection[3] database. We downloaded whole genome fasta files of 1,213 strains isolated from "cheese", "drain raw meat", "seafood processing environment", "hass avocados", "ice cream", "lettuce", "milk", "plain cream cheese spread", "potato", "raw meat", "raw milk", "raw cut vegetables", "retail meat", "salami", "shrimp", "slaughterhouse environment", and "smoked salmon" on 02/24/2020 (**Supplementary Table S1**). The non-clonally identical isolates of *L. monocytogenes* originated from 19 countries (Canada = 24, United States = 272, Mexico = 10, Brazil = 2, Chile = 12, Uruguay = 5, Denmark = 1, France = 7, Germany = 1, Greece = 1, Ireland = 3, Italy = 64, Norway = 3, Poland = 2, Switzerland = 10, United Kingdom = 62, South Korea = 1, Australia = 1 and New Zealand = 19) and six major geographic regions (North America (NA) = 296, Central America (CA) = 10, South America (SA) = 19, Europe (EU) = 154, Asia (AS) = 1, and Oceania (OC) = 20). To assess

[1]https://www.cdc.gov/listeria/index.html

[2]https://www.cdc.gov/listeria/outbreaks/index.html

[3]https://www.ncbi.nlm.nih.gov/pathogens/

genome assembly quality, we used BUSCO v3.1.0 to quantify the percentage of complete single copy orthologs present in each *L. monocytogenes* genome using the "bacillales_odb9" dataset[4] (Simão et al., 2015).

Relationship of *L. monocytogenes* Isolates

We initially examined the relationship of the *L. monocytogenes* isolates using an alignment of 1,92,465 polymorphic sites across the 1,213 genomes. SNPs were extracted from whole genome assemblies using the Phylogenetic and Molecular Evolution (PhaME) analysis tool v1.0.2 with default settings (Ahmed et al., 2015), using *L. monocytogenes* serotype 4b str. F2365 as the reference (Nelson et al., 2004). We then identified and removed all but one occurrence of isolates with identical genotypes (i.e., isolates with zero SNP differences were considered clones). After this clonal-correction, we removed sites where minor allele frequency was <0.5%. Our final dataset consisted of 504 isolates and 66,698 SNPs. We used Principal Component Analysis (PCA) to investigate the relationship between the *L. monocytogenes* isolates. PCA was performed in TASSEL with eigenvalue decomposition on the covariance matrix (Bradbury et al., 2007). Additionally, we used RheirBAPS (Tonkin-Hill et al., 2018) and Discriminant analysis of principal components (DAPC) (Jombart et al., 2010) to further analyze population structure, using a subset of 661 SNPs that were spaced evenly throughout the genome. In RheirBAPS we used the parameters max.depth = 2, n.pops = 100, n.extra.rounds = Inf, and assignment.probs = TRUE. RheirBAPS predicts the most likely population number given two levels of clustering (max.depth argument), for which we considered the first level of clustering the optimal population number. For DAPC, the number of distinct populations was predicted using the "find.clusters" *k*-means clustering algorithm and by calculating the Bayesian Information Criterion (BIC) value for each *K* between?1 and 100. Predicting the optimal population number is often unclear and complex in panmictic natural populations, and we considered the optimal population number as the first local BIC minimum. We evaluated the population assignments between RheirBAPS and DAPC with the PCA patterns to define 10 "consensus populations". From here forward, we describe populations 1–10 as P1, P2, P3, P4, P5, P6, P7, P8, P9, and P10.

Lastly, we constructed an approximately maximum likelihood tree from the alignment of 66,698 SNPs using FastTree2 (Price et al., 2010) with 100 bootstrap replicates. For visualization and annotation of the phylogenetic tree, the R packages "ggtree", "ggplot2", and "APE" (Wickham, 2016; Yu et al., 2017; Paradis and Schliep, 2019). These packages were used to produce a tree that displays lineage, serovar and population structure assignments. To better visualize these characteristics, the genetic clusters and various serovars were differentially color coded using a custom color palette created in RColorBrewer using the hexadecimal color picking tool from the (Html Color Picker, 2020).

[4]https://busco-data.ezlab.org/v4/data/lineages/

Genomic Prediction of *L. monocytogenes* Serotypes

In order to assign isolates to major *L. monocytogenes* serotypes, we ran PhaME v1.0.2 again with the 504 isolates and an additional 166 *L. monocytogenes* isolates of known serotypes from Hingston et al. (2017). The genomes from the Hingston et al. (2017) isolates were obtained from NCBI Bioproject PRJNA329415. The *L. monocytogenes* EGD-e reference genome was used as the reference genome in PhaME and was acquired from the NCBI database under RefSeq accession number NC_003210.1. We used PCA to infer the relationships between individuals as described above, and we constructed an approximately-maximum-likelihood phylogenetic tree using FastTree v2.1.10 with 100 bootstrap replicates (Price et al., 2010). Serotyping was inferred from the proximity of each isolate to the isolates with known serotypes in the phylogenetic tree and PCA plot. The 166 additional isolates and EGD-e reference genome were only used for serotyping and were not included in subsequent analyses.

Overrepresentation of Isolates by Population, Geographical Origin, Serotype and Food Source

We used a χ^2 test of independence to test the null hypotheses that variables were randomly distributed between each other (population assignment, geographical origin, serotype, and food source). We considered χ^2 standardized residuals ≥ 2 and ≤ -2 as indicative of an overrepresentation and underrepresentation, respectively, of a particular category. These values represent two standard deviations from the mean. Statistical analysis was conducted in R (R Core Team, 2017).

Identification of Lineage Specific Genes

Because P2 and P1 are so closely related and because P2 displayed an overrepresentation for cheese (**Figures 1**, **2**), we identified genes specific to P2 and genes specific to P1 + P2 using two different approaches. First, we used the LS-BSR pipeline (Sahl et al., 2014) to identify genes present in all isolates (P2, or P1 + P2) and absent in all other isolates by requiring BSR values ≥ 0.8 for gene presence in all isolates, and BSR values ≤ 0.4 for gene absence in all remaining isolates. Specifically, we used the "compare.py" command within LS-BSR to identify genes specific to these particular groups of isolates, with the BSR matrix and a FASTA file of all coding sequences across samples as inputs. We also used a less stringent approach by identifying all genes with an average BSR value ≥ 0.9 in P2 or P1 + P2, and an average BSR value ≤ 0.2 in all other isolates. This approach allows for some variation in gene presence/absence within populations. Second, we used the Roary pipeline to identify genes specific to P2 and P1 + P2 (Page et al., 2015). In this approach, we first used Prokka to annotate each of the 504 *L. monocytogenes* genomes using "−−kingdom Bacteria" and default settings (Seemann, 2014). Next, we used Roary to generate a pan-genome from the Prokka gff files, and used MAFFT (−e and −n options) to generate a core gene alignment (Katoh and Standley, 2013). Finally, we used the "gene_presence_absence.Rtab" output file to determine the

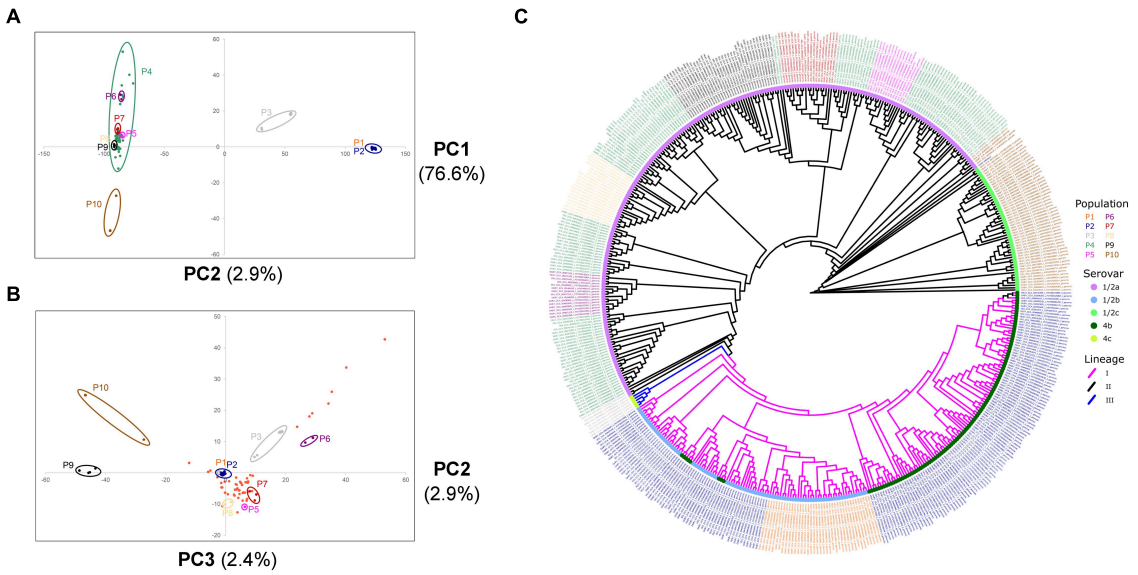

FIGURE 1 | Population structure of 504 *L. monocytogenes* strains isolated from food. Principal component analysis (PCA) of 66,698 SNPs for PC1 and PC2 **(A)** and PC2 and PC3 **(B)**. Percent of variance explained by each PC is reported in parentheses. Isolates are colored and circled based on the consensus population assignment predicted through hierBAPS and DAPC (**Supplementary Figure S2**). P4 is not circled in the PC2 vs. PC3 plot for clarity. **(C)** FastTree2 approximately maximum likelihood phylogenetic tree inferred from the SNP alignment generated by PhaME. Taxon labels, external nodes, and branches are colored according to population assignment, serovar, and lineage, respectively.

presence and absence patterns of genes in P1 and P1 + P2 versus all other populations.

Antimicrobial Resistance and Virulence Gene Typing

We investigated the gene presence/absence patterns of 30 previously characterized antimicrobial resistance genes (Granier et al., 2011; McMillan et al., 2019) across the 10 *L. monocytogenes* populations. We obtained sequences for each gene from the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013). To investigate the distribution of virulence genes across the genetic clusters, a library of 92 *L. monocytogenes* virulence genes were obtained from the virulencefinder database (Joensen et al., 2014). The set of antibiotic resistance genes and virulence genes were independently used as a reference with the "gene screen" method implemented in LS-BSR using the default parameters.

RESULTS

Quality Assessment of *L. monocytogenes* Genome Assemblies

To assess the quality of genome assemblies, we used BUSCO to quantify the percentage of complete Bacillales single copy orthologs found in each of the 504 *L. monocytogenes* isolates analyzed (Simão et al., 2015). The average and median complete BUSCO gene presence percentages were 96.58 and 96.9%, respectively (minimum = 90.3 and maximum = 97%), and 97% of isolates had ≥95% recovery of complete BUSCO genes (**Supplementary Table S1**). These results suggest that the vast majority of analyzed genome assemblies are high quality.

Population Structure of *Listeria monocytogenes* Isolates From Food

Using a number of approaches, we inferred the population structure of 504 non-clonally identical isolates of *L. monocytogenes* from food. First, we used the Bayesian Analysis of Population Structure (BAPS) software (Jombart et al., 2010) to predict population structure using a subset of 661 SNPs that were evenly spaced across the genome to minimize physical linkage between markers. BAPS analysis suggested the presence of 12 populations. Next, we used DAPC with the 661 SNP marker set to predict population structure (Jombart et al., 2010). With DAPC, we observed a local minimum BIC value at $K = 12$, before BIC increased at $K = 13$ and $K = 14$ then decreased again at $K = 15$ (**Supplementary Figure S1**). For this reason, we chose to analyze DAPC population structure at $K = 12$. Individual population assignment between BAPS and DAPC were mostly agreeable with a few exceptions: (1) BAPS population 2 was divided into three DAPC populations (5, 6, and 12), (2) BAPS populations 3 and 4 were combined into DAPC population 1, and (3) BAPS populations 5 and 6 were made up of DAPC populations 7, 8, and 11 (**Supplementary Figure S2**). Next, we performed PCA on the entire set of 66,698 SNPs to visualize population structure. We evaluated the BAPS, DAPC, and PCA population structure to define 10 consensus populations (P1−P10) (**Figures 1A,B** and **Supplementary Figure S2**). PC1 explained 76.6% of variance and separated populations P1, P2, P3, and P6 (**Figure 1A**), PC2 explained 2.9% of variance

and separated populations P4, P5, P6, and P7 (**Figures 1B,C**), and PC3 explained 2.4% of variance and separated population P9 (**Figure 1B**).

Additionally, we constructed an approximately-maximum likelihood phylogenetic trees using FastTree2 (Price et al., 2010) with the 504 isolates and the *L. monocytogenes* serotype 4b str. F2365 reference genome, and with the 504 isolates and 166 previously sequenced and serotyped isolates (Hingston et al., 2017) in order to bioinformatically predict serovar and lineage for each isolate. Importantly, the phylogenetic results are in strong agreement with the population structure results (**Figure 1**). All but two of the 504 isolates were assigned with confidence to a serovar based on their phylogenetic proximity to the isolates that were previously serotyped (**Figure 1C** and **Supplementary Figure S3**). The two exceptions were isolates MEAT_GCA_004724945_1_PDT000123584_4_genomic and VEG_GCA_004726465_1_PDT000272419_3_genomic which were assigned to serovar 1/2a. We identified three lineages and five distinct serovars within the 504 isolates. Lineage I is exclusive to P1 and P2 which consists of 206 isolates from serotypes 4b and 1/2b. P3 consists of seven isolates from lineages II and III, and serovars 1/2a and 4c. The remaining populations were made up of lineage II isolates. P4-9 consist of 236 isolates of serovar 1/2a. P10 consists of 51 isolates from serovar 1/2c and 4 isolates from serovar 1/2a. The low occurrence of lineage III isolates and complete absence of lineage IV isolates is in line with the observation that these two lineages are rarely isolated from food-associated environments (Orsi et al., 2011).

Overrepresentation of Populations From Geographic Origin, Serotype, and Food Source

Next, we evaluated whether isolates from each population were associated with geographical origin (North America (NA), Central America (CA), South America (SA), Europe (EU), Asia (AS), and Oceania (OC)) or any of the major food sources (cheese, dairy non-cheese, meat, seafood, and fruit/vegetable). We rejected the null hypothesis that populations were uniformly distributed across the six geographic regions (X^2 = 151.37, df = 45, p-value < 2e-13). P1 was overrepresented with isolates from NA and underrepresented with isolates from EU, P2 was overrepresented with isolates from SA and underrepresented with isolates from OC, P4 was underrepresentated with isolates from SA, P7 was overrepresentated with isolates from CA, P8 and P9 were overrepresentated with isolates from OC and underrepresentated with isolates from OC, and P10 was overrepresented with isolates from EU and underrepresentated with isolates from NA (**Supplementary Figure S4**).

Additionally, we rejected the null hypothesis that isolates from the 10 populations (P1–P10) were evenly distributed across food sources (X^2 = 190.16, df = 36, p-value < 2.2e-16) (**Figure 2A**). P2 showed an overrepresentation of isolates from cheese and fruit/vegetable, P5, P8, and P9 showed overrepresentations of isolates from seafood, and P10 showed an overrepresentation of isolates from meat (**Figure 2B**).

We additionally tested the null hypothesis that serovars are evenly distributed across food types. Again, the null hypothesis was rejected (X^2 = 135.49, df = 16, p < 2.2e-16) indicating that there is a nonrandom distribution of serovars amongst the food sources. Serovar 1/2a was overrepresented in seafood, 1/2b was overrepresented in dairy non-cheese, 1/2c was overrepresented in meat, and 4b was overrepresented in cheese (**Supplementary Figure S5**).

Genes Unique to Genetic Clusters Associated With Dairy

We sought to identify genes unique to P2 because this population displayed an overrepresentation of strains isolated from cheese (**Figure 2**). We used LS-BSR and Roary to identify genes present in P2 and absent in all other populations (Sahl et al., 2014; Page et al., 2015). Using LS-BSR and Roary, we identified zero genes and one gene, respectively, that were uniquely present in P2 and absent in all other populations. The gene identified by Roary was annotated as *cobD*, which encodes a protein with L-Threonine-O-3-phosphate Decarboxylase activity that is involved in cobalamin biosynthesis (Fang et al., 2017). Because P1 and P2 are so closely related (**Figure 1**), we repeated our analysis by comparing the combined gene content of P1 and P2 versus all other isolates. This analysis yielded 4 genes with LS-BSR and 51 genes with Roary that were uniquely present in all P1 and P2 genomes but absent in all other isolates. Three of genes identified with LS-BSR encode predicted TetR/AcrR family transcriptional regulators, and the forth gene encodes a protein that contains a LPXTG cell wall-anchoring domain that is commonly found in pathogenic strains of *Listeria* (Reis et al., 2010). Because the LS-BSR analysis is stringent in requiring that all isolates in a population have BSR values meeting presence/absence cut-offs, we also examined genes with average BSR scores $\geq$ 0.90 in P1 and P2 and average BSR scores $\leq$0.20 in P3–P10 (**Figure 3A**). Using these less stringent, but still conservative cut-offs, we identified 40 genes that were present in the majority of P1 and P2 isolates and absent in the majority of P3-P10 isolates (**Figure 3B** and **Supplementary Table S2**). These genes include significant BLAST hits to Crp/Fnr family transcriptional regulators, GntR family transcriptional regulators, TetR/AcrR family transcriptional regulators, GNAT family *N*-acetyltransferases, a methyltransferase, an *N*-acetylmuramic acid 6-phosphate etherase, genes encoding cell wall proteins containing the LPxTG motif, and genes encoding proteins with the MucBP mucin-binding domains (**Figure 3** and **Supplementary Table S3**). Using Roary, we identified 51 genes unique to P1 and P2 but absent in all other populations. This collection of genes was highly similar to those identified using the less conservative parameters with LS-BSR (**Supplementary Table S3**).

Antimicrobial Resistance and Virulence Gene Profiles of *Listeria monocytogenes* Isolates

To understand the prevalence of antimicrobial resistance genes and virulence genes across the 504 isolates, we used the gene-screen method in LS-BSR to characterize the

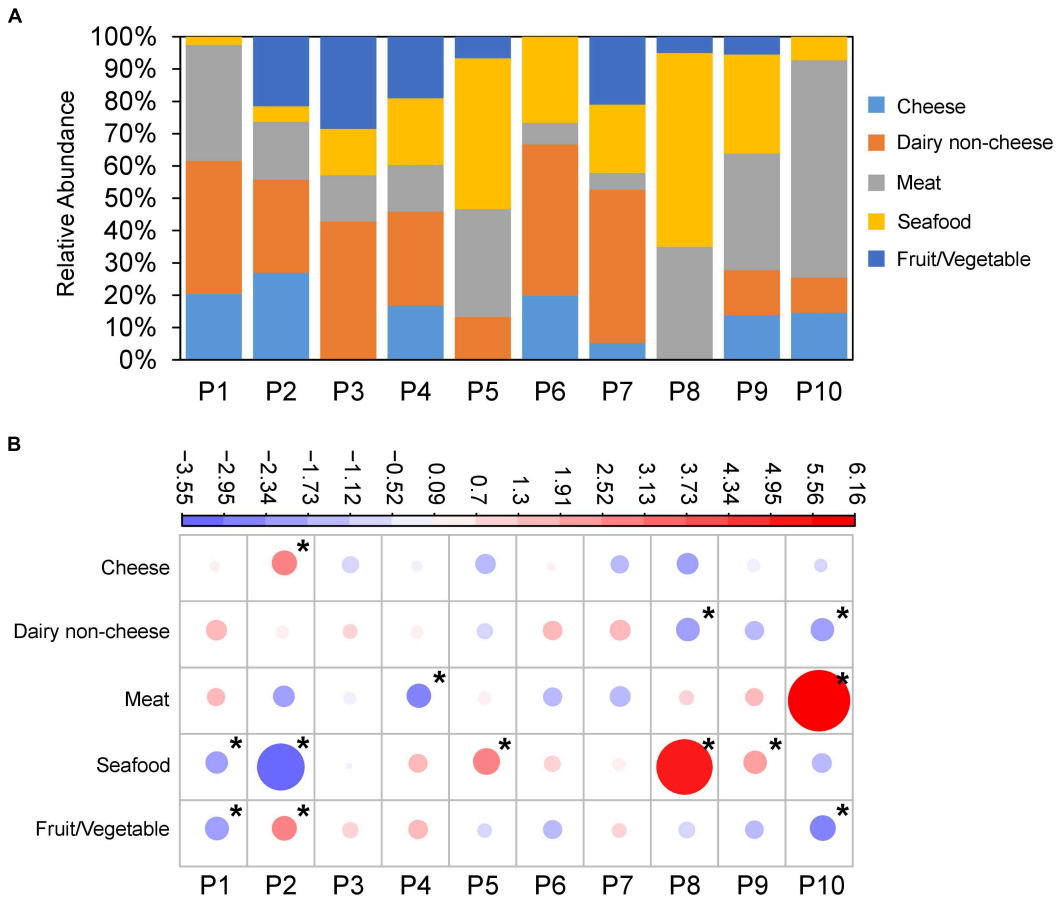

FIGURE 2 | *L. monocytogenes* populations are associated with food source. **(A)** Relative abundance of food source *L. monocytogenes* were isolated from (*Y*-axis) across populations (*X*-axis). **(B)** Overrepresentation and underrepresentation of isolates from populations versus food type. Plots represent standardized residuals from χ^2 analysis. Circle size represents absolute value of the standardized residual, while color represents positive (red) and negative (blue) values. Stars indicate standardized residuals with absolute values ≥2 (two standard deviations). P2 was overrepresented with strains isolated from cheese and fruit/vegetables, P5, P8, and P9 with seafood, and P10 with meat.

presence/absence patterns of 30 antimicrobial resistance genes and 92 *L. monocytogenes* virulence genes. The results of this analysis suggest that the vast majority of antimicrobial resistance and virulence genes do not have an association with a particular population (**Figure 4**). However, several virulence genes show population specific patterns of presence/absence. For instance, *vip*, a cell surface protein required for entry into some mammalian cells (Cabanes et al., 2005) was absent in P7, P8, P9, and some isolates of P4. *lmo2026* encodes a class I internalin that is involved in adhesion and colonization of host tissue (Popowska et al., 2017) and BSR scores suggest this gene is partially deleted in P1, P2, P3, P5, P9, and ∼60% of P4 isolates. *btlB* plays a role in intestinal persistence (Begley et al., 2005) and is absent from P3, but present in almost all other isolates. *aut*, which encodes an autolytic protein necessary for cell invasion (Cabanes et al., 2004), is variably absent within P2 but present in the vast majority of other isolates. Finally, *ami*, which also encodes for an autolysin (Milohanic et al., 2004), is variably absent in P1, P2, P3, P4, P9, and P10, and mostly present in P5, P6, P7, and P8. Of the 30 antimicrobial resistance genes surveyed, only *tetM* and *tetS* were detected. These genes are involved in tetracycline resistance (Charpentier and Courvalin, 1999) and were present in only 10 isolates (∼2% of isolates) and spread across four populations (P2 = 1, P4 = 6, P6 = 1 and P10 = 2) (**Figure 4B**).

DISCUSSION

We analyzed the population genomics of 504 *L. monocytogenes* isolates from the food environment and identified 10 distinct populations spanning isolates from lineages I, II and III and serovars 1/2a, 1/2b, 1/2c, 4b, and 4c (**Figure 1**). The low occurrence of lineage III isolates and complete absence of lineage IV isolates is in line with the observation that these two lineages are rarely isolated from food-associated environments (Nightingale et al., 2008; Orsi et al., 2011). PC1 and PC2 explain a large portion of observed variation (81.9%), and the remaining variation can potentially be attributed to recombination by horizontal gene transfer (HGT). Though natural transformation has not been directly observed in

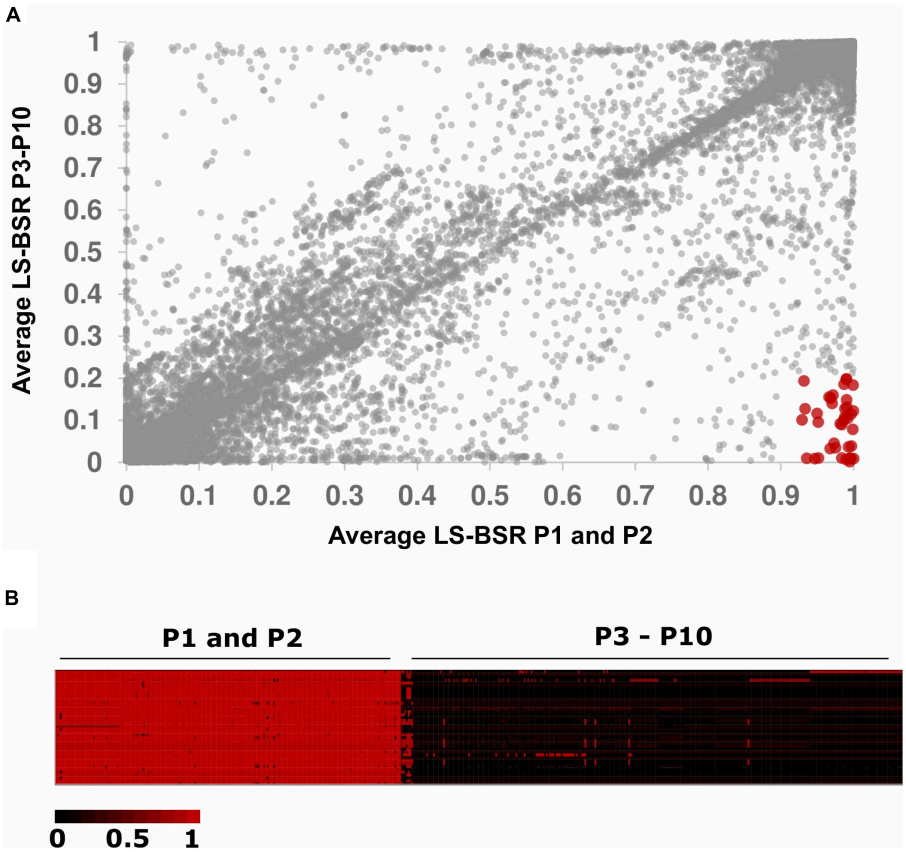

FIGURE 3 | Genes unique to populations 1 and 2. **(A)** Dot plot of average BSR values for P1 and P2 (*X*-axis) versus P3–P10 (*Y*-axis). Forty genes had BSR scores ≥0.90 in P1 and P2 and BSR scores ≤0.20 in P3–P10 (shown in red). **(B)** Heat map of BSR scores for the 40 genes uniquely present in P1 and P2 compared to P3–P10. Rows represent genes, and columns represent individuals.

L. monocytogenes (Borezee et al., 2000) the genome contains homologues for the competence machinery encoding genes (*ComG*, *ComEA*, *ComEC*, and *ComFA*) (Claverys and Martin, 2003; Chen and Dubnau, 2004), suggesting the potential for this mechanism of HGT. Additionally, hundreds of *L. monocytogenes* bacteriophages have been identified and several are capable of generalized transduction (Chen and Novick, 2009; Upham et al., 2019). Lastly, a number of studies have demonstrated that *L. monocytogenes* can receive genetic information through conjugative transfer (Poyart-Salmeron et al., 1992; Charpentier and Courvalin, 1999; White et al., 2002; Bertsch et al., 2013). At the population level, we observed several associations between populations and particular food sources (**Figure 2**). Here, we focus primarily on P2, which is composed of lineage I isolates, because this population displayed an overrepresented of isolates from cheese.

Between the 10 populations, we identified population specific gene presence/absence patterns for several key virulence genes (**Figure 4**). Among these genes was *vip*, which was absent in P7, P8, P9 and some isolates of P4. *vip* encodes a LPXTG protein that utilizes the host Gp96 endoplasmic reticulum receptor to gain entry into the mammalian cell (Cabanes et al., 2005). Additionally, *aut* was variably absent from P2 while the gene was primarily present in all other populations (**Figure 4A**). *aut* is a cell surface autolysin that is required for entry into eukaryotic cells (Cabanes et al., 2004). In line with our observations, a study of 121 *L. monocytogenes* isolates revealed that 60% of lineage I isolates contained the *aut* gene compared to 98% in lineage II (Kim et al., 2018). Lastly, almost all isolates of P2 possessed intermediate BSR scores for *ami,* an autolysin-encoding gene, suggesting a partial deletion (**Figure 4A**). *ami* knockouts displayed significantly less adhesion to eukaryotic cells compared to the wild-type, indicating *ami* functions in the attachment to host cells (Milohanic et al., 2004).

P2 was comprised of isolates from Lineage I, which was also the most common lineage isolated from milk, milk filters, and milking equipment collected from bovine dairy farms in the United States over a 12-year period (Kim et al., 2018). Further, an assessment of hazards associated with the spread of *L. monocytogenes* in Switzerland over 10 years, observed a significant positive association between serotype 1/2b isolates with hard and semi-hard cheeses (Pak et al., 2002). Finally, 91% of *L. monocytogenes* strains isolated from more than 4,000 dairy samples from England and Wales were from serotype 1/2 (58%) and 4b (33%) (Greenwood et al., 1991). Cheese population P2

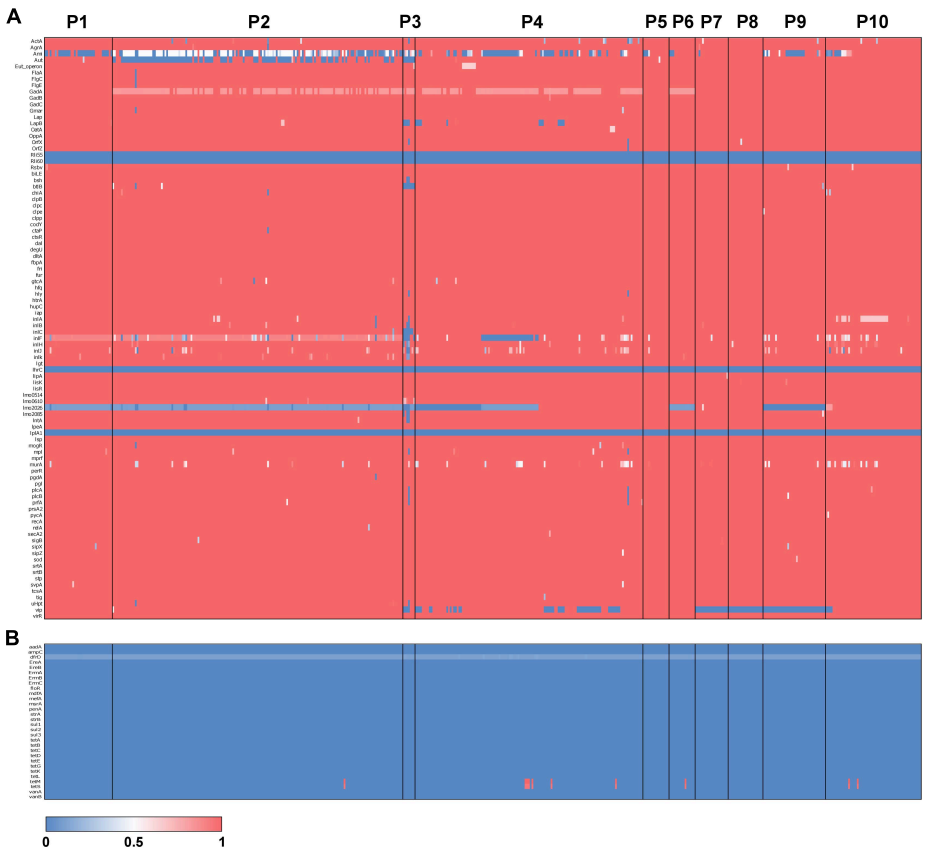

FIGURE 4 | Antimicrobial resistance and virulence gene typing across *L. monocytogenes*. Heatmaps of BSR scores for 90 *L. monocytogenes* virulence genes **(A)** and 30 antimicrobial resistance genes **(B)**. Rows represent genes, and columns represent individuals with populations labeled.

was comprised of 33% of isolates from serovar 1/2b and 67% from serovar 4b.

Despite the overrepresentation of cheese in P2 and dairy in P1 and P2 combined, we identified few genes that were present in high frequency in P1 and P2 and low frequency or absent in all other isolates (**Figure 3**). P1 and P2 are very closely related (as indicated by the lack of separation between P1 and P2 by PC1, PC2, or PC3 in **Figures 1A,B**). Interestingly, Doumith et al. (2004) identified five serovar 4b-specific genes in their investigation of lineage-specific genes between epidemic serovar 4b and a non-epidemic serovar 1/2a. Two of these five genes were transcriptional regulators, while the remaining three genes encoded LPXTG anchoring proteins. Similarly, we identified four genes that were present in all P1 and P2 isolates and absent in all other isolates. Three of these genes encode transcriptional regulators while the other encodes a LPXTG cell wall-anchoring protein. Using a less stringent approach, we identified > 40 genes with high gene presence frequency in P1 and P2 that were present in low frequency in P3–P10 (**Figure 3** and **Supplementary Tables S2, S3**). These genes also contained transcription factors and cell surface LPXTG encoding genes. Proteins containing leucine rich repeat (LRR) and LPXTG domains function in the attachment and invasion of host cells (Kuenne et al., 2013). It is important to note that other mutations, such as short indels and SNPs, can also contribute to genomic and phenotypic divergence between populations. For instance, a number of mutations cause a premature stop codon variant in the *inlA* internalin encoding gene, which results in a truncated protein that is secreted instead of anchored to the cell wall (Nightingale et al., 2005, 2006, 2008). Our results suggest that particular *L. monocytogenes* genotypes may be associated with the colonization of and persistence in certain food environments, such as dairy and cheese (**Figure 2**). To further address this hypothesis, physiological and biochemical assays as well as functional analysis of candidate genes in the fermented dairy environment will be required.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

TB and JG designed the study, conducted the analysis, and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.620033/full#supplementary-material

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The Cheese Production Facility Microbiome Exhibits Temporal and Spatial Variability

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A primary goal of modern cheese manufacturing is consistent product quality. One aspect of product quality that remains poorly understood is the variability of microbial subpopulations due to temporal or facility changes within cheese production environments. Therefore, our aim was to quantify this variability by measuring day-day and facility-facility changes in the cheese facility microbiome. In-process product (i.e., milk and cheese) and food-contact surfaces were sampled over the course of three production days at three cheese manufacturing facilities. Microbial communities were characterized using 16S rRNA metabarcoding and by plating on selective growth media. Each facility produced near-identical Cheddar cheese recipes on near-identical processing equipment during the time of sampling. Each facility also used a common pool of *Lactococcus* starter cultures which were rotated daily as groups of 4–5 strains and selected independently at each facility. Diversity analysis revealed significant facility-facility and day-day differences at each sample location. Facility differences were greatest on the food contact surfaces (i.e., draining-matting conveyor belts), explaining between 25 and 41% of the variance. Conversely, daily differences within each facility explained a greater proportion of the variance in the milk (20% vs. 12%) and cheese (29% vs. 20%). Further investigation into the sources of these differences revealed the involvement of several industrially relevant bacteria, including lactobacilli, which play a central role in flavor and texture development during Cheddar cheese ripening. Additionally, *Streptococcus* was found to contribute notably to differences observed in milk samples, whereas *Acinetobacter*, *Streptococcus*, *Lactococcus*, *Exiguobacterium*, and Enterobacteriaceae contributed notably to differences on the food contact surfaces. Facility differences in the cheese were overwhelmingly attributed to the rotation of *Lactococcus* starter cultures, thus highlighting circumstances where daily microbial shifts could be misinterpreted and emphasizing the importance of repeated sampling over time. The outcomes of this work highlight the complexity of the cheese facility microbiome and demonstrate daily and facility-facility microbial variations which might impact cheese product quality.

Keywords: facility microbiome, cheese, built environment, fermentation, food

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INTRODUCTION

The industrialization of food production has required that modern food manufacturing practices be highly regimented in order to create consistent products that meet consumer expectations. Despite this highly controlled approach, food manufacturers still face challenges in producing products with consistent quality, especially when producing the same product at multiple facilities. Food quality is complex and can be impacted by a combination of factors including raw ingredient heterogeneity and process changes (Gram et al., 2002). The impact of microbial populations and their dynamics in the food processing environment remain understudied.

Microbial variability in the production environment is particularly important for fermented foods, which rely on the action of microorganisms for their production. Many modern fermentation practices employ starter cultures as a means of standardizing the fermentation process; however, non-starter bacteria, which enter the food by way of the raw ingredients or the food processing environment, can also participate during fermentation and are sometimes attributed with quality changes in the finished product (Gram et al., 2002; Kandasamy et al., 2018). A prime example being Cheddar cheese production, where non-starter lactic acid bacteria (NSLAB) originating from the milk and the cheesemaking environment are sometimes associated with positive or negative quality outcomes that primarily occur during cheese ripening (Fox et al., 2017; Blaya et al., 2018).

Though decades of research have focused on identifying the microorganisms that cause these quality changes in cheese and other fermented foods, our understanding of how microbial communities vary in the production environment remains limited (Gram et al., 2002; Petruzzi et al., 2017). High throughput sequencing has opened the door to the exploration of microbial variability at the community-level and thus has allowed for initial investigations into these complex but fundamental questions. Bokulich and Mills (2013) were among the first to explore the cheese facility microbiome and revealed a complex and diverse microbial landscape that was, in large part, shaped by the environmental conditions at each processing stage (e.g., milk handling, curd processing, packaging, etc.). Despite the influence of processing stage, underlying facility-facility differences were still observed by Bokulich and Mills (2013). This led the authors to postulate that cheese facilities can harbor facility-specific microbiomes which potentially influence product quality.

Farm-level differences have likewise been observed in the milk microbiome, further supporting the idea that dairy processors can harbor site-specific microbial communities (Kable et al., 2016; Skeie et al., 2019). However, the milk microbiome can also exhibit considerable temporal variation, changing both seasonally and throughout the production day during milk processing (Kable et al., 2016, 2019; Skeie et al., 2019). Given the close relationship between milk and cheese production, it is anticipated that microbial communities would likewise change over time in the cheesemaking environment. Thus, our aim was to evaluate temporal and spatial variation of bacterial communities in the Cheddar cheese production environment by performing 16S rRNA metabarcoding and enumeration on selective growth media of samples from in-process product (i.e., milk and cheese) and swabs of food contact surfaces from three manufacturing facilities over the course of three consecutive production days. Our results show that there is significant temporal and spatial variation in these communities, and that comprehensive sampling is required to accurately characterize a cheese facility microbiome.

METHODS

Commercial Facilities

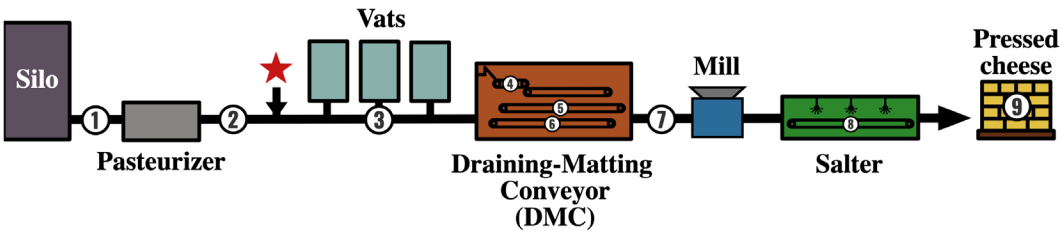
Three cheese processing facilities (A, B, C) served as the testing sites for this research. Each facility produces more than 25,000 tons of cheese per year and produces Cheddar cheese using identical formulations and processes, on nearly identical equipment. All three facilities follow a similar production schedule—i.e., 21-h production shift with a midday wash (caustic, acid, and water rinse between the milk balance tank and the DMC) followed by a 3-h sanitation shift. These facilities source raw milk from dairy farms within 250 miles (402 km) of each facility. These facilities also produce other types of cheeses (other Cheddar recipes, other semi-hard cheeses); therefore, sampling visits were coordinated to ensure that comparable Cheddar cheese recipes were being produced at all three facilities for three consecutive production days (September 10–12, 2019). Cheddar cheese was not produced at Facility C on the first day of sampling; therefore, only two production days (days 2 and 3) were sampled at Facility C.

Starter Rotations

Each of the participating facilities rotate 6 blends of *Lactococcus* starter cultures, each consisting of 4–5 *Lactococcus lactis* subsp. *cremoris* strains. A *Lactobacillus rhamnosus* adjunct starter is also used at each facility but is not rotated. *Lactococcus* starters are inoculated at $\sim 10^6$ CFU/mL via pH adjusted batch cultures injected directly into the milk-to-vat line, whereas the *Lactobacillus* starter is inoculated at $\sim 10^6$ CFU/mL but using a direct vat inoculation (**Figure 1**). *Lactococcus* blends are rotated every 24–48 h at each facility. Facilities B and C use the same *Lactococcus* starter blends, whereas half of the starter blends used at Facility A are unique. Blends used during the 3-day sampling period are shown in **Supplementary Figure 1**.

Sample Collection and Processing

A total of 288 samples were collected from cheese facilities A, B, and C during three consecutive production days. All samples were collected within 15–21 h from the start of production at the sampling locations listed in **Figure 1**. Milk samples (50 mL) were collected from a sanitary port using a sterile syringe or from balance tanks or vats using a sanitized stainless-steel ladle. Food contact surfaces (i.e., conveyor belts) were sampled using sponge-stick swabs stored in neutralizing buffer (Solar Biologicals, Newark, DE). Curd samples (approximately 150 g) were collected directly into an inverted Whirl-Pak® bag (Nasco, Fort Atkinson, WI). All samples were immediately stored on ice and shipped to



#	Name	Description	Sampling Method
1	Raw Milk	Raw milk taken before the pasteurizer either in-line (Facility B) or from the balance tank (Facilities A & C)	Syringe (Facilities A & B); Ladle (Facility B)
2	Thermized Milk	Heat-treated milk taken in-line, after the pasteurizer regeneration section	Syringe (Facilities A & B); Ladle (Facility B)
3	Vat Milk	Milk taken right before entering the vat (Facilities B & C) or from the vat (Facility A), and prior to starter culture addition	Syringe (Facilities B & C); Ladle (Facility A)
4,5,6	Upper, Middle, Lower DMC Belts	Surface swab of the upper, middle, or lower draining-matting conveyor belts	Sponge-stick swab
7	Matted Curd	Matted curd taken from the draining-matting conveyor belt, prior to the mill	Inverted Whirl-Pak® bag
8	Salting Belt	Surface swab of the salting machine conveyor belt	Sponge-stick swab
9	Pressed Cheese	Curd taken from a 40-lb, vacuum-sealed cheese block, after pressing	Inverted Whirl-Pak® bag

FIGURE 1 | Flow diagram showing the generic Cheddar cheesemaking process and sample collection points. Numbers denote sampling locations within the cheese production facilities (A, B, C). Descriptions of each sample location and the method used are listed. The red star indicates the approximate location of starter addition. Thermization conditions used at each facility are 67–70°C for 26–28s.

Oregon State University within 36 h of collection. Samples were collected repeatedly from each location every 3 min over a 12-min period (n = 4). The continuous nature of the cheesemaking process means that each repeated sample represents a new section of the in-process product or processing environment. For this reason, repeated samples taken over the 12-min period were treated as biological replicates.

Upon arrival at OSU, liquid samples (milk and swab solution) were vortexed, and a 1.8 mL aliquot was taken for DNA extraction. Curd samples (10 g) were homogenized by stomaching in 90 mL of 1% sodium citrate buffer (pH 5.2). Aliquots were centrifuged at 9,500 × g for 2 min, the supernatant was removed, and the resulting pellets were stored at −80°C for later DNA extraction.

Bacterial Enumeration and Isolation

A subset of samples (n = 72) was enumerated by standard serial dilution and spread plating on selective media: de Man, Rogosa and Sharpe agar [MRS; generic lactic acid bacteria (LAB)] (Neogen), Rogosa SL agar (SL; non-*Lactococcus* LAB) (HiMedia, Mumbai, India), m-Enterococcus agar (m-EA; presumptive enterococci/streptococci) (Neogen), and MacConkey agar (MAC; coliforms) (Neogen). This subset included one of the four replicate samples taken from each sampling location on each day from each facility. MRS and SL plates were incubated at 35°C for 5 days under microaerophilic conditions (5% O_2, 10% CO_2) in a hypoxic chamber (Bactrox, Sheldon Manufacturing, Inc., Cornelius, OR). MAC and m-EA plates were incubated at 37°C for 24 or 48 h, respectively.

Representative isolates of unique colony morphologies from MAC (n = 34 isolates) and m-EA (n = 17 isolates) plates from the draining matting conveyor (DMC) swab samples (locations 4, 5, and 6; **Table 1**) were streaked for isolation on tryptic soy agar supplemented with 0.6% yeast extract (TSAYE) and incubated under the same conditions as the original source media. Isolates were cultured in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) for 48 h and diluted 1:1 in 50% glycerol for long-term storage at −80°C.

Isolate Identification by 16S rRNA Sequencing

Bacterial isolates were identified by 16S rRNA sequencing. DNA was extracted from pure cultures of each isolate using either a crude extraction (boil in water for 10 min) or the

TABLE 1 | *P*-values from the Kruskal-Wallis test for differences in Shannon's alpha diversity between facilities and between days within each facility.

Sample location	Facility	Day (Facility A)	Day (Facility B)	Day (Facility C)
Raw milk	ns	ns	ns	ns
Thermized milk	ns	ns	0.021	ns
Vat milk	0.018	0.039	ns	0.021
Upper DMC belt	<0.001	0.024	0.024	0.021
Middle DMC belt	<0.001	0.012	ns	0.021
Lower DMC belt	<0.001	0.007	0.012	ns
Salting belt	0.023	0.007	0.007	ns
Matted curd	0.002	0.007	0.023	ns
Pressed cheese	0.018	0.007	0.023	0.021

DNeasy Blood and Tissue Kit (Qiagen, Carlsbad, CA). PCR was performed in 25 μL reactions using Platinum Hot-Start Master Mix (Thermo Fisher, Waltham, MA) and 0.2 μM the 27F/1492R universal primers (Frank et al., 2008). PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR quality and fragment size were verified by gel electrophoresis (1% agarose gel, 10 V/cm). PCR products were cleaned with the Gel/PCR DNA fragment extraction kit (IBI Scientific, Dubuque, IA) and the DNA concentration was quantified using the Qubit 4 (Invitrogen, Carslbad, CA). Cleaned PCR products were sequenced using both 27F and 1492R primers on an ABI 3730 capillary sequencer (Thermo Fisher) by the Oregon State University Center for Genome Research and Biocomputing (CGRB, Corvallis, OR). Consensus sequences were generated from the forward and reverse sequences for each isolate using SeqTrace (Stucky, 2012). Taxonomy was assigned using the EZBioCloud Database (Yoon et al., 2017).

DNA Extraction and High Throughput Sequencing

DNA was extracted from previously frozen milk, swab, and curd subsamples (n = 288) using the PowerFood Microbial DNA Isolation Kit (Qiagen, Carlsbad, CA) following manufacturer's instructions. A single thermized milk sample from Facility C was lost due to human error during this process. Subsamples were homogenized with the Bead Ruptor 24 (Omni, Kennesaw, GA) using 10 cycles of a 15 s pulse at 8 m/s with a 55 s rest between cycles. DNA extractions and PCR reactions were validated using the ZymoBIOMICS Microbial Community Standard and Microbial Community DNA Standard (Zymo Research Corp., Irvine, CA). Non-template controls were also included on each PCR reaction plate.

PCR libraries for 16S rRNA metabarcoding were prepared in the manner described by Comeau et al. (2017) with modifications. Indexed libraries of the milk, belt swab, and cheese samples (n = 287) were prepared using pairwise combinations of 515F and 926R fusion primers containing Illumina i5 and i7 indices and P5 and P7 sequence adapters (**Supplementary Table 1**). Duplicate PCR reactions were performed using Platinum Hot-Start Master Mix. PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. PCR product quality and fragment size were verified by gel electrophoresis (1% agarose gel, 10 V/cm) and then normalized using SequalPrep 96-well plates (Applied Biosystems, Foster City, CA). Amplicon sequencing was carried out in two sequencing runs on a Miseq 3000 (Illumina, San Diego, CA) with 2 × 300 bp v3 chemistry at the CGRB.

Sequence Processing

High throughput sequence data were processed using workflows in QIIME2 v2019.1.0 (Bolyen et al., 2019) and R v4.0.0 (R Core Team, 2020). Initial processing began with demultiplexed sequence files from combined sequence runs. Residual forward and reverse primer sequences were removed from the demultiplexed sequences using the QIIME2 cut-adapt plug-in (Martin, 2011). Reads were trimmed to 250 nt (forward) and 220 nt (reverse), quality filtered, merged, and chimeras were removed using the QIIME2 DADA2 plug-in (Callahan et al., 2016). The average merged contig length was 373 bp. Sequence taxonomy was assigned twice, both times using a Naïve-Bayes trained QIIME2 classifier trained to the 16S rRNA 515F/926R region using the Greengenes 99% OTU database (downloaded January 28, 2020). The first assignment of taxonomy was used for the identification and removal of sequences identified as "mitochondrial" or "chloroplast" DNA using the "filter-table" and "filter-seq" options of the QIIME2 taxa plug-in. Taxonomy was then reassigned to the resulting dataset and used for all subsequent processing in R.

QIIME2 artifacts were loaded into R using the qiime2R v0.99.23 (Bisanz, 2018) and phyloseq v1.32.0 (McMurdie and Holmes, 2013) packages. Suspected DNA contaminants were removed using the "prevalence" method in decontam v1.8.0 (Davis et al., 2018) with threshold (P*) 0.495. The resulting "decontaminated" sequence files were used for all downstream analysis.

Data Analysis

High throughput sequence data and bacterial enumeration data were analyzed in R. Rarefaction curves were produced using the "ggrare" function in ranacapa v0.1.0 (Kandlikar et al., 2018). Species richness and Shannon's alpha diversity were estimated from rarefied abundance tables using the "estimate_richness" function in vegan v2.5.6 (Oksanen et al., 2019). Beta diversity was estimated following a center log-ratio (clr) transformation. Principle component analysis was performed on clr transformed data using the "prcomp" function in stats v3.6.2 (R Core Team, 2020). Group centroids were determined by taking the average component scores for PC1 and PC2. Individual contributions of SVs to the PC1 and PC2 variance were determined by squaring the loadings generated by prcomp. Significant differences in beta diversity between facilities and between production days nested within each facility were determined by PERMANOVA based on the Aitchison's distance, using the "adonis2" function in vegan (formula = ∼facility*day). Heterogeneity of within-group dispersion for facility and day-per-facility was determined

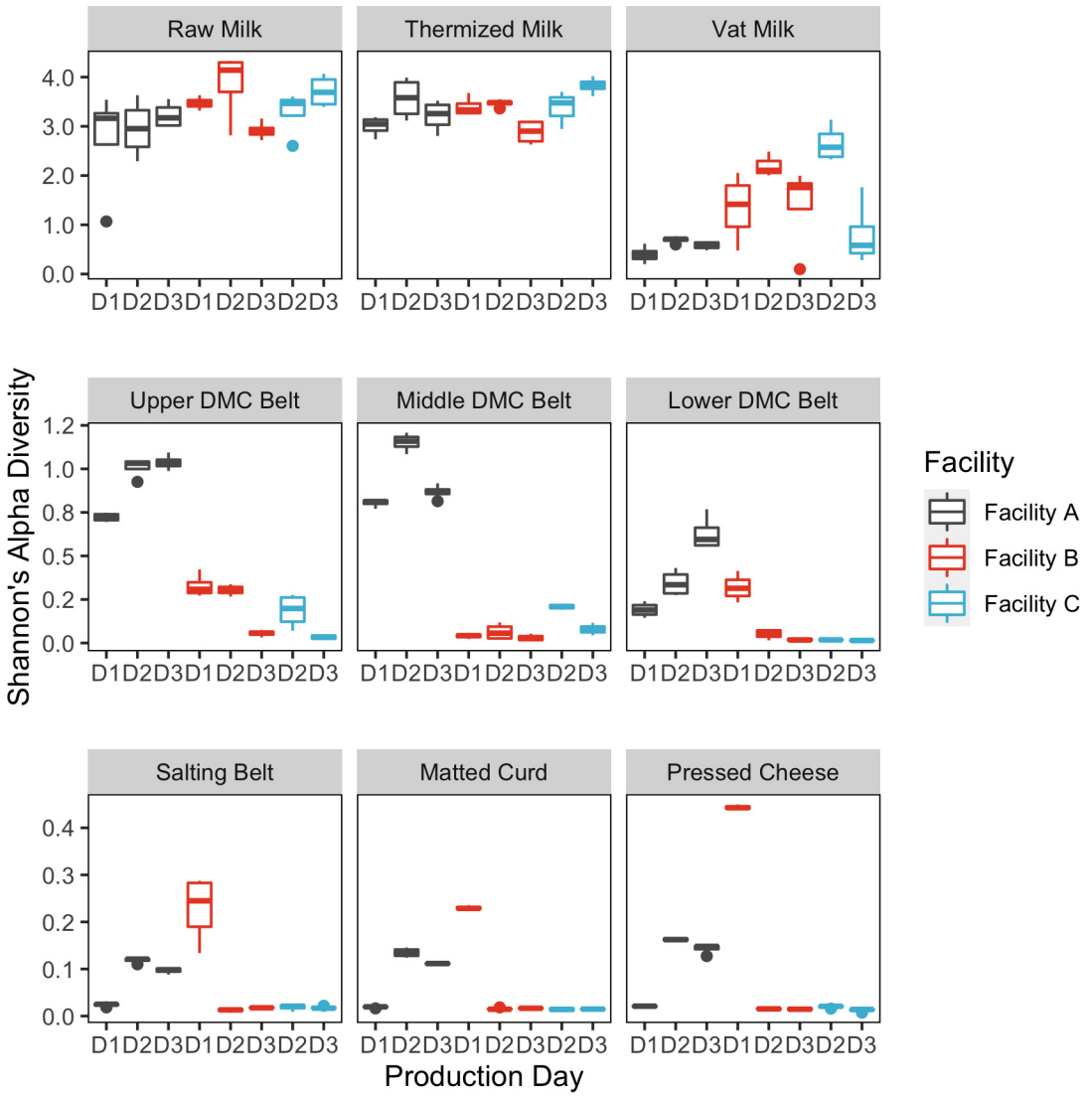


FIGURE 2 | Box plots showing Shannon's alpha diversity estimates by sample location. Bars are colored by cheesemaking facility. Repeat sampling days within each facility are displayed across the *x*-axis (n = 3 Facilities A and B; n = 2 Facility C). Decline in alpha diversity in samples collected post-thermization is partially explained by starter addition which occurs in the vat.

by PERMDISP using the "betadisper" function in vegan. Core microbial species were determined using the microbiome v1.10.0 package (Lahti and Shetty, 2019) with threshold 0.90. Figures and tables were generated in R and Microsoft Excel.

RESULTS

Sequencing Results and Alpha Diversity

Microbial diversity was investigated at each Cheddar cheese production facility using 16S rRNA metabarcoding. Large differences in sequencing depth were observed between sample types (i.e., milk, belts, cheese). Consequently, each sample type was rarefied to different depths accordingly prior to analysis of alpha diversity (**Supplementary Figure 2**).

Alpha diversity was greatest in the milk samples and generally declined through the cheesemaking process (**Figure 2**). Median species richness for milk, belt, and cheese samples were 56, 8, and 6.5 sequence variants (SVs) per sample, respectively. Residual starter from previous cheese makes in the milk-to-vat line reduced species richness from a median value of 66 SVs per sample in the thermized milk to 22 SVs per sample in the vat milk. Significant differences in alpha diversity were observed between facilities at all sample locations, with the exception of thermized and raw milk samples, and between days in at least one facility for all sample locations, except raw milk (**Figure 2** and **Table 1**). Alpha diversity at Facility A was generally greater in sample locations where significant facility differences were observed (i.e., DMC belts, salting belt, matted curd, and pressed cheese).

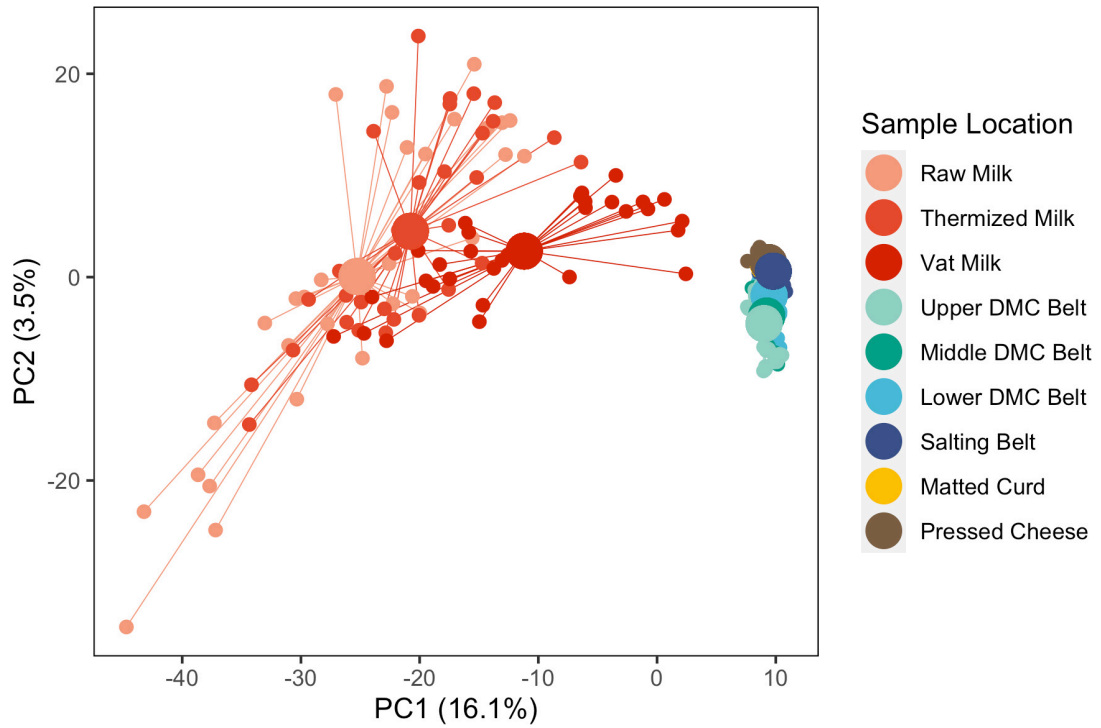


FIGURE 3 | Principle component analysis of microbial communities from all sample locations and all facilities, following clr transformation. Samples are grouped by sample location. Individual samples (small points) are connected by segments to their corresponding group centroids (large points). Sequence variants which explained at least 5% of the component variance include *Clostridium* (2 SVs), Peptostreptococcaceae (1 SV), *Streptococcus* (2 SVs), and *Turicibacter* (1 SV) for PC1, and *Acinetobacter* (1 SV) and *Streptococcus* (1 SV) for PC2.

Comparing Between Samples (Beta Diversity)

Beta diversity was evaluated at two levels: (i) between sample locations and (ii) within sample locations. Between sample locations, milk samples (raw, thermized, and vat milk) grouped distinctly from belt (DMC and salting belts) and cheese (matted curd and pressed cheese) samples across PC1, whereas belt and cheese samples were only weakly separated across PC2 (**Figure 3**). Milk samples exhibited greater within group variability (dispersion) than belt and cheese samples.

Within sample locations, significant day-day and facility-facility differences were detected at each location (**Figure 4**, **Supplementary Figure 3**, and **Table 2**). Evidence of significant dispersion effects were also observed in many cases, though differences in group centroid locations for facility and production days were still clearly visible (**Supplementary Table 2**, **Figure 4**, and **Supplementary Figure 3**). The proportion of variance explained by the first two principle components was greatest for the belt swab samples, followed by cheese samples and then milk samples (**Figure 4**). Facility A was generally distinct from facilities B and C at each sample location, while facilities B and C became distinct only following thermization and were again mostly indistinguishable in the curd, salting belt, and cheese samples.

Regarding day-day differences, all production days formed distinct clusters within each facility in the milk and DMC belt samples but only formed distinct clusters on production day 1 within facilities A and B for the salting belt, matted curd, and pressed cheese (**Figure 4**). These last three sample locations exhibited similar grouping patterns and were attributed to daily starter rotations that occurred at each facility (**Supplementary Figure 1**). Within-day replicates were highly similar for belt and cheese samples but displayed moderate variability in the milk (**Figure 4**).

SVs that commonly explained at least 5% of the variance in the first or second component of the within-location PCAs included *Streptococcus* and *Lactobacillus* for milk samples; *Lactococcus*, *Acinetobacter*, and *Streptococcus* for belt samples; and *Lactococcus* for cheese samples (**Figure 5**). Enterobacteriaceae and *Exiguobacterium* also contributed to >5% of the component variance for the upper and lower DMC belts, respectively. Further examination revealed that many of these SVs exhibited variations in their centered log-ratios according to facility and day of sampling (**Figure 6**). This agreed with the PERMANOVA models which found that both facility and production day nested within facility explained a large proportion of the community variance at each sample location (**Table 2**). Comparing these factors, facility explained a greater proportion of the variance in the DMC samples, whereas production day explained more variance in the remaining locations. Milk samples contained a large proportion of unexplained variance compared to the other sample types, again owing to their increased diversity.

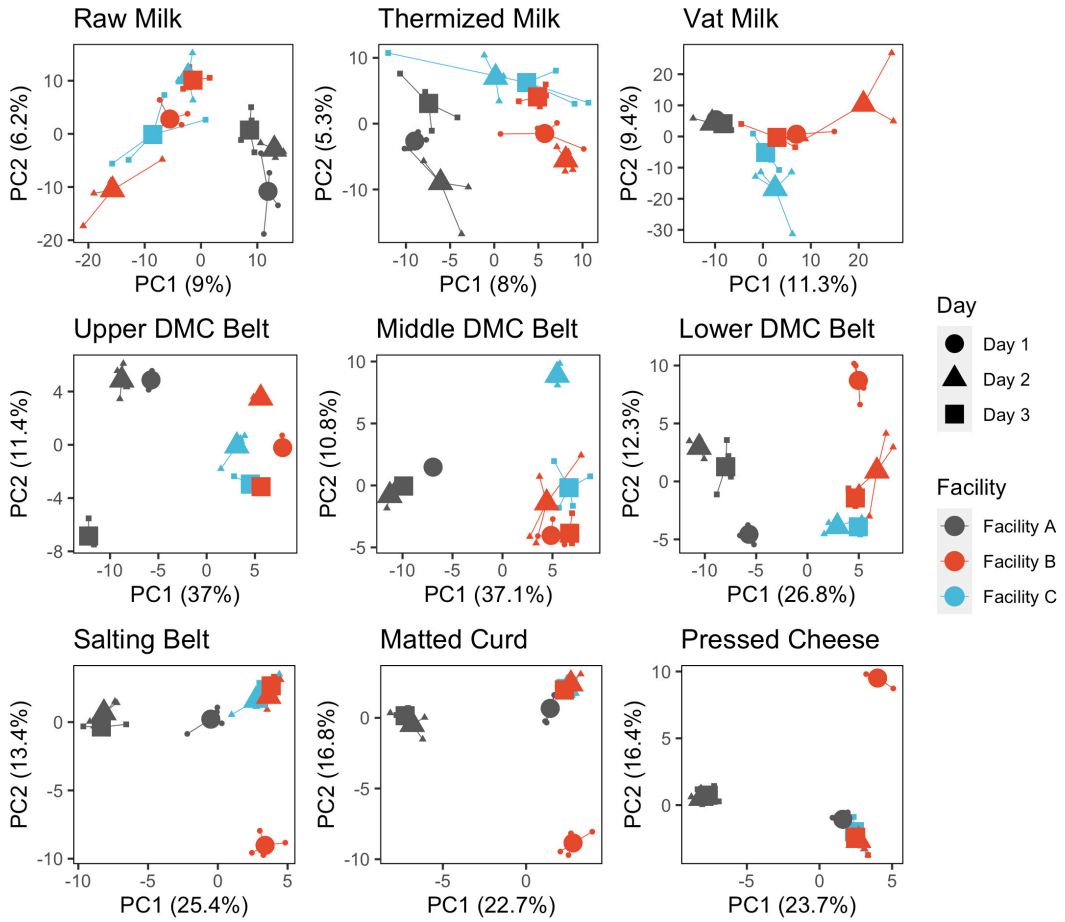


FIGURE 4 | Principle component analysis of microbial communities following clr transformation and separated by sample location. Points are colored by facility and shaped by production day. Samples are grouped by production day nested within facility. Individual samples (small points) are connected by segments to their corresponding group centroids (large points).

Changes in Relative Sequence Abundance and Bacterial Load

To gain greater insight into the factors that contributed to facility or daily differences in the alpha and beta diversity, changes in the relative abundance of starter and non-starter bacteria and their associated bacterial loads were assessed per sample location. The DMC bacterial community was also further evaluated by isolation and identification using16S rRNA sequencing.

In agreement with beta-diversity analyses, clear differences in species richness/evenness and the relative abundance of dominant/subdominant taxa were observed between sample types, sample locations, facilities, and production days (**Figure 7**). Conversely, changes in bacterial load did not always reflect these differences (**Figure 8**). Milk samples were visibly more diverse than belt or cheese samples in terms of the number of unique colony morphologies observed on MRS (data not shown). Samples collected after the vat were typically dominated by *Lactococcus* sequences and had greater CFU counts of generic LAB, as determined on MRS agar (**Figures 7**, **8**). CFU counts on Rogosa SL agar were comparably larger at Facility A in the vat milk and may be a consequence of residual starter lactobacilli remaining on the ladle used for collection at this facility (**Table 1** and **Figure 8**). The decrease in relative abundance of Gammaproteobacteria and increase in Firmicutes following thermization (**Figure 7**) corresponded with

TABLE 2 | Significance and explained variance of facility and production day nested within facility as determined by PERMANOVA of the Aitchison's distance.

	Proportion of variance (R^2)			Significance (p-value)	
Sample location	**Facility**	**Day (Facility)**	**Residual**	**Facility**	**Day (Facility)**
Raw milk	0.12	0.20	0.68	0.001	0.001
Thermized milk	0.11	0.19	0.70	0.001	0.001
Vat milk	0.16	0.18	0.66	0.001	0.003
Upper DMC belt	0.41	0.30	0.29	0.001	0.001
Middle DMC belt	0.43	0.21	0.35	0.001	0.001
Lower DMC belt	0.32	0.25	0.43	0.001	0.001
Salting belt	0.25	0.29	0.46	0.001	0.001
Matted curd	0.20	0.32	0.48	0.001	0.001
Pressed cheese	0.20	0.29	0.51	0.001	0.001

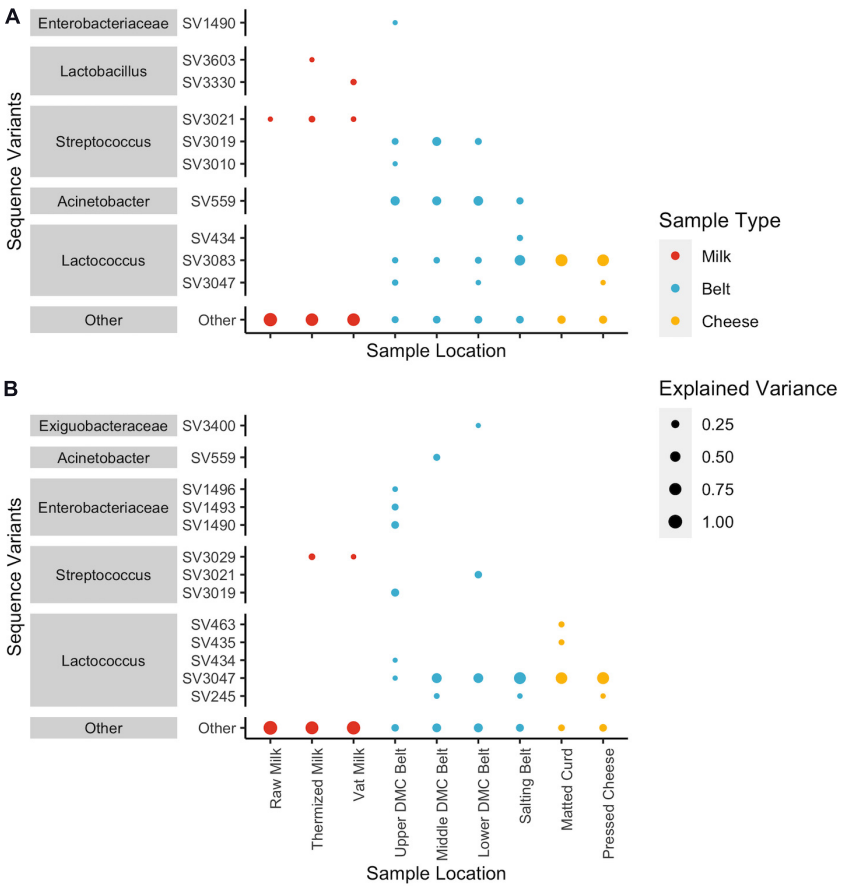


FIGURE 5 | Dot plots showing squared loadings for **(A)** component 1 and **(B)** component 2 of the location-wise principle component analyses. Dot size represents the proportion of component variance explained by each SV. Dot color represent the general sample type (i.e., milk samples: raw, thermized, and vat milk; belt samples: DMC and salting belts; and cheese samples: matted curd and pressed cheese). SVs that explain < 5% of the component variance are grouped as "Other".

a comparable decrease in presumptive coliforms (MAC agar) and increase in generic LAB cell densities (MRS agar) (**Figure 8**).

A resurgence of non-starter bacteria, including Gammaproteobacteria and *Streptococcus*, was observed in the DMC (**Figures 7**, **8**), though the extent of which was not always reflected in the sequence data (**Figure 7**) and further differed by facility, production day, and the specific DMC belt (i.e., upper, middle, or lower) (**Figures 7**, **8**). Most notable was the cell density observed on MAC agar in the DMC at Facility A, which exceeded 5.3, 4.2, and 2.7 log cfu/mL each day on the upper, middle, and lower DMC belts, respectively (**Figure 8**). Further investigation into the identities of bacterial isolates from the DMC revealed that *Acinetobacter* was the predominant genus on MAC agar, while *Streptococcus* was predominant on m-EA (**Supplementary Table 3**). Other genera observed at lower frequencies and/or lower cell densities included *Escherichia*, *Klebsiella*, *Pseudomonas,* and *Enterobacter* on MAC agar and *Enterococcus* on m-EA agar. Species and subspecies repeatedly isolated from the DMC included *Acinetobacter baumanii*, *Escherichia fergusonii*, *Enterococcus faecalis*, and *Streptococcus gallolyticus* subsp. *macedonicus* at Facility A; *Escherichia fergusoni* and *Escherichia* CP040443/*Escherichia* LFH at Facility B; and *Acinetobacter baumannii* and *Pseudomonas mosselii* at Facility C (**Supplementary Table 3**). Following the DMC, bacterial cell densities on m-EA and MAC were mostly below the detection limit (1 log CFU/mL), supporting observations that the *Lactococcus* starter dominated these samples (**Figures 7**, **8**). Core SVs present in ≥ 90% of samples included *Lactococcus*, *Streptococcus*, *Clostridium*, *Turicibacter,* and Peptostreptococcaceae for milk samples; *Lactococcus* and *Enterococcus* for belt samples; and *Lactococcus* and *Lactobacillus* for cheese samples.

DISCUSSION

Few studies have attempted to characterize the temporal and facility-facility variability of microbial communities in the food production environment. A fundamental understanding of this variability is essential for answering questions related to the microbial ecology of food production systems and the consequences for food quality and safety. A primary goal of this research was to improve our understanding of microbial variability in the cheesemaking environment by

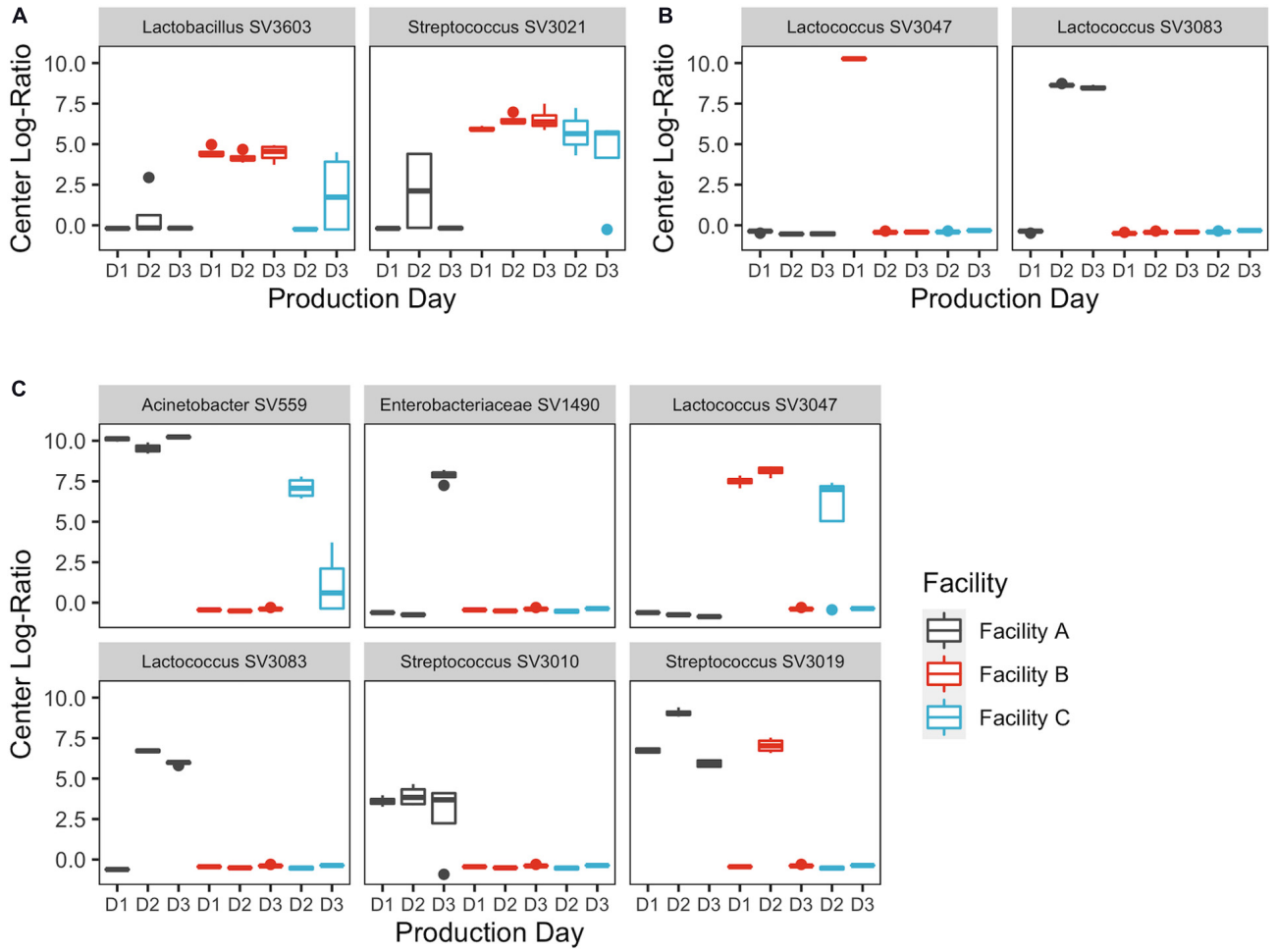


FIGURE 6 | Centered log-ratios of SVs that explained ≥5% of the variance in the first component of the **(A)** thermized milk, **(B)** pressed cheese, and **(C)** upper DMC belt principle component analyses. Bars are colored by cheesemaking facility. Repeat sampling days within each facility are displayed across the *x*-axis.

quantifying facility-facility and day-day differences in the cheese facility microbiome.

Microbial Diversity Throughout Cheddar Cheese Production

Production stage has been previously identified as a principle driver of community assembly in the cheese production environment (Bokulich and Mills, 2013; Falardeau et al., 2019) and likewise had a clear impact on microbial compositions in the present study. Production stages were defined as milk processing (raw, thermized, and vat milk), cheddaring (DMC swabs and matted curd), salting (salting belt swabs), and pressing (pressed cheese), and were further separated based on their sample type: milk, food contact surfaces, or cheese.

Microbial dynamics in milk are complex, as they are influenced by several factors including the farm and milk handling practices (Kable et al., 2016; Falardeau et al., 2019). As a result, microbial communities in the milk were highly diverse, leading to greater ambiguity in these samples, as evidenced by the large proportion of unexplained variance in the PERMANOVA models (66–70%). In contrast, microbial differences on food contact surfaces and in the cheese were comparably simple. Differences on the DMC belts were driven by large disparities in bacterial load and species richness, both of which were distinctly greater at Facility A, while cheese and salting belt samples were driven by differences in the composition and abundance of *Lactococcus* SVs and were an attributed consequence of starter addition which occurred in the vat.

The interpretation of microbial diversity in the cheesemaking environment was also dependent on the sample type. While both the matted curd and lower DMC belt swabs were collected from essentially the same location in the production environment, belt swabs generally had greater proportions of non-starter bacteria, particularly *Acinetobacter* at Facility A, whereas the matted curd was almost entirely dominated by *Lactococcus* SVs. These differences likely arose from two factors: (1) greater concentration of *Lactococcus* starter in the curd, as compared to the surrounding food contact environment, and (2) growth of non-starter bacteria on the

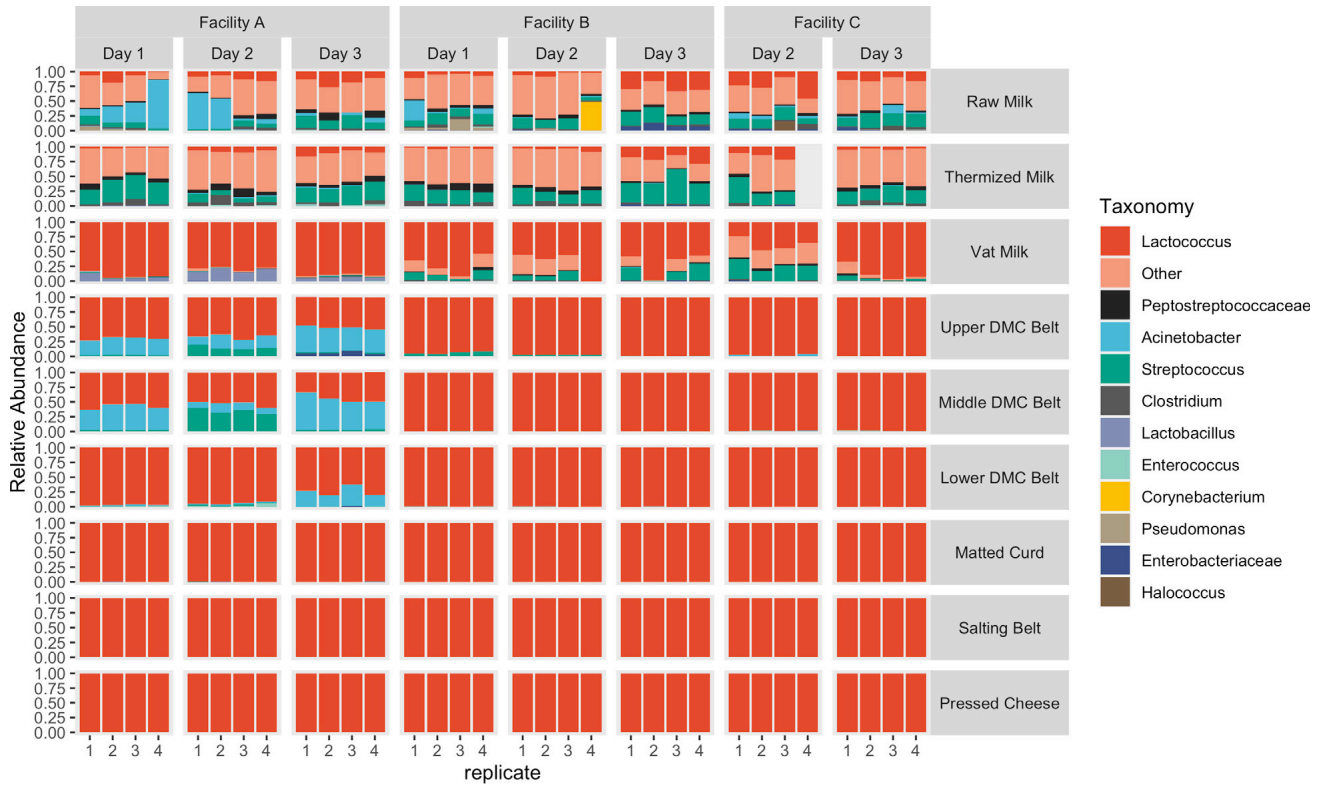


FIGURE 7 | Relative abundance of taxonomic features separated by sample location. Features with a relative abundance >10% in the milk samples and >1% in the remaining locations were grouped as "Other."

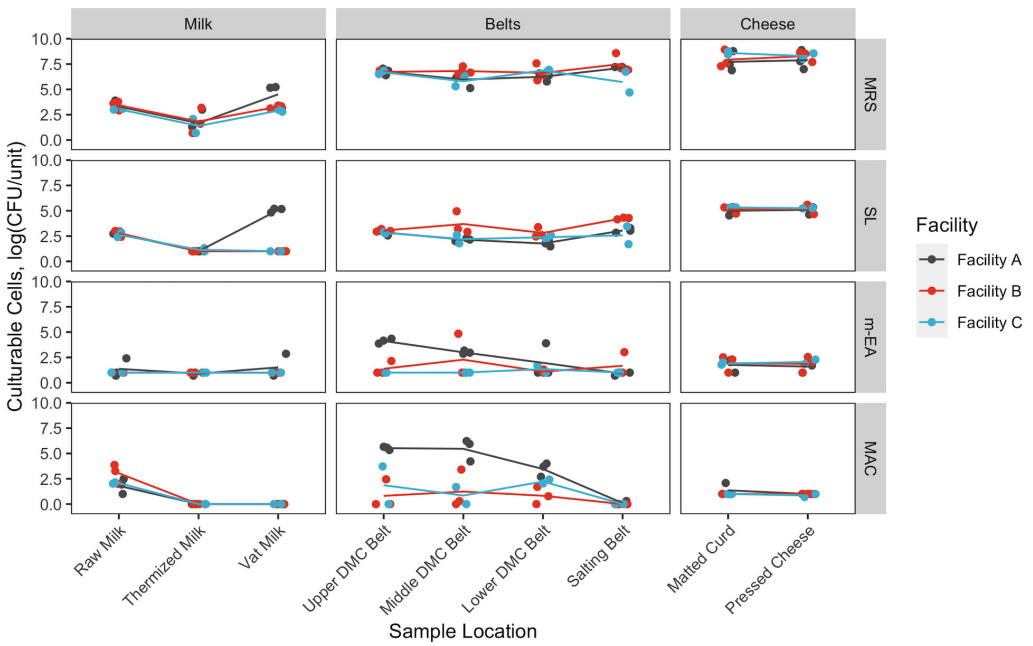


FIGURE 8 | Changes in cell density throughout production. Cell density estimates are based on De Man, Rogosa and Sharpe agar (MRS), Rogosa SL agar (SL), m-Enterococcus agar (m-EA), and MacConkey agar (MAC). Density units vary by sample location: log CFU/mL for milk samples, log CFU/swab for belt samples, and log CFU/g for cheese samples. Samples are colored by facility. Points represent individual samples. Lines represent facility averages.

lower DMC belt surface throughout the production day (Selover et al., 2021).

The co-inhabitance of starter and non-starter bacteria in the cheese production environment is regularly discussed (Stellato et al., 2015; Falardeau et al., 2019) and thus, it was not surprising to find *Lactococcus* starter at high abundance at all sample locations post-starter addition (i.e., those following thermization). However, an important consequence of this commingling of starter and non-starter bacteria was the obfuscation of low-level community members that could contribute importantly to cheese quality during the later production stages (i.e., ripening). This obfuscation was most apparent in the vat milk, where residual starter culture present in the milk-to-vat line resulted in a lower estimate of species richness as compared to the thermized milk, despite the same underlying community members being expected at both locations. Absence of samples collected prior to starter addition, these low-level microbial communities would likely go undetected and thus their industrial relevance may be overlooked. As such, future investigations into the cheese facility microbiome should consider taking samples both pre- and post-starter addition with the intention of capturing these underlying communities.

Sources of Facility-Facility Microbial Diversity

Previous investigations into facility-facility differences in the cheese facility microbiome have relied on comparisons between facilities that were producing different cheese recipes on very different production equipment (Bokulich and Mills, 2013; Quijada et al., 2018). In the present study, facility differences were based on comparisons between facilities producing near-identical cheese recipes on near-identical processing equipment. This approach allowed for greater explanation of facility differences and their potential sources. One trend that was consistent throughout production was the distinction of Facility A from Facility B and Facility C. While several factors likely contributed to this distinction, some of the most apparent sources included the shared milk source at Facility B and Facility C, and the differences in age of equipment used at each facility.

Facilities B and C are neighboring facilities that share common milk sources from predominantly large-scale dry-lot dairy operations, whereas Facility A is approximately 250 miles (402 km) away and sources their milk from numerous smaller pasture-based dairy farms. As a result, microbial communities in the raw milk from facilities B and C shared greater similarities than those from Facility A. These differences were seemingly driven in large part by *Streptococcus* SV 3021, which was absent at Facility A, but accounted for up to 6.7 and 6.9% of the sequences at facilities B and C. This adds to the growing evidence that milk can exhibit farm-level microbial differences and also supports previous observations that streptococci are a primary contributor to farm-level differences (Kable et al., 2016; Skeie et al., 2019). Streptococci are among the most common bacteria isolated from the dairy environment and are notable for their roles in human health, animal health, and fermented dairy products (De Vuyst and Tsakalidou, 2008). Further investigation into the species identity of SV 3021 revealed it to most likely be *Streptococcus thermophilus*, which is associated with fermented dairy products, including yogurt and Swiss cheese, and has been used experimentally to produce low-fat Cheddar cheese (Broadbent et al., 2003; Iyer et al., 2010). While the impact of *Streptococcus* in Cheddar cheese production is understudied, further support of *Streptococcus* being a primary contributor to microbial variability in milk suggests it could be a source of quality variability in other dairy products, where its impact is better understood. The actual quality implications of these farm-level differences in the milk microbiome should be the focus of future studies.

Despite the shared milk source at facilities B and C, differences in their microbiota were evident following thermization. This was unexpected, given that each facility was following identical thermization protocols, and seems to suggest that subtle differences in processing equipment can result in noticeable changes in the facility microbiome. Among the SVs that contributed to differences in the thermized milk at facilities B and C was *Lactobacillus* SV 3603. Ratios of SV 3603 were similar in the raw milk at facilities B and C but became distinctly different following thermization. Lactobacilli play a central role in flavor and texture development in Cheddar cheese and are thus considered an important contributor to Cheddar cheese quality (Montel et al., 2014).

Facility differences in the DMC, while simpler in their presentation, were more difficult to attribute to any one factor and likely originated from several factors including the milk and the processing equipment. Indeed, the processing equipment used at each facility, while mostly the same in function and design, did contain subtle differences, particularly regarding their age. The DMC at Facility A has been in operation for approximately 10–15 years longer than those at facilities B and C, possibly explaining the increased bacterial load and species richness observed in the Facility A DMC. The likelihood of bacterial fouling increases with prolonged equipment use, in large part due to the roughening of food contact surfaces resulting from natural wear-and-tear (Van Houdt and Michiels, 2010; Selover et al., 2021). This hypothesis is supported by the observation that many bacterial species isolated from the DMC belts at Facility A are often associated with biofilm formation in dairy manufacturing environments (Cherif-Antar et al., 2016; Zou and Liu, 2018). These species included *Acinetobacter*, *Escherichia*, *Klebsiella*, and *Enterococcus*, many of which were repeatedly isolated on all 3 days of sampling.

The Importance of Daily Changes in the Cheese Facility Microbiome

Evidence of widespread day-day variations in the cheese facility microbiome calls into question the importance of these daily changes for cheese product quality and their influence on the interpretation of spatial diversity. Many of the SVs that differed between facilities also differed between days within facilities. This included *Lactobacillus* SV 3603, which varied between facilities B and C in the thermized milk, along with others including

Streptococcus, *Acinetobacter*, and Enterobacteriaceae. While all four bacteria are commonly isolated from the dairy environment, the quality implications of *Streptococcus* and *Acinetobacter* in Cheddar cheese are mostly unknown (Riquelme et al., 2015; Kable et al., 2016).

Detection of Enterobacteriaceae, which includes coliforms such as *Escherichia coli*, can be indicators of insanitary conditions in dairy manufacturing, in some cases leading to non-compliance with good manufacturing practices (Martin et al., 2016). The sudden increase in Enterobacteriaceae on the final day of sampling in the DMC at Facility A presents a possibly troubling scenario, where problematic bacteria may sporadically increase in cell density in the cheesemaking environment, thus leading to a corresponding increase in the cheese. This observation supports accounts described by the cheese manufacturer at Facility A, where coliforms are sporadically detected in the fresh cheese at low levels (Selover et al., 2021).

A popular belief for many fermented foods is that production facilities can harbor facility-specific microbial communities which impart unique "house" characteristics to the finished product. Evidence of the facility-specific microbiome has been based on observations that microbial communities can differ between facilities (Bokulich and Mills, 2013; Quijada et al., 2018). We argue that facility-facility differences do not necessarily imply specificity and that temporal variations in the facility microbiome may confound the interpretation of facility-facility variation when sampling efforts are insufficient. This argument is based on the recognition of significant day-day variability in the cheese facility microbiome and is supported by trends observed in the matted curd and pressed cheese.

Facility differences in the curd and cheese samples were attributed to an asynchronous rotation of starter blends, which occurred approximately every 24–48 h at each facility. While not all starter blends are shared between facilities, and therefore could be considered facility-specific, the facility-facility differences observed in the curd and cheese in the present study were driven by starter blends that are shared between facilities. Specifically, starter blend III, which was used at Facility A on days 1 and 2 but never at facilities B or C during the sampling period, appeared to be the primary driver of facility differences in the curd and cheese, despite this blend being common to all three facilities. It is expected that greater sampling efforts over a larger timespan would resolve this issue by showing that starter blend III is in fact common to each facility. Future investigations into the facility-specific microbiome should include larger timespans with the intent of differentiating temporal changes from facility changes, particularly those originating from the resident microbiome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI PRJNA687545.

AUTHOR CONTRIBUTIONS

JJ, CC, and JW-C conceived and designed the work. JJ collected the data, conducted data analysis and interpretation, and drafted the article. CC and JW-C critically revised the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Brazilian Artisanal Cheeses: Diversity, Microbiological Safety, and Challenges for the Sector

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Artisanal cheeses made with raw milk are highly appreciated products in Brazil. Most of these cheeses are produced in small facilities across different production regions in the country, some of which have been granted a protected designation of origin and are award winners. The most prominent state that manufactures these products is Minas Gerais (MG), but production is also gaining strength in other Brazilian states. The major challenge faced by artisanal cheese production is related to microbial risks associated with foodborne pathogens when the quality of the raw milk is unsatisfactory. Regulations created for the dairy industry are constantly been revised and adapted, considering the small-scale production of Brazilian artisanal cheeses, in order to guarantee safety at all steps of cheese production and commercialization. This text presents a summary of the huge diversity of artisanal cheeses produced in the country, grouped by geographical regions, and reviews the current challenges faced by producers and government considering the safety of these cheeses.

Keywords: food safety, ripening, foodborne pathogens, fermentation, good manufacturing practice

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INTRODUCTION

The history of artisanal cheese production in Brazil dates to the second half of the 18th century. After the arrival of Europeans in Brazil in 1500, Portuguese settlers brought cattle from Serra da Estrela, Portugal, to the region which now corresponds to the state of Bahia, in the Northeastern part of the country. Herds of domesticated cattle expanded southward along the São Francisco River, reaching the region of Serro, in Minas Gerais (MG) state, where gold explorers started the production of artisanal cheeses, using raw bovine milk and rennet from parts of the stomach of calves (Sertão Brás, 2017a). When gold mining and sugar cane exploration lost strength, the manufacture of artisanal cheeses gained economic importance, leveraged by the construction of a highway in 1929–1930 connecting the region of Serro with other municipalities in MG state, and Belo Horizonte, the state's capital city. A great expansion of the market occurred in the following years, and artisanal cheese production became an autonomous element of the economy, not only for the region, but also for the entire state of Minas Gerais (Pires, 2013a,b). Although having started only recently in several parts of the country, artisanal cheese production is gaining increasing

importance as economic income for thousands of families in rural areas, contributing to the local economies. This is especially due to the new demands from consumers who are increasingly seeking differentiated products, less processed, culturally rich and with a unique identity.

According to the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística - IBGE), Brazil encompasses 26 states and the Federal District, grouped into five geographical regions: Southeast, Northeast, South, North and Central-West (IBGE, 2020). In addition to Minas Gerais in the Southeast region, which is by far the largest cheese producer state, artisanal cheese production is growing rapidly in other states (**Figure 1**), with cheeses for all tastes and purchasing power. Two recent reviews show complementary aspects of artisanal cheese production in Brazil: Kamimura et al. (2019) characterized the technological, physical-chemical, and microbiological features of the main types of artisanal cheeses in the country, and Camargo et al. (2021) discussed the quality and safety of these products and presented insights into the regulatory aspects of the production chain.

With many research advances, several recent regulatory developments throughout the country and an increased appreciation for artisanal cheese consumption, an updated view of the Brazilian production chain is still required. The current review highlights the diversity of artisanal cheese production in Brazil, discussing aspects that have not been evaluated previously, presenting an updated outlook of artisanal cheese production in the country. We underscore the areas in which more research needs to be conducted and indicate how Brazilian scientists have contributed to advancements in the field. We finally present a perspective for how research and extension collaborative efforts could drive quality and safety improvements in artisanal cheese production.

DIVERSITY OF ARTISANAL CHEESES PRODUCED IN BRAZIL

Artisanal cheese production is performed by small rural facilities across the nation, with historical, cultural, and technological aspects that are specific to the various producing regions.

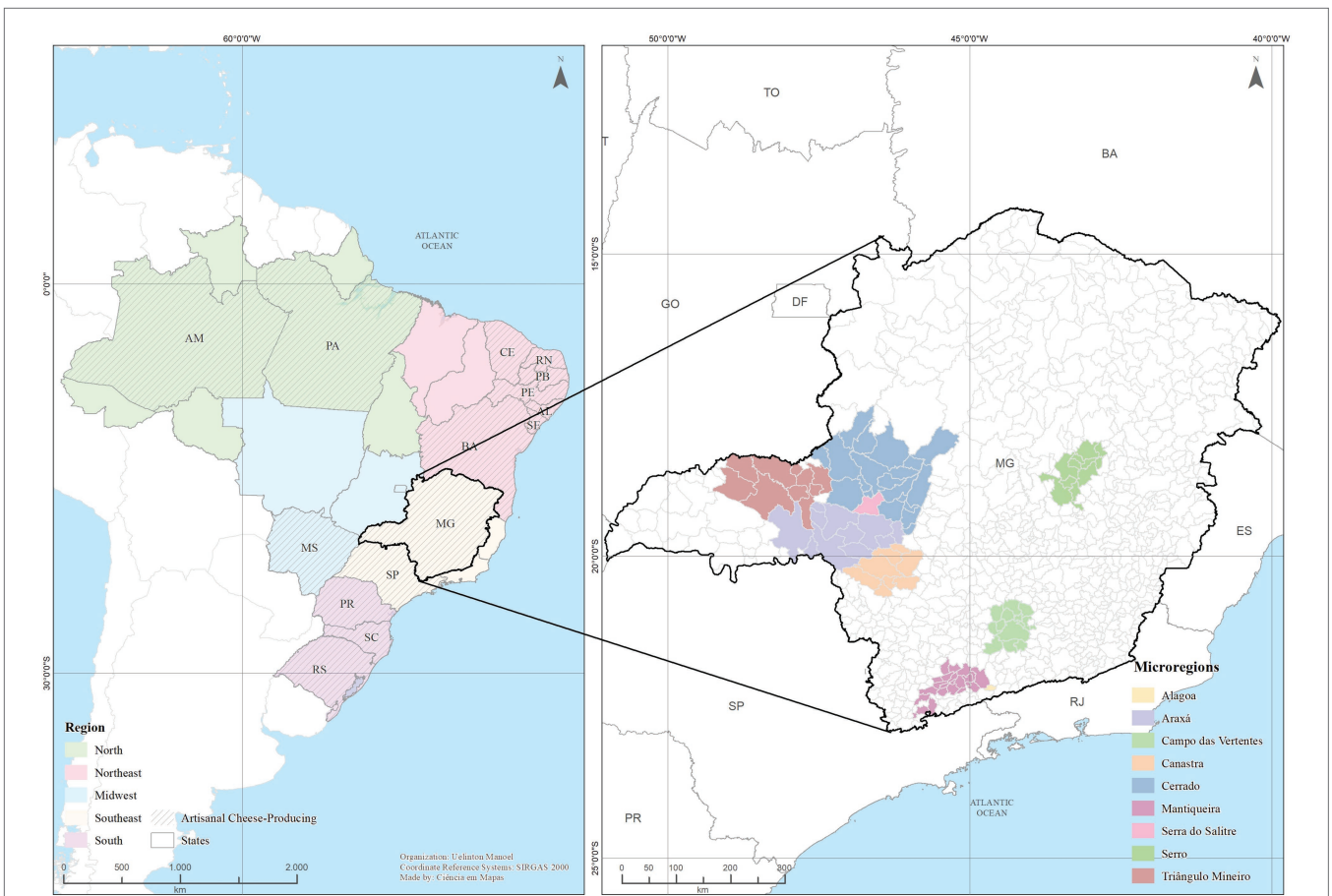

FIGURE 1 | States within each Brazilian region where the main Brazilian artisanal cheeses are produced. **North**: AM, Amazonas; PA, Pará; **Northeast**: AL, Alagoas; BA, Bahia; CE, Ceará; PB, Paraíba; PE, Pernambuco; RN, Rio Grande do Norte; SE, Sergipe; **Southeast**: MG, Minas Gerais; SP, São Paulo; **Central-West**: MS, Mato Grosso do Sul; **South**: PR, Paraná, RS, Rio Grande do Sul; SC, Santa Catarina. The inset highlights the nine artisanal cheese producing microregions in the state of Minas Gerais (MG).

The best-known types of Brazilian artisanal cheeses, according to the producing region and state, are listed in **Table 1**. **Figure 1** shows an updated view of the geographical location of these producing regions.

Artisanal Cheeses From the Southeast

Cheeses From the State of Minas Gerais

The state of Minas Gerais in Brazil is historically recognized for its secular tradition in cheese making. This state is the largest cheese producing in Brazil and stands out because of the production of a large variety of artisanal cheeses, collectively named as *Minas artisanal cheese*.

The manufacture of *Minas artisanal cheese* started with the Portuguese colonizers in the 18th century, becoming the most popular and consumed artisanal cheese in the country. These products have a great socio-economic importance in the state, as thousands of rural families depend on them for their survival (Meneses, 2006; Bemfeito et al., 2016). These cheeses are conventionally produced in seven spatially limited regions in the state harboring peculiar geomorphological and cultural characteristics: Araxá, Campo das Vertentes, Canastra, Cerrado, Serra do Salitre, Serro, and Triângulo Mineiro (IMA, 2016; **Figure 1**). The manufacturing procedure in these regions is similar and follows the Portuguese tradition, differing mainly in the curd pressing stage: in Serro, the curd is pressed with bare hands while in Canastra, Serra do Salitre and Cerrado cheese cloth is used. Thus, depending on the pressing method, more whey can be retained in the curd and, consequently, the product will present significant differences in flavor and texture. It is worth mentioning that there are other regions recognized as cheese producers in Minas Gerais, but the production process is carried out differently from the *Minas artisanal cheese* process, as it will be described below (EMATER, 2004; Minas Gerais, 2020a,b).

Figure 2 shows a flowchart of the production of *Minas artisanal cheese*, the most studied Brazilian artisanal cheese. This product is manufactured with unpasteurized cow milk, added liquid or powdered rennet, salt and a type of endogenous starter culture usually referred to as "*pingo*," known as the back-slopping method. *Pingo* is composed of fermentative

TABLE 1 | Types of Brazilian artisanal cheeses.

Cheese type	Production region	Production state	Type of milk	Starter culture type	Classification	Ripening time (minimum period, in days)
Araxá	Southeast	MG	Cow	*Endogenous**	Medium moisture	14
Campo das Vertentes			Cow			
Canastra			Cow			22
Canastra Real or Canastrão			Cow			60
Cerrado			Cow			
Serra do Salitre			Cow			
Triângulo Mineiro			Cow			
Serro			Cow			17
Cabacinha			Cow	*Endogenous*	Medium to high moisture	15
Alagoa			Cow	*Endogenous*	Low moisture	14
Mantiqueira de Minas			Cow	*Endogenous*	Low moisture	14
Different Types of Cheese		SP	Goat/Cow/Buffalo/ Goat/Sheep	Varies according to the type of cheese		
Porungo	Northeast	AL, BA, CE, MA, PB, PE, RN, SE	Cow	*Endogenous*	High moisture	Not ripened
Coalho			Cow/Goat/ Buffalo/ Sheep	Commercial	Medium to high moisture	Fresh or ripened up to 10 days.
Manteiga (Sertão Cheese)		CE, PE, RN BA, PB,	Cow/Goat	Endogenous	Medium to high moisture	Not ripened
Flor de Mandacaru		PE	Cow	No information available		60
Cariri		PB	Goat	Commercial		7
Dom Ariano		PB	Goat	No information available		180
Dom Manelito			Cow	No information available		120
Colonial	South	PR, RS, SC	Cow	Commercial	Medium moisture	10
Serrano		SC, RS	Cow	Commercial	Medium moisture	60
Kochkäse		SC	Cow	Not used	High moisture	Not ripened
Marajó	North	PA	Buffalo/Cow	*Endogenous*	High moisture	Not ripened
Caipira	Central West	MS	Cow	*Endogenous*	Medium to low moisture	Up to 60

**Endogenous culture is the fermented whey collected in the manufacture of the cheese from the previous day.*

AL, Alagoas; BA, Bahia; CE, Ceará; MG, Minas Gerais; MA, Maranhão; MS, Mato Grosso do Sul; PA, Pará; PB, Paraíba; PE, Pernambuco; PR, Paraná; RN, Rio Grande do Norte; RS, Rio Grande do Sul; SP, São Paulo; SC, Santa Catarina; SE, Sergipe.

microorganisms, obtained from the whey that drains from freshly manufactured cheeses during the molding stage, and it is used to make the next day's batch (Perin et al., 2017). The microbial diversity of *pingo* is characteristic of each production region, explaining the unique sensorial characteristics (taste, texture, color, and aroma) that develop during ripening of cheeses produced in these regions (Meneses, 2006).

The Minas Gerais State Law 23157 (Minas Gerais, 2018a) defines *Minas artisanal cheeses* as those manufactured with fresh and raw whole cow milk, harboring specific identity and quality characteristics. Due to the traditional, cultural and economic importance, *Minas artisanal cheeses* were granted with the Cultural Property of Immaterial Nature status, conferred by the National Historic and Artistic Heritage

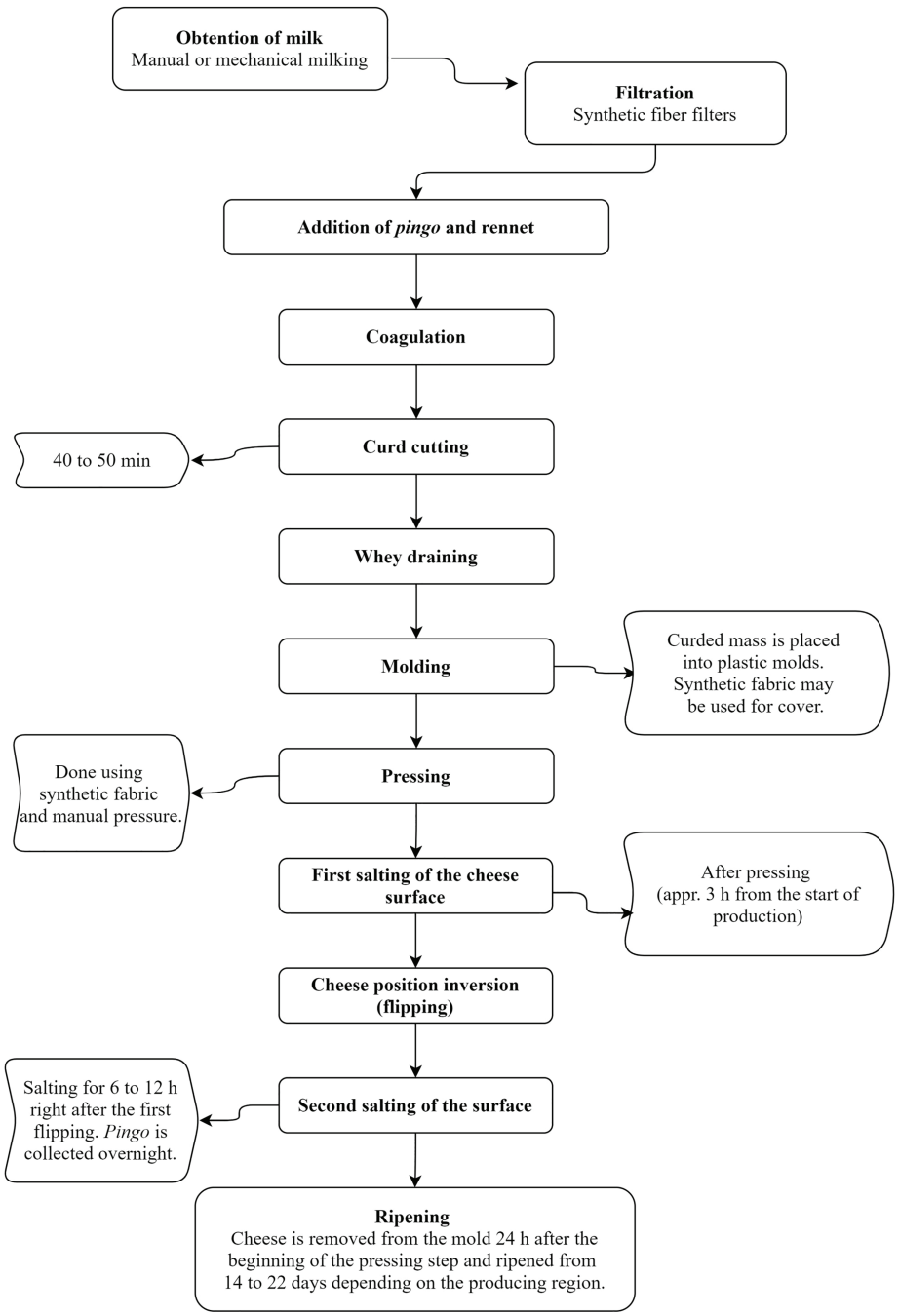

FIGURE 2 | Flowchart of *Minas artisanal cheese* production. *Pingo* is a type of endogenous starter culture composed of fermentative microorganisms. It is collected from freshly manufactured cheeses during the molding stage, and it is used to make the next day's batch (backslopping method).

Institute (*Instituto do Patrimônio Histórico e Artístico Nacional* - IPHAN) in 2008 (IPHAN, 2008).

The most relevant types of *Minas artisanal cheeses* are described below:

Canastra Cheese

The *Canastra cheese* is a typical product from the Serra da Canastra region, covering Delfinópolis, São Roque de Minas, Vargem Bonita, Tapiraí, Bambuí, Medeiros, Piumhi, São João Batista do Glória and Córrego D'Anta municipalities (Minas Gerais, 2018b). In this region, there are 4,813 dairy farms and approximately 264,000 animals, resulting in an average of 55 animals per farm (EMATER, 2004). Approximately 800 of these farms are dedicated to production of *Canastra cheese*. Average milk production of matrices is about 1,400 liters/lactation, and the fat content is close to 3%, i.e., excellent for cheese production (EMATER, 2004; Meneses, 2006). In 2002, around 60 producers joined efforts and created the Canastra Cheese Producers Association (*Associação dos Produtores de Queijo da Canastra* - APROCAN), aiming at increasing their reach and protecting their products. The total turnover of this group of producers is around R$ 60 million (11 million USD) per year, with an average of 25 cheeses produced every day in each farm (Folhapress, 2019). After the official recognition of APROCAN in 2005, several new producers have joined the association and many others are in the process of joining (Folhapress, 2019).

The *Canastra cheese* has the following characteristics: cylindrical shape, flat or slightly curved at the sides and a slightly acidic and non-spicy flavor. It has a yellowish-white color and a thin yellowish crust that may darken with ripening. The required ripening time is a minimum of 22 days, resulting in a semi-hard or slightly soft, buttery and compact cheese (APROCAN, 2011). There are some variants of *Canastra cheese*:

- *Traditional*: cheeses produced in bottomless cylindrical containers, presenting 6–9 cm height, 17 cm diameter, 900–1,300 g weight, and 22 days of ripening (APROCAN, 2011; Borges et al., 2019).
- *Merendeiro*: smaller cheeses, presenting 10 cm diameter, 6 cm height, 300–400 g weight, and 22 days of ripening (APROCAN, 2011).
- *Real* (also called *Canastrão*): larger in size (28–35 cm diameter and 10–18 cm height), these cheeses have 5,000–7,000 g weight and are ripened for at least 60 days (APROCAN, 2011, 2014). Their flavor is sweet and light, with a bitter taste at the end, reminiscent of Dutch cheeses. According to the local memory, this type of *Canastra cheese* was formerly produced for special occasions, such as visits of authorities from the church, the government or the military. The main characteristic of *Canastra Real* is the presence of propionic bacteria, responsible for the curing process, and production of gas, which contributes to the puffiness and formation of round holes in the cheese, similar to Emmental and Gruyère cheeses.

In May 2008, the *Canastra cheese* was recognized as a Brazilian Intangible Cultural Heritage (IPHAN, 2008). In 2012, the National Institute of Industrial Property (*Instituto Nacional de Propriedade Industrial* - INPI) granted these cheeses with the "Geographical Indication" seal (SEBRAE, 2018; INPI, 2019) and the Serra da Canastra region was recognized as a reference in production of cheeses (EMBRAPA, 2019a). *Canastra cheeses* are gaining international recognition, winning many international awards, such as the 24 super gold, gold, silver and bronze medals in the "*Mondial du Fromage et des Produits Laitiers*" competition, in France, in June 2019 (EM, 2015; G1, 2019).

The unique features of *Canastra cheese* can be attributed to the milk that comes from mixed-breed of *Bos taurus*, *Bos indicus*, and other variants and to the altitude and characteristic climate, in addition to natural pastures for cattle feeding, comprised by *Capim meloso* (*Melinis minutiflora*) and native grasses, which are being gradually replaced by more productive ones, such as *Brachiaria* spp. and *Panicum* (IMA, 2013; Meneses, 2006).

Serro Artisanal Cheese

This type of cheese corresponds to a group of cheeses produced by approximately 760 cheese producers, located in the municipalities of Alvoradas de Minas, Conceição do Mato Dentro, Dom Joaquim, Materlândia, Paulistas, Rio Vermelho, Sabinópolis, Santo Antônio do Itambé, Serra Azul de Minas, Coluna and Serro. The average daily production in Serro and neighboring municipalities is around 10,000 cheeses, with a volume of 110 L of milk per property (Sertão Brás, 2017b).

According to Minas Gerais State Ordinance 1305/2013 (Minas Gerais, 2013), *Serro cheese* must be ripened for 17 days, resulting in products with firm consistency and mild and slightly acid flavor. They present a thin crust and a yellowish white color and a natural sheen. The shape is cylindrical, with 13–15 cm diameter and 4–6 cm height and weight varies from 700 to 1,000 g (EMATER, 2003). *Serro cheeses* have also been recognized as a Brazilian Intangible Cultural Heritage in 2008 (Minas Gerais, 2018a). In 2011, INPI granted the "Geographical Indication" seal in the item of "Origin Indication" (SEBRAE, 2018).

The peculiar sensorial properties of *Serro cheeses* are devoted to milk coming from European cow breeds such as Dutch, Jersey and Swiss Pardo. The Serro region has about 124,000 animals, distributed in 2,581 properties, usually small cattle rancher families, with an average of 50 animals per property (EMATER, 2002). Average production is 110 L of milk per day, per property. It is estimated that *Serro cheese* is produced in 6,000 properties, but only 756 are registered in this activity, where 10,000 pieces are produced per day. The cattle in this region is fed with *Brachiaria* spp., *Panicuns*, Jaraguá grass (*Hyparrhenia rufa*), Meloso grass (*Melinis multiflora*), and leguminous plants such as Carrapicho (*Cenchrus echinatus* L.), Beiço de boi (*Desmodium* sp.), Calopogônio (*Calopogonium mucunoides*), among others (APROCAN, 2014).

Araxá Artisanal Cheese

The *Araxá cheese* is a typical product from the micro-region comprising the municipalities of Araxá, Tapira, Pratinha,

Conquista, Ibiá, Campos Altos, Perdizes, Pedrinópolis and Sacramento. These cheeses present semi-hard consistency, with a tendency to soft, and a butter-like nature. They have a thin crust and are yellowish without cracks and generally present cylindrical size of 13–15 cm diameter and weigh between 1.0 and 1.2 kg. The smell and taste are acid, pleasant and not spicy (EMATER, 2003).

The local herd for milk production is composed mostly by mixed-breed animals, mainly Dutch, with around 476,000 animals, divided into roughly 7,000 farms, with an average of less than 60 heads per farm. The average production is 2,400 L of milk per lactation, with fat content above 3.0%. Around 88% of the area is pasture lands, and the remaining has unmodified vegetation cover (EMATER, 2003). Physical and environmental conditions such as altitude, soil and microclimate, provide special pastures, mainly *Capim gordura* (also known as *Capim meloso*), *Capim Jaraguá*, and *Macega* (EMATER, 2003). There are approximately 1,336 cheese producers in this micro-region.

Other Types of Artisanal Cheese Produced in Minas Gerais State

This group encompasses cheeses named *Parmesão da Mantiqueira* (also known as *Queijo Artesanal Mantiqueira de Minas*), *Parmesão de Alagoa* (also known as *Queijo Artesanal de Alagoa*), *Cabacinha* and *Requeijão Moreno*. The region of Mantiqueira and the municipality of Alagoa were recently recognized as artisanal cheese producer regions in the state of Minas Gerais (Minas Gerais, 2020a,b; **Figure 1**).

Despite being produced with raw milk, *Parmesão da Mantiqueira* and *Parmesão de Alagoa* are different from the traditional *Minas artisanal cheeses* because they are made with commercial starter culture and are submitted to a thermal process during production (Minas Gerais, 2019; SEAPA, 2020).

The *Cabacinha cheese*, a local version of the Italian Caciocavallo cheese, is produced in the Vale do Jequitinhonha, in the North of the state. The curd is cooked in boiling water, shaped into natural gourd or teardrop shape, tied in pairs by strings and hung to dry. Due to its peculiar shape that reminds the Brazilian porongo (or porungo) fruit (*Lagenaria siceraria*), the *Cabacinha cheese* is also known as *Porongo cheese* (or *Porungo cheese*). The cheese can be smoked or filled with butter (Filho et al., 2017; Vasek and Filho, 2019).

The *Requeijão Moreno cheese* is mechanically pressed, with high salt content (EM, 2018; Sobre Queijos, 2020). This cheese is produced in Jequitinhonha and Mucuri Valleys, located in the North of Minas Gerais.

Moldy Artisanal Cheeses

Production of moldy cheeses, similar to the French Brie cheese, is gaining importance in the state of Minas Gerais, particularly in Serra da Canastra and Serro. These cheeses present a white mold rind, are firm to the touch but creamy in the mouth, with striking aroma and taste that can be delicate or intense (EM, 2018). Fungi that proliferate in the surface of the cheese introducing their digestive enzymes into the curd, breaking down fat and proteins, turning it softer (Hui et al., 2004). Even though Brazilian artisanal moldy cheeses do not have a specific legislation, they have gained a precious status in specialized stores and fine restaurants in several Brazilian big cities. Little information is available on the production techniques of artisanal moldy cheeses in Brazil (Tristão, 2015), but it is known that the fungi come from the ripening rooms, as producers do not add a specific mold to their product. The production conditions are not controlled and the contamination, in a way, occurs at random.

Cheeses From the State of São Paulo

Artisanal cheeses produced in the state of São Paulo are distinct from those of the Minas Gerais state. As producers in the state of São Paulo cannot rely on centuries of tradition, they invest in innovation, using European fine cheeses as models. However, little is known about the production processes, annual turnover, market, and production practices. Even the "artisanal" concept in the state of São Paulo differs from that used in Minas Gerais state for raw milk cheeses. In São Paulo, this nomenclature refers to cheeses produced in small producing properties, using a large array of technological processes and ingredients, some of them quite sophisticated. These cheeses may be produced with raw or pasteurized milk from different types of animals (cow, goat, buffalo, and sheep), resulting in unique cheeses not found in other parts of the country. There is no current legislation that specifically deals with artisanal cheese production in the state of Sao Paulo and research is needed to characterize the products and production processes.

Aiming at strengthening the artisanal cheese sector in the state of Sao Paulo, and removing these cheeses from clandestinity, a group of local producers created, in 2017, the São Paulo Association of Artisanal Cheese (*Associação Paulista do Queijo Artesanal* - APQA). Currently, the APQA affiliates around 80 cheese producers from across the state (Pereira, 2018). APQA includes not only producers with more than 20 years of history in cheese production, but also new cheese makers starting ventures in the sector.

For the purpose of this review, artisanal cheeses produced in the state of São Paulo were divided into two groups:

Cheeses From the Paulista Artisanal Cheese Path (Caminho do Queijo Artesanal Paulista)

Currently, the *Paulista Artisanal Cheese Path* (Caminho do Queijo Artesanal Paulista) comprises 10 cheese producers from the municipalities of Joanópolis, Amparo, Porangaba, Itapetininga, São João da Boa Vista, São José do Rio Pardo, Pardinho, Cabreúva, Bofete and Porto Feliz, in the state of São Paulo. These producers use raw or pasteurized milk from cow, buffalo, goat and sheep, and cure the cheeses in ripening chambers or subterranean caves, and some add spices (REPEQUAB, 2020). For instance, over 150 cheese varieties are produced in these dairies, highlighting the potential for innovation in cheese production in the state of São Paulo. The tropical climate during hot and rainy summers favors

the growth of protein-rich pastures, such as *Capim elefante* (*Pennisetum purpureum*) and *Capim Tanzânia* (*Panicum maximum* cv. Tanzania), while in dry and relatively cold winter, equally rich oat (Avena sp), azevém (*Lolium multiflorum* Lam), sugarcane (*Saccarum officinarum*) and silage are used (EMBRAPA, 2002a; São Paulo, 2017, 2018). The cheese producers from the *Paulista artisanal Cheese Path* are also affiliated to APQA.

Porungo Cheese

The *Porungo cheese*, also called *porongo*, *cabacinha, cabaça, porunguinho, nozinho, cabecinha, enforcado* or *pescocinho*, is similar to the *Cabacinha cheese* produced in the state of Minas Gerais, described above. This type of cheese is produced in the southwest of the São Paulo state, mainly in the municipalities of Angatuba, Buri, Campina do Monte Alegre, Itapetininga and Pilar do Sul (Vasek and Filho, 2019; Silva et al., 2021). Porungo is an unripe *pasta filata* cheese, manufactured with raw milk coagulated with commercial rennet and added of fermented whey, collected in the previous day production, that contains the autochthonous microbiota of milk, responsible for the peculiar sensorial characteristics of the cheese, that resemble the mozzarella cheese. *Porungo cheese* producers are not part of the *Paulista Artisanal Cheese Path* or even affiliated to APQA, but commercialization of this product has great economic importance and is a source of income for numerous small producers. These cheeses are sold formally in supermarkets but also informally, directly to consumers or in free markets (Vasek and Filho, 2019).

Cheeses From the Northeast (States of Alagoas, Bahia, Ceará, Maranhão, Paraíba, Pernambuco, Rio Grande do Norte, and Sergipe)

Coalho Cheese

Coalho cheese is the most typical artisanal cheese produced in the Northeast region of Brazil, widely consumed by the local population and throughout the country. This is a firm, lightweight yellowish white fresh cheese, prepared with raw milk of cow, buffalo, goat, or sheep and rennet, presenting 35.0–60.0% fat content. It has a slightly salty and acidic flavor and elastic texture and it is used for preparation of the popular "roasted cheese" as it does not melt when heated.

The most relevant *Coalho cheese* producers are located in Batalha, state of Alagoas; Antas, Chapada Diamantina National Park Juazeiro, Feira de Santana and Irecê, state of Bahia; Quixadá and Sobral, state of Ceará; Riachão do Jacuípe, state of Maranhão; Garanhuns and Riacho das Almas, state of Pernambuco; Seridó, state of Rio Grande do Norte; Nossa Senhora da Glória, state of Sergipe and around 50 municipalities in the state of Paraíba (**Figure 1**).

This cheese has great economic importance for the Northeast region of Brazil, significantly impacting the income of milk suppliers, especially those who lack access to milk processing plants (Brazil, 2001a; Silva et al., 2012; Fontenele et al., 2017; Pernambuco, 2018). It is estimated that about 40–50% of milk production in the region is destined to the production of *Coalho cheese* (Silva et al., 2012; Da Cruz, 2016). In this region, the use of genetically modified animals, such as the F1 hybrid (Dutch/Zebu), is common. These animals present profitable characteristics such as high resistance against lack of rain, and high productivity (Da Cruz, 2016).

Manteiga Cheese (Sertão Cheese)

Also called *butter cheese* and produced in many states of the Northeast region of the country, *Manteiga cheese* is soft and has a fat content ranging between 25 and 55%. The taste is light, slightly acidic, and salty, and the color is light yellow (Brazil, 2001b; Leite et al., 2019). Its production consists of coagulating whole or skimmed cow's milk, draining the curd obtained by acidification, melting and addition of butter or vegetable oil to the melted curd, cooking at 85°C for 15 min and pressing. The butter (*Manteiga de Garrafa*, *Manteiga da Terra* or *Manteiga do Sertão*) used in the manufacture is artisanal as well.

Other Artisanal Cheeses of This Region

Other award-winning but not so well characterized goat milk cheeses produced in this region are *Dom Ariano* and *Dom Manelito*, created to honor two famous Brazilian poets (Ariano Suassuna and Manuel Bandeira) and *Cariri cheese*, that honors the religious mysticism of the Cariri region, in the state of Paraíba. *Requeijão Pernambucano*, a soft cheese and *Flor de Mandacaru*, that reminds the French Camembert cheese, are popular cow milk cheeses produced in the state of Pernambuco (Sertão Brás, 2013; Taperoa, 2016).

Cheeses From the South (States of Paraná, Rio Grande do Sul and Santa Catarina)

The South region of Brazil is characterized by subtropical climate with temperature ranging between 0 (occasionally below 0°C) and 32°C (EPAGRI, 2015). The arrival of European immigrants, mainly Italian and German, to this region in the 19th and 20th centuries had strong influence on the cheese-making culture (Wilkinson et al., 2017). The most prominent artisanal cheeses produced in the South region are the *Serrano*, *Colonial*, and *Kochkäse cheeses*. Less known are the *Diamante* cheese, from the municipality of Major Gercino, *Contestado cheese* from Contestado Valley and *Queijinho* from Itajaí Valley, all located in Santa Catarina state.

Serrano Cheese

The *Serrano cheese* is the main type of cheese produced in Serrana Region in the state of Santa Catarina and in Campos de Cima da Serra Region in the state of Rio Grande do Sul (Pretto and Sant'Anna, 2017; Slow Food, 2018). *Serrano cheese* is a semi-fat cheese of medium moisture, made with raw cow milk and ripened for 60 days. Its color is yellowish or straw yellow. It has a compact curd and elastic consistency, tending to the greasiness, and may contain small mechanical and/or propionic eyes, lacking a standard for shape, weight, moisture and

salt content (Rio Grande do Sul, 2014, 2016, 2018). The milk comes from mixed breeds, mainly Charolais, Dutch, Devon, Norman, Angus and Hereford, fed on pasture (Slow Food, 2018).

Serrano cheese production in the state of Santa Catarina is widespread, with approximately 2,000 producers and 1,600 tons of cheese traded every year, and total gross sales around R$ 21 million (U$ 3.8 million). In Rio Grande do Sul, there are around 1,500 producers that trade 800 tons of *Serrano cheese* per year, with sales of approximately R$ 10 million (U$ 1.8 million; EPAGRI, 2015). In March 2020, the INPI granted the Geographical Indication "*Campos de Cima da Serra*," in terms of "*Origin Appellation*" to the *Serrano cheese* produced in this location (INPI, 2020).

Colonial Cheese

Colonial cheese is produced by many rural families in the South of Brazil, especially in Santa Catarina state (Carvalho et al., 2019). Traditionally made from raw cow milk, production had to change to pasteurized milk due to legal requirements. The cheese curd is heated to 30°C and can be added with spices or vegetable products and the minimum ripening period of 10 days is required (Santa Catarina, 2018). The cheeses present square and round shapes, and the weight of each piece varies from 1.0 to 1.2 kg (Fava et al., 2012). The cattle used in milk production belong to Dutch and Jersey breeds, fed on pastures with the addition of corn, sweet potato leaves and forage (Slow Food, 2016a).

Kochkäse Cheese

This cheese is an unripened cheese, made from raw or pasteurized milk and the curd is cooked. It is produced in the Itajaí Valley in the state of Santa Catarina, mainly in the cities of Indaial, Timbó, Pomerode, Blumenau, Caminhos do Príncipe, and Joinville. This cheese has a mild flavor and is light yellow (Slow Food, 2016b). Milk comes from Jersey breed, fed with *Capim gramão novo*, *Capim gordura, Catamão branco* and silage. Many families produce *kochkäse* for their own consumption and commercialization, but current health and safety standards have forced some producers to stop producing this cheese (Meisen et al., 2019).

Artisanal Cheeses From the North

Marajó Cheese

The *Marajó cheese* is the most famous artisanal cheese from the North of Brazil. It is an unripened cheese produced for over 200 years in the Marajó archipelago, in the state of Pará, using buffalo milk or a mix of buffalo and cow milk. The buffalo herd in this region is the largest in the country, around 800,000 animals, and among these, 450,000 in state of Pará state (Seixas et al., 2015). The buffalo herd is made up of the *Carabao, Baio, Mediterraneo, Múrrah, Jaffarabadi*, and crossbred breeds (Cassiano et al., 2003). The main milk and buffalo cheese producers are in the municipalities of Soure and Cachoeira do Arari, in the state of Pará (Agência Pará, 2019). There are 60 cheese producers in the Marajó Island, which make 60–100 kg of cheese per day and this activity is very important for the local economy (CEAD, 2014).

The Marajó Island has a rainy tropical climate and an average temperature of 27°C. The rainiest months are January to June and the less rainy ones are September to November (Lima et al., 2005). The climate contributes to the presence of native pastures such as *Capim canarana verdadeira* (*Echinochloa polystachya*) and *Capim quicuio* (*Brachiaria humidicola*), which are used to feed the herd (Meisen et al., 2019).

There are two types of *Marajó cheeses*: one is butter-type, made with whole milk and added butter, and the other is cream-type, made with skimmed milk and cream from skimmed milk. For manufacturing, the curd is drained and washed with water or milk. The product has a light-yellow color and presents slightly acidic and salty flavor and semi-hard consistency (ADEPARÁ, 2013; Vasek and Filho, 2019).

Other Artisanal Cheeses From This Region

The *Manteiga* and *Coalho cheeses*, produced in the Northeast region of the country, are also manufactured in Manaus and surroundings, in the Amazonas state, following the same cheese making techniques (Vasek and Filho, 2019).

Artisanal Cheeses From the Central-West

Caipira Cheese

Caipira cheese is manufactured in Mato Grosso do Sul state, traditionally recognized as a cattle production state. Nevertheless, in 1980, rural families started producing cheese as an option of income. The majority of producers are located in São Gabriel do Oeste, Corguinho, Rochedo, Jaraguari, Terenos, Ribas do Rio Pardo, Aquidauana, Bandeirantes, Camapuã, Santa Rita do Pardo and Sidrolândia municipalities, besides Campo Grande, the state's capital. *Caipira cheese* is made with raw, fresh whole cow milk, following historical and cultural tradition of the region of production. The product must be manufactured in the original rural property and must be submitted to 60 days of ripening. The climate of this region is tropical, characterized by hot and rainy weather. The main breeds of cattle used for milk production are Dutch, Swiss-Parda, Schwyz, Jersey, Guernsey, Ayrshire or crossbred, and the feed is based on a mixture of silage, hay, chopped green grass, added with energy and protein concentrates, minerals and vitamins (EMBRAPA, 2002b).

Unlike *Minas artisanal cheese*, the sensorial and physicochemical characteristics of *Caipira cheese* are not well defined, so the producers belonging to the Association of Artisanal Cheese Producers in Mato Grosso do Sul (*Associação dos Produtores de Queijo Artesanal de Mato Grosso do Sul* - AQUEIJART) have recently partnered with the State Agency for Animal and Plant Sanitary Defense (*Agência Estadual de Defesa Sanitária Animal e Vegetal* - AGRAER) and State Secretariat for the Environment, Economic Development, Production and Agriculture (*Secretaria de Estado de Meio Ambiente, Desenvolvimento Econômico, Produção e Agricultura Familiar* - SEMAGRO) in order to establish quality and production parameters, by means of technical-scientific and microbiological studies (Campo Grande News, 2018).

SAFETY OF ARTISANAL CHEESES MADE WITH RAW MILK

The microbial communities in cheeses manufactured with raw milk play an important role during ripening. Besides determining the sensorial and physicochemical properties of the final products, they may inhibit the growth of pathogens (Fuka et al., 2013). High humidity and short ripening cheeses are at greater risk of harboring pathogens, in comparison to lower moisture and slower ripening varieties (Ozturkoglu-Budak et al., 2016). In the beginning of ripening, there is a higher prevalence of lactic acid bacteria, mainly *Lactococcus*, *Streptococcus*, and *Lactobacillus*, responsible for the fermentation of sugars and development of the unique sensorial characteristics of artisanal cheeses (aroma, flavor, and texture). Their capability to produce organic acids from sugars during ripening causes pH drop and lowers the oxy-reduction potential. In addition, production of hydrogen peroxide, carbon dioxide, and bacteriocins may inhibit the growth of pathogens (Camargo et al., 2021).

A compilation of studies conducted from 1973 to 2006 in Switzerland, United States, Sweden, Canada, France, Brazil, United Kingdom, Spain, Malta, Scotland, England, and Finland (FSANZ, 2009), based on 84 outbreaks attributed to cheese consumption, concluded that 69% of the outbreaks were associated to raw milk cheeses, while 7.2% were caused by cheeses with no information about heat treatment. Among the outbreaks, two were attributed to cheeses produced in Minas Gerais state, Brazil. However, none of these cheeses fit the category of artisanal cheeses, as defined by the Brazilian regulatory standards (Do Carmo et al., 2002; Brazil, 2019b).

In Brazil, artisanal *Coalho cheese* was incriminated in 55 foodborne disease outbreaks in the Amazonas state between 2005 and 2009. According to the Department of Epidemiological and Environmental Surveillance of Manaus, Amazonas, 14 (25%) of these outbreaks were due to coagulase positive *Staphylococcus aureus*, four (8%) due to *Bacillus cereus*, two (4%) due to *Salmonella* spp. and one due to *Clostridium perfringens*. In one outbreak, both coagulase-positive *Staphylococcus* and *B. cereus* were found. The etiological agent was not determined in the remaining outbreaks (Ruwer et al., 2011).

Even though there are some reports of outbreaks due to consumption of raw milk and raw milk cheeses around the world, accurate and official information on this issue is lacking in Brazil. Data from the National Health Surveillance Agency of the Brazilian Ministry of Health indicate that milk and dairy products were responsible for 2.75% of the foodborne outbreaks reported in the 2000–2018 period (Brazil, 2019a; Finger et al., 2019). Even considering that the type of dairy product associated with the reported outbreaks is unknown and that the number of outbreaks is possibly underreported, the relevance of artisanal cheeses prepared with raw milk as causes of foodborne diseases should not be ignored.

One of the major concerns in dairy products made with unpasteurized milk are *Brucella* spp. and *Mycobacterium bovis*. *Brucella* spp. causes brucellosis, a zoonosis that can be transmitted from animals to humans and vice versa. In cattle, this disease can cause abortion and congenital related problems, while in humans it is usually manifested as a general infection. This pathogen can be present in the mammary gland of infected animals and eliminated through milk. Brucellosis presents economic losses due to the reproductive problems caused by the disease in cattle (Paulin and Ferreira Neto, 2008). Another important zoonotic disease is tuberculosis caused by *M. bovis*, which contributes to the development of nodular lesions in tissues or organs of the animal, and causes abortion, fever, drop in milk production and weight reduction, which can lead to the death or slaughter of animals. In humans, the disease can be transmitted through the consumption of unpasteurized milk and dairy products, the consumption of uncooked meat or by contact with the infected animal and may present symptoms such as fever, weight loss, lung problems, cough, shortness of breath, among others (Kuria, 2019). The Brazilian regulations, by means of the National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis, set requirements for the control of *Brucella* spp., *M. bovis* and pathogens in dairy products manufactured with raw milk, establishing that herds must be certified as brucellosis and tuberculosis free (Brazil, 2019c).

Staphylococcus. aureus is another relevant microbial pathogen in unpasteurized milk, as it causes mastitis, an infectious process that affects the mammary gland and causes changes in the secretion and composition of milk, resulting in great economic losses in milk production. *Staphylococcus aureus* in contaminated milk can be transmitted to dairy products, and cause intoxication, through production of enterotoxins. In addition, this pathogen is also present in cheese due to improper handling (Dias, 2007; Neto and Zappa, 2011; Dittmann et al., 2017). High counts of *S. aureus* are the main nonconformities found in Brazilian artisanal cheeses, but there is a general lack of data related to staphylococcal intoxication in these products (Finger et al., 2019; Camargo et al., 2021). It is possible that microbial interactions in the cheese matrix suppress synthesis of enterotoxins or the strains that contaminate these products are not enterotoxin producers. Additional studies are needed to evaluate the behavior of native *S. aureus* strains in these products.

Other relevant pathogens that can be found in artisanal cheeses are *Salmonella* and *Listeria monocytogenes*. These pathogens may originate from raw materials (milk) or from the factory environment, especially from the processing area, including equipment, personnel or cross contamination between finished products and raw materials (Williams, and Withers, 2010; McIntyre et al., 2015; Muhterem-Uyar et al., 2015; Aragon-Alegro et al., 2021). The occurrence of *Salmonella* and *L. monocytogenes* in cheeses with higher humidity is more common, suggesting that lower water content could be less favorable to their survival. Also, low pH values, low water activity (a_w) and the presence of lactic acid bacteria that have antimicrobial activity may decrease or eradicate the presence of these pathogenic microorganisms, and probably because of that, the studies conducted so far indicate that prevalence of *Salmonella* spp. and *L. monocytogenes* in Brazilian artisanal cheeses is low (**Table 2**).

TABLE 2 | Summary of studies on pathogenic bacteria in Brazilian artisanal cheeses.

Type of cheese	Number of tested samples	Results highlights	Reference
Coagulase positive *Staphylococcus* (CPS)			
Canastra	10	Seven samples presented counts of *S. aureus* above 4.8 log CFU g^{-1} with 5 days of ripening. Nine *S. aureus* isolates were positive for at least one toxin (SEA, SEB, SEC, SED and TSST-1)	Borelli et al., 2006
Serro	100	Counts of CPS were lower than 3 log CFU g^{-1} after 15 days of ripening in the rainy season and 45 days of ripening in the dry season*. SEB and SEC were found in some samples of cheese.	Cardoso et al., 2013
Araxá	30	28 samples presented CPS counts above 3 log CFU g^{-1}* (no information about ripening time)	Souza et al., 2015
Serro	53	40 samples presented CPS counts above 3 log CFU g^{-1}* (no information about ripening time). None of the isolates produced SEA, SEB, SEC, SED and SEE nor had SE related genes	Andretta et al., 2019
Canastra	78	33 samples presented CPS counts above 3 log CFU g^{-1}* after 22 days of ripening	Campos et al., 2021
Listeria* spp. and *L. monocytogenes			
Coalho (Manaus, AM)	58	*Listeria* sp. was detected in 2 samples	Ramos and Costa, 2003
Canastra	32	*Listeria* sp. was not detected	Dores et al., 2013
Serro (17 days of ripening)	256	*L. monocytogenes* was not detected	Martins et al., 2015
Coalho (Pernambuco)	60	*Listeria grayi* detected in 2 samples	Aragão et al., 2019
Canastra	78	*L. monocytogenes* detected in 1 sample.	Campos et al., 2021
Pathogenic *Escherichia coli*			
Raw milk cheeses from Paraná, São Paulo, Minas Gerais, Mato Grosso do Sul and Bahia	147	*E. coli* was found in 28 cheeses. One among 39 *E. coli* isolates was positive for *eae* gene, and negative for *bpf* and *efa1/lifA* genes, being classified as atypical EPEC (aEPEC).	Campos et al., 2017
Serrano (Santa Catarina)	109	22 among 109 *E. coli* isolates presented the *eae* (EPEC), *st* and *lt* (ETEC) or *aggR* (EAEC) genes	Parussolo et al., 2019
***Salmonella* spp.**			
Coalho (Pernambuco)	127	*Salmonella* spp. was detected in 7 samples	Duarte et al., 2005
Serro	100	*Salmonella* spp. was not detected	Cardoso et al., 2013
Coalho (*artisanal and non-artisanal samples*, Northeast)	104	*Salmonella* spp. was detected in 1 artisanal cheese sample	Sousa et al., 2014
Serro	256	*Salmonella* spp. was detected in 1 sample	Martins et al., 2015
Minas artisanal (Montes Claros, MG)	18	*Salmonella* spp. was detected in 2 samples	Pinto et al., 2016
Canastra	78	*Salmonella* spp. was not detected	Campos et al., 2021
***Brucella* spp.**			
Minas artisanal (4 and 8 days of ripening) (Serro, MG)	55	*Brucella* spp. was detected in 17 samples	Silva et al., 2018

Counts of CPS must be lower than 3 log CFU.g-1 according to Minas Gerais (2008).

Besides the above cited microorganisms, other pathogens that have been associated to outbreaks caused by cheeses manufactured with unpasteurized milk in other parts of the world are Shiga Toxin-producing *E. coli* (Deschênes et al., 1996), *Salmonella* Muenster (van Cauteren et al., 2009), *Salmonella* Typhi (FSANZ, 2009), *Brucella* spp. (Linnan et al., 1988; FSANZ, 2009), *Campylobacter* (Gould et al., 2014), and *Campylobacter jejuni* (Oliver et al., 2009). These etiological agents have caused outbreaks of different intensity and severity, showing that they deserve the attention of those responsible for the safety of artisanal cheeses.

Some studies have evaluated the occurrence of pathogenic microorganisms in different types of Brazilian artisanal cheeses. Silva et al. (2018) evaluated the presence of *Brucella* spp. in 55 samples of Serro artisanal Minas cheese and observed that 17 tested positive. The study was conducted with samples ripened for 4 and 8 days only, using a culture independent method (Nested-PCR). Additional studies with cheeses ripened for

longer periods, as required by state regulation for this particular type of cheese, are still pending.

Dores et al. (2013) evaluated the effect of the ripening temperature (8 and 25°C) on the counts of pathogenic and indicator bacteria in *Canastra* cheeses. They observed that ripening at 25°C for 22 days was sufficient to decrease the counts of total coliforms, *Escherichia coli* and *Staphylococcus aureus* to the levels required by the legislation (< 1,000 CFU g^{-1} for *E. coli* and coagulase positive staphylococci), while ripening at 8°C required 64 days to reach these levels. None of the tested cheeses presented *Listeria monocytogenes* or *Salmonella* spp. Lower values of a_w and higher pH and sodium chloride were detected in cheeses ripened at 25°C, suggesting that these characteristics may have had a positive effect on the control of pathogens. In a similar study conducted by Martins et al. (2015) with *Serro cheese* ripened at room temperature (25 ± 4°C) and under refrigeration (8 ± 1°C), the authors observed that ripening at room temperature for 17 days was the ideal condition to reduce the counts of *S. aureus* to safe limits (< 1,000 CFU g^{-1}). *Listeria monocytogenes* was not detected in the 256 tested cheese samples, but *Salmonella*, present in one initial sample, was no longer detected after 22 days of ripening.

Mata et al. (2016) evaluated the effect of "*pingo*" collected in the *Serro* region on the survival of *Listeria* sp. during ripening of cheeses produced in laboratory conditions. Cheeses were prepared with raw milk experimentally contaminated with *Listeria innocua* ATCC 33090 (10 CFU ml^{-1}). Results indicated that *L. innocua* was not eliminated even after 60 days of ripening at 30°C, showing that the physicochemical changes and activity of the competitive microbiota during ripening may not be enough to guarantee the absence of *L. monocytogenes* in the final product under tested conditions.

Campagnollo et al. (2018) conducted a quantitative risk assessment of *L. monocytogenes* in semi-hard cheeses prepared with raw milk experimentally contaminated with 6 log CFU ml^{-1} of *L. monocytogenes* and ripened up to 22 days at 22 ± 2°C. The authors concluded that these cheeses presented lower risk of listeriosis than a soft cheese produced with pasteurized milk containing 1 log CFU ml^{-1} of *L. monocytogenes*, observing that the longer the ripening time the lower the risk. This study reinforced that mitigation of listeriosis risk is related to the cheese ripening period, when pH decreases, sodium chloride concentration increases, a_w decreases and interactions with lactic acid bacteria control the survival of *L. monocytogenes*.

Recently, Campos et al. (2021) evaluated safety indicators and pathogens from Canasta cheeses during the production process, including ripening. They confirmed that 22 days of ripening are sufficient to control the populations of hygiene indicator microorganisms (total coliforms, coagulase-positive *Staphylococcus* and *E. coli*) in *Canastra cheese* samples in accordance with the levels established by the current regulations, provided that the producers adopt good manufacturing practices. *Salmonella* was not detected in the study, but *L. monocytogenes* was detected in one sample, reinforcing the importance of the good hygiene and manufacturing practices.

Several studies have evaluated the presence of coagulase positive *Staphylococcus* (CPS), *Listeria* and *L. monocytogenes*, pathogenic *E. coli*, *Salmonella* and *Brucella* spp. in artisanal cheeses manufactured in Brazil. Results of these studies are summarized in **Table 2**.

GOOD HYGIENE AND MANUFACTURING PRACTICES

Brazilian regulations on artisanal cheese production indicate that the good hygiene and manufacturing practices must start at the farm level, i.e., cattle feeding, vaccination, and milking (Paulin and Ferreira Neto, 2008; Brazil, 2013, 2018, 2019b; Minas Gerais, 2017). Brazil has extensive legislation on hygiene and manufacturing practices for artisanal cheeses. Over the years, there have been major changes such as allowing marketing of raw milk cheeses provided they are ripened for 60 days, through a federal resolution (Brazil, 2000). Years later, a state normative allowed Minas artisanal cheeses such as *Canastra cheese* to be marketed with less than 60 days of ripening, reducing this requirement to 22 days (Minas Gerais, 2013). The reduction in the ripening period was based on a study that concluded that 22 days are enough to ensure safety as long as the cheeses are ripened at room temperature (Dores et al., 2013). The mentioned study was based on counts of hygiene and food safety indicator microorganisms only; thus, additional studies are vital to validate shorter ripening times, including the detection of hazards, such as *Brucella, Mycobacterium*, and *Staphylococcus* enterotoxins.

One of the most important changes in the Brazilian legislation regarding artisanal products of animal origin occurred in 2018, when the ARTE Seal (ARTE, short for *artesanal* – artisanal in Portuguese) was created. Cheeses with the ARTE Seal can be marketed interstate without restrictions, provided they are inspected by State or Federal Agencies (Brazil, 2018). The Brazilian scientific sector played a relevant role in defining the minimum ripening period for artisanal cheeses, with the involvement of many researchers across the country. These scientists highlighted the importance of the good manufacturing practices in the production chain and played an important role in disseminating this knowledge to cheese producers around the country.

The milking process is a critical control point. Guidelines of EMATER (State Technical Assistance and Rural Extension Company) determine that before milking, udders must be washed with chlorinated water containing 2–3 ppm of free chlorine and disinfected properly by pre-dipping with a chlorinated solution (50–100 mg L^{-1}). After milking, the udders must be disinfected with iodine solution at 20–30 mg L^{-1} or another recommended disinfectant. Animals with mastitis should be milked last and their milk cannot be destined for cheese production. Milk intended for artisanal cheese production should be filtered in 10–16 mesh filters before entering the cheese making facility and again using 60–90 mesh filters before entering the production tank (EMATER, 2009).

Cheese production sites must be well structured and maintained. The quality and safety of the water must be controlled, and corrals and milking rooms must

be well-finished, with easy disposal of water and organic waste. Walls must be painted with washable paint and floors must be sturdy, waterproof and from non-slippery material. Walls, floors, utensils, and equipment should be subjected to cleaning and disinfection with chlorinated solution, before and after milking. The cleaning of milk reception and raw material storage areas must be monitored, and manufacturing areas must be adequately ventilated (SEBRAE, 2015).

Workers at the cheese production sites must keep updated health certificates and wear clean and appropriate clothing, rubber boots, masks, and hat. Workers with health problems (cuts, wounds, cold, among others) cannot have access to the cheese manufacturing area. Hand washing with disinfectants is mandatory, before and after milking. Bad practices, such as smoking, sneezing, coughing, scratching the head, etc., and visitors must be avoided (EMATER, 2009; SEBRAE, 2015).

ANIMAL HEALTH PROTECTION

The Brazilian Ministry of Agriculture, Livestock and Food Supply and state agencies of animal health protection require vaccination for brucellosis by means of the National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis (MAPA, 2019). Vaccination against brucellosis is mandatory for females in ages between 3 and 8 months. Vaccination against foot-and-mouth disease must be applied directly by the health authorities of each state. The vaccine against symptomatic anthrax must be applied to all animals on the third month of age and must be repeated every 6 months until 2 years of age. Another important vaccination is against rabies that should be applied annually, especially in outbreak regions when much of the herd can be affected by the disease (MAPA, 2019). There are some vaccines that can be used to control some animal diseases such as botulism, clostridium disease, leptospirosis, and infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), mastitis, campylobacteriosis and colibacillosis (EMBRAPA, 2019b).

An effective feeding system is also relevant for animal health protection. It must provide energy, protein, minerals, and vitamins to meet the nutritional needs of each category of animal and at each stage of the life cycle of animals in the herd (Salman et al., 2011). The planning of a balanced diet is an indispensable strategy for a positive impact on the economy and production in the livestock sector (EMBRAPA, 2003).

PERSPECTIVES FOR IMPROVEMENTS

Brazil is a country with continental dimensions and hence with a great diversity of climate, vegetation, topography, and culture that directly reflects the diversity of cheeses produced in the country, as indicated in this review. The popularity of artisanal cheeses in the national market has been growing. In terms of flavor, these cheeses have already proven their attributes and value in international contests. The regulation for artisanal cheese production is numerous, and producers, particularly the small ones, consider it too rigorous and sometimes confusing and not well accepted or understood. The recent improvements in the regulations, at local, state, and national levels, that revised old and obsolete laws, have contributed to combat clandestinity, bringing significant economic turn over for producers. The role of consumers demanding better quality and safety is also important. These actions, alongside with technical qualification of producers and incentives for research projects, will contribute to elevate Brazilian artisanal cheeses to worldwide recognition. In this sense, producers' associations play an important role, as they protect the identity of the products, promote educational actions that improve production practices and assist in the proper publicity of artisanal products throughout the national territory (APROCAN, 2021). This process of cooperativism is gaining strength, being a reasonable solution to increase the market share of these products and improve their overall quality.

The challenges to assure absence of pathogens in Brazilian artisanal cheeses are no different than those in similar cheeses produced elsewhere: they are attributed to the use of unpasteurized milk and to disruptions in the production/trade chain regarding the failure in good hygiene and good manufacturing practices. The good hygiene practices must be adopted at all stages, from proper vaccination of the herd to milking and manufacturing up to consumption, in order to guarantee microbiological safety and avoid public health problems. For the effective application of these practices, the proper training of cheese producers and food handlers is mandatory.

One issue that seems to be unique in the country is the minimal ripening time necessary to guarantee microbiological safety of artisanal cheeses produced with raw milk. Brazilian legislation, that follows international norms, require a minimum of 60 days of ripening, but recent state regulations allow shorter ripening time such as 14–22 days, depending on the geographical origin of the cheese. These new regulations were based on local studies that evaluated hygiene microbiological indicators, primarily focusing on counts of coliforms, *E. coli*, and *S. aureus* and the detection of *Salmonella* and *L. monocytogenes* in the final product. Some studies have confirmed the safety of these products regarding these microbiological criteria, but additional research, including the detection of other microorganisms, such as *Brucella* spp. and *Mycobacterium* sp., as well as *Staphylococcus* enterotoxins in the product, would increase information on the safety of these cheeses. It is important to point out that there is a lack of data related to the detection of microbial pathogens in artisanal cheeses as well as on beneficial microbiota, especially considering the huge diversity of artisanal cheeses produced in the nation.

Most of the artisanal cheeses produced in the country have been characterized by culture dependent methods and traditional chemical approaches. Besides including the diverse types of artisanal cheeses produced nationwide, it is important that new studies use state of the art genomic and metabolomic approaches that could reveal the singularities of each producing region, helping define the unique microbiological and chemical profiles of these products. Finally, studies considering the microbial interaction in the cheese making environment, including the cheese making facility and the product during ripening, will

reveal the kinds of interactions that take place in products that have desirable safety and sensorial features. For instance, a study conducted with cocoa beans has revealed the role of quorum sensing and cross-feeding in shaping microbial succession during fermentation, as discussed by Almeida et al. (2020).

Recently, an initiative known as Brazilian Artisanal Cheese Research Network (REPEQUAB – Rede de Pesquisas em Queijos Artesanais Brasileiros[1]) was created with the aim of integrating scientists from all Brazilian producing regions in order to promote the exchange of knowledge, samples, databases, and especially to stimulate collaborative research to solve regional and national issues related to artisanal cheese production. The network has already connected 70 researchers, and several collaborative investigations are ongoing, especially in the Canastra region in Minas Gerais and in the state of Sao Paulo. Future meetings aiming to discuss the advancements in the field and to build new collaborative investigations will drive quality and safety improvements in artisanal cheese production in the country.

[1]http://repequab.com.br/

AUTHOR CONTRIBUTIONS

UP planned the manuscript. AP and GC wrote and revised the drafts of the manuscript. AP, GC, NP-F, BF, and UP contributed to write and revise the drafts of the manuscript. UP and BF edited the manuscript. All authors contributed to the article and approved the submitted version.

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Evaluation of the Relationships Between Microbiota and Metabolites in Soft-Type Ripened Cheese Using an Integrated Omics Approach

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Cheese ripening is effected by various microorganisms and results in the characteristic flavors of cheese. Owing to the complexity of the microbiota involved, the relationship between microorganisms and components during ripening remains unclear. In this study, metagenomics and metabolomics were integrated to reveal these relationships in three kinds of surface mold-ripened cheeses and two kinds of bacterial smear-ripened cheeses. The microbiota is broadly divided into two groups to correspond with different cheese types. Furthermore, surface mold-ripened cheese showed similar microbiota regardless of the cheese variety, whereas bacterial smear-ripened cheese showed specific microbiota characterized by marine bacteria (MB) and halophilic and alkaliphilic lactic acid bacteria for each cheese variety. In the metabolite analysis, volatile compounds suggested differences in cheese types, although organic acids and free amino acids could not determine the cheese characteristics. On the other hand, Spearman correlation analysis revealed that the abundance of specific bacteria was related to the formation of specific organic acids, free amino acids, and volatile compounds. In particular, MB was positively correlated with esters and pyrazines, indicating their contribution to cheese quality. These methodologies and results further our understanding of microorganisms and allow us to select useful strains for cheese ripening.

Keywords: cheese, metagenomics, microbiota, metabolome, marine bacteria, correlation analysis

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INTRODUCTION

Cheese is one of the most common fermented foods in the world and has many varieties. In general, cheesemaking involves several processes, the coagulation of milk, separating whey from curd, salting, and ripening. In detail, these process are different depending on the cheese varieties (Almena-Aliste and Mietton, 2014). For example, three methods are used for milk coagulation as follows: acid, which is derived from starter lactic acid bacteria (SLAB), rennet, and acid/heat (McSweeney et al., 2017). In draining the whey, there are various methods such as cutting, cooking, stirring, and pressing (Kindstedt, 2014). Among these, ripening is a crucial process that is responsible for the characteristic flavor, texture, and appearance of each cheese type (Fox and McSweeney, 2017). Examples of how differences in ripening affect the characteristics include

surface mold-ripened cheeses, such as Brie de Meaux, and bacterial smear-ripened cheese, such as Maroilles. The flavor and appearance of these two types of cheeses are significantly different after ripening, although they are classified as soft uncooked unpressed cheeses, which are similar in the manufacturing process from milk to curd (Almena-Aliste and Mietton, 2014). To make surface mold-ripened cheese, a suspension of *Penicillium camemberti* is sprayed onto the surface before ripening at low temperatures (11–13°C) for at least 21 days (Kindstedt, 2014; Spinnler, 2017). In contrast, bacterial smear-ripened cheese are washed with brine every few days during ripening under cold conditions (4–20°C) for up to 63 days (Kindstedt, 2014; Mounier et al., 2017). At the beginning of ripening, the pH value is typically approximately 5 because of acidification by SLAB (Monnet et al., 2015). When ripening starts, a variety of yeast and *P. camemberti* is developed on the surface of mold-ripened cheese, whereas halotolerant yeasts are developed on bacterial smear-ripened cheese. In general, it is known that these fungi raise the pH by metabolizing lactate to CO_2 and H_2O oxidatively and producing alkaline metabolites such as NH_3 from amino acids in cheeses (McSweeney, 2004; Gori et al., 2007; Fröhlich-Wyder et al., 2019). The pH at the cheese surface can reach a value higher than 7.5 at the end of ripening, allowing not only starters but also non-starters, such as acid-sensitive bacteria, to grow (Monnet et al., 2015). Some non-starter bacteria and fungi have been shown to play a role in cheese ripening. For example, non-starter lactic acid bacteria (NSLAB), such as hetero-fermentative *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, and *Carnobacterium*, establish themselves in ripened cheese from raw milk, cheesemaking environments, and natural starter cultures, and contribute to the final characteristics of cheese (Afzal et al., 2010; Settanni and Moschetti, 2010; Pogačić et al., 2016; Gobbetti et al., 2018; Eugster et al., 2019; D'Angelo et al., 2020). On the other hand, *Brevibacterium* has been recognized as a ubiquitous microorganism in bacterial smear-ripened cheese and plays an important role in the development of characteristic orange color and flavor (Rattray and Fox, 1999; Anast et al., 2019). As for fungi, *Geotrichum candidum* and *Debaryomyces hansenii* are commonly found in ripened cheeses and contribute to cheese flavor and texture because of their deacidifying, proteolytic, and/or lipolytic activity (McSweeney, 2004; Irlinger et al., 2015; Fröhlich-Wyder et al., 2019). Thus, various microorganisms present during the ripening period affect the cheese characteristics, especially with respect to the formation of flavor. Moreover, specific microbiota in each cheese is considered to be caused by a difference in ripening methods. Traditionally, this secondary microbiota that develop during ripening is adventitious and is acquired from the milk and/or environment. Since this method is likely to bring about variable microbiota, inconsistencies in cheese quality often occur (Fox et al., 2017). Therefore, in modern cheese technology, the adventitious microbiota are selected as adjunct cultures and are artificially added during cheesemaking (Fox et al., 2017). However, the role of some microorganisms that adventitiously grow during cheese ripening is still unclear. Furthermore, due to the diversity and complexity of microbiota and components that constitute ripened cheese, the overall relationship between flavor development and existing microorganisms during ripening remains unclear.

Recently, metagenomics approach that employed high-throughput sequencing technologies revealed the microbiota of various ripened cheeses in detail. Further, it has been revealed that various bacteria such as *Actinobacteria*, *Proteobacteria*, and halophilic and alkaliphilic lactic acid bacteria (HALAB) that did not originate from starter and artificial secondary cultures were found to dominate the cheese microbiota (Irlinger et al., 2015; Dugat-Bony et al., 2016). Furthermore, the application of metabolomics enables the comprehensive analysis of the components of ripened cheese, including the characteristic metabolites (Ochi et al., 2012; Pisano et al., 2016). Over the past few years, the integration of meta-omics technologies has gained attention (Chen et al., 2017; Afshari et al., 2020a). In fermented foods, such as table olives and fermented dairy beverages, metagenomic and metabolomic analyses have revealed a correlation between microorganisms and metabolites (Walsh et al., 2016; Randazzo et al., 2017; Wurihan et al., 2019). These methods could be powerful tools to provide comprehensive and in-depth insight into the complex associations between microbiota and components in ripened cheese. The obtained data by applying this approach will contribute to elucidating the role of non-starter microorganisms and to facilitating targeting to screen microorganisms that are candidates for adjunct culture. Therefore, this information could provide new insights into efficient and sustainable cheesemaking and control cheese quality. Nevertheless, integrating metagenomics and metabolomics approach has not been applied to the verification of cheese, except for that of cheddar cheese (Afshari et al., 2020b).

Therefore, an integrated approach combining metagenomics and metabolomics were applied to reveal the comprehensive relationships between microorganisms and components in the two different types of soft uncooked unpressed cheeses, surface mold-ripened cheese, and bacterial smear-ripened cheese, which are similar in the manufacturing process from milk to curd but differ in the ripening processes. In this study, the microbiota and metabolites were analyzed using metagenomic amplicon sequencing, high-performance liquid chromatography (HPLC), and headspace gas chromatography mass spectrometry (HS-GC/MS).

MATERIALS AND METHODS

Cheese Samples

Three types of surface mold-ripened cheeses (Brie de Meaux, Brie de Melun, and Coulommiers) and two kinds of bacterial smear-ripened cheeses (Langres and Maroilles) were used in this study. Each type of cheese was purchased three times in the food market in Tokyo at different times. After dividing these cheeses into rind and core, a total of 30 samples (e.g., five kinds of cheeses, $n = 3$, and rind and core) were used for the metagenomic sequencing analysis, and to analyze various organic acids, free amino acids, and volatile compounds. The NaCl concentration and pH were determined using a C-121 salinity meter (HORIBA, Ltd., Kyoto,

Japan) and an HM-25G pH meter (DKK-TOA Corporation, Tokyo, Japan), as described previously (Unno et al., 2020).

Metagenomic Sequencing

Total DNA was extracted from cheese samples using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, United States) with some modifications. Cheese samples (150 mg) from the rind or core were homogenized in 750 μL Bashing Bead Buffer using BioMasher SP (Nippi, Tokyo, Japan). After transferring the suspension to a ZR BashingBead™ Lysis Tube, DNA was extracted according to the manufacturer's instructions. The extracted DNA was quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, United States). 16S rRNA and ITS2 gene amplicon libraries were prepared following the Illumina protocol (Illumina Inc, 2013). For bacteria, the V3 and V4 regions of the 16S rRNA gene were amplified using the forward primer (5′-CCTACGGGNGGCWGCAG-3′) and the reverse primer (5′-GACTACHVGGGTATCTAATCC-3′) with an overhang adapter (Klindworth et al., 2013). For fungi, the ITS2 regions were amplified using the forward primer (5′-GCATCGATGAAGAACGCAGC-3′) and the reverse primer (5′-TCCTCCGCTTWTTGWTWTGC-3′) with an overhang adapter (White et al., 1990; Toju et al., 2012). After amplification, dual index and Illumina sequence adapters were added to the amplified products using the Nextera XT Index Kit (Illumina, San Diego, CA, United States). PCR products were purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, United States) and read size was checked using an Agilent 2200 Tape Station (Agilent, Santa Clara, CA, United States). The libraries were diluted to 5 nM with 10 mM Tris (pH 8.5) based on qPCR results using the Kapa Library Quantification Kit (Kapa Biosystems, Wilmington, MA, United States), and 2 × 300 bp paired-end sequences were performed using the MiSeq Reagent Kit v3 on an Illumina MiSeq sequencing platform (Illumina, San Diego, CA, United States).

Assign Taxonomy by QIIME2

The QIIME2 pipeline was used for quality control and taxonomic classification (Bolyen et al., 2019). Sequence reads were imported into QIIME2 and DADA2 (Callahan et al., 2016) to perform denoising and merging to generate amplicon sequence variants (ASVs). The bacterial sequences were subjected to paired-end read analysis. Regarding fungi, the sequence reads were processed with single-end reads using only the forward reads because of insufficient read overlap. With the obtained ASV as a query sequence the bacterial and fungal taxonomy was assigned using Naive Bayes classifier pre-trained on Silva 16S rRNA gene database 132 (Quast et al., 2013) and UNITE database version 8.0 (Nilsson et al., 2019), respectively.

Organic Acids and Free Amino Acids Analysis

One gram of cheese sample was homogenized in 2 mL ultrapure water with BioMasher SP (Nippi, Tokyo, Japan) and centrifuged for 5 min at 20,000 × *g* at 20°C. The solution was treated with a twofold volume of 5% trichloroacetic acid solution for deproteinization and centrifuged for 10 min at 20,000 × *g* at 20°C. The supernatant was filtered through an Ultrafree®-MC Centrifugal Filter Unit (pore size, 0.2 μm; EDM Millipore, Billerica, MA, United States) by centrifuging for 5 min at 16,000 × *g* at 20°C prior to analysis. Organic acids and free amino acids were analyzed using HPLC, as previously described (Suzuki et al., 2021).

Volatile Compounds Analysis

Volatile compounds were determined using HS-GC/MS using a GCMS-TQ8040 NX trap system (Shimadzu Corporation, Kyoto, Japan) with an Agilent J&W GC-DB-WAX column (Agilent, Santa Clara, CA, United States). Samples (0.5 g) were placed in TORAST HS vials (Shimadzu GLC, Tokyo, Japan), and the temperature program was executed by agitating the vials at 50°C for 30 min. Subsequently, the temperature was maintained at 50°C for 5 min, increased to 250°C at a rate of 10°C/min, and then kept at 250°C for 10 min. The mass spectrometry range was set between 33 and 400 *m/z* in the scan mode. Peak identification was performed by a similarity search of the NIST17 mass spectral library[1] and retention time comparison. In this study, the peaks detected from the 1.5–20 min were adopted for the analysis.

Statistical Analysis

All statistical analyses and graphical plotting were performed using *R* (R Core Team, 2019). Principal component analysis (PCA) and correspondence analysis (CA) were performed using the *ropls* package (Thévenot et al., 2015) and the *ca* package (Nenadić and Greenacre, 2007), respectively. Biplots were generated using the *plot* function. Correlation analysis was performed for the sequence data and metabolomics data. Correlation was computed using the *cor* function (method = "spearman") and tested for *p*-value using the *cor.test* function (p-value < 0.05, regarded as statistically significant). Heat maps and dendrograms were generated using the *gplots* package (Warnes et al., 2020).

RESULTS

Salinity and pH of Sample Cheeses

The salinity and pH of five kinds of cheese samples were confirmed. Each sample was divided into the rind and core before measurements. The NaCl concentration of rind (2.0–3.0%) was slightly higher than that of the core (1.0–2.0%) in all samples. The pH of rind (6.7–8.3) was higher than that of the core (5.0–7.4). Overall, the rind can be regarded as a slightly alkaline environment with a pH of approximately 8.0, except for Langres samples. The obtained data are shown in **Table 1**.

Identification of Bacteria Dominating Ripened Cheeses

When 30 cheese samples were subjected to 16S rRNA gene V3-V4 amplicon sequencing analysis, 7,856,332 reads were

[1]http://www.nist.gov

TABLE 1 | The salinity and pH of five kinds of cheese samples.

Sample	NaCl (%)		pH	
	Core	Rind	Core	Rind
Brie de Meaux	2.0	2.0–3.0	6.6–6.8	7.8–8.0
Brie de Melun	2.0	3.0	7.3–7.4	7.5–7.6
Coulommiers	2.0	2.0–3.0	5.6–6.5	7.6–7.8
Langres	1.0	2.0	5.0–5.2	6.7–6.8
Maroilles	2.0	2.0	5.9–6.0	8.2–8.3

Each sample was divided into rind and core before measurement.

obtained. Denoising yielded 3,350,955 reads, from which 579 ASVs were identified. To verify the bacterial microbiota between the two types of cheese with different ripening methods (surface mold-ripened cheese and bacterial smear-ripened cheese), ASVs generated from 48,953 reads per sample were identified at the phylum level (**Figure 1**). All cheese samples were dominated by *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, or some of them. Among these, bacterial smear-ripened cheese had a higher proportion of *Proteobacteria* than surface mold ripened cheese. Langres hardly had *Actinobacteria*. Maroilles also had other phyla, including *Epsilonbacteraeota* and *Bacteroidetes*, along with the above-mentioned phyla.

At the genus level, 76 bacteria were identified using ASVs. Among them, 29 bacteria were present with a relative abundance > 0.5% in at least one sample. Hierarchical cluster analysis based on relative abundance at the genus level showed that cheese samples were separated into characteristic clusters corresponding to the existence of specific bacterial communities (**Figure 2**). The cheese samples were divided into three major clusters (I, II, and III). Clusters I and II were composed of Langres and Maroilles samples, respectively, which belong to bacterial smear-ripened cheese. Cluster III was composed

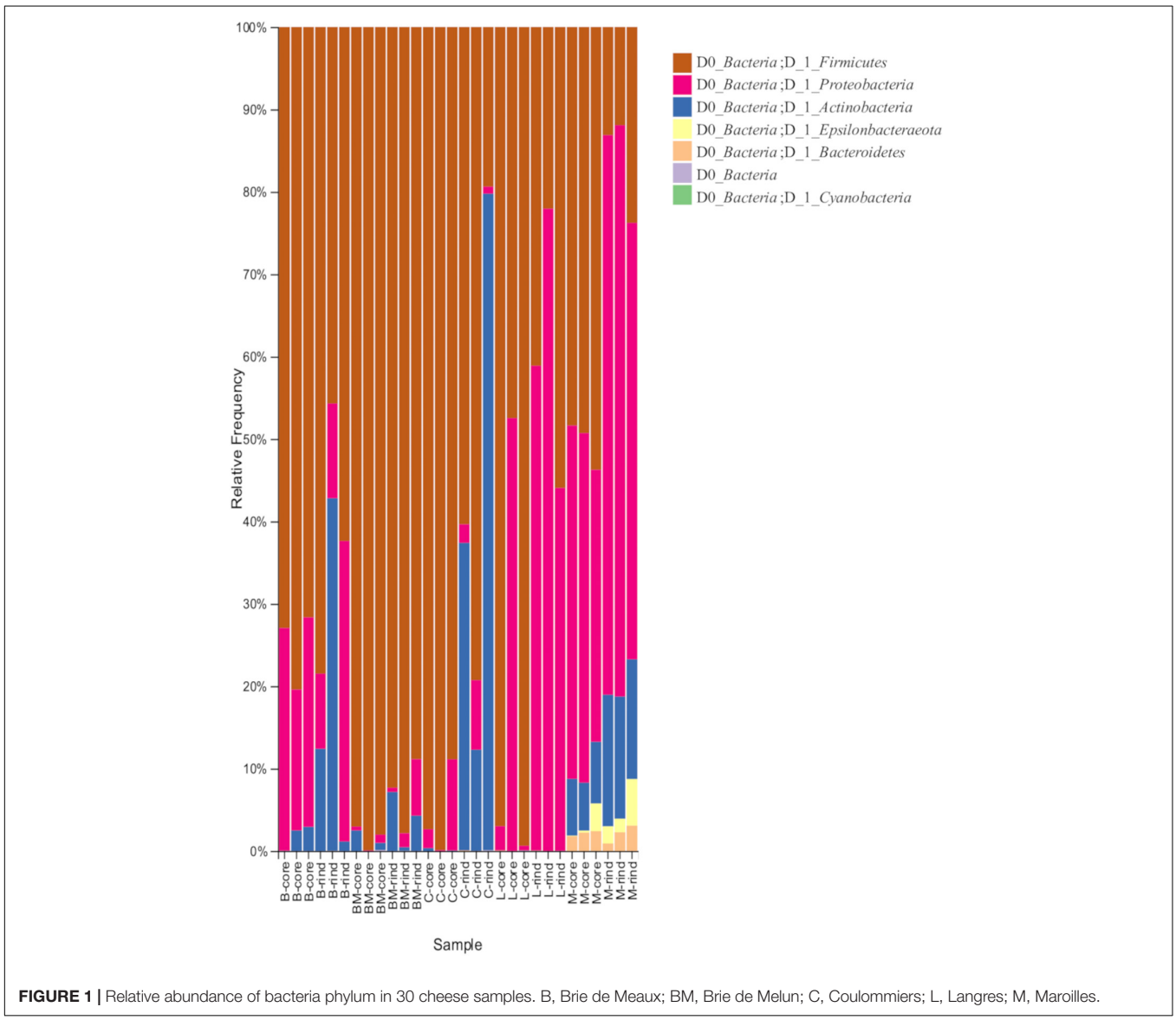

FIGURE 1 | Relative abundance of bacteria phylum in 30 cheese samples. B, Brie de Meaux; BM, Brie de Melun; C, Coulommiers; L, Langres; M, Maroilles.

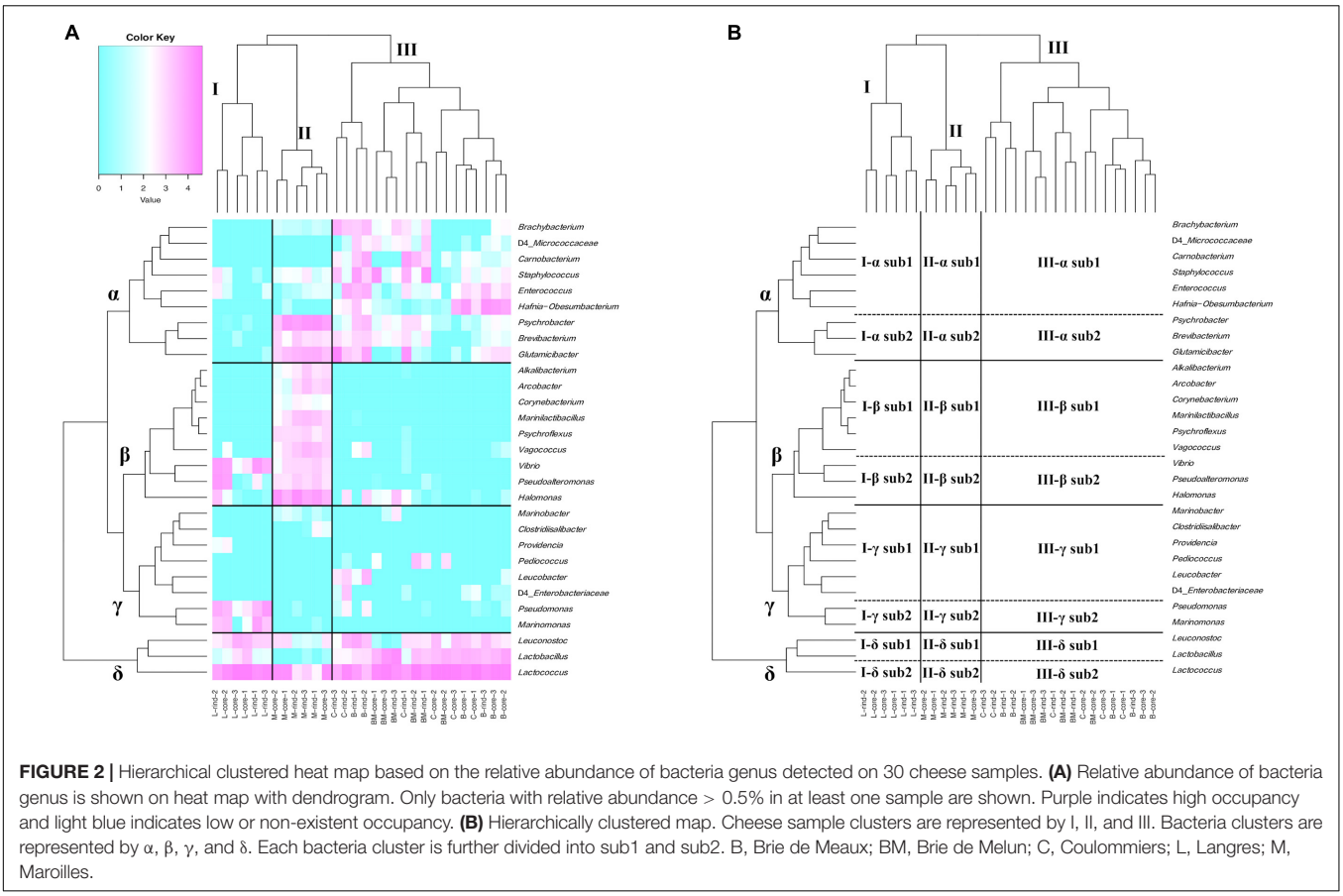

FIGURE 2 | Hierarchical clustered heat map based on the relative abundance of bacteria genus detected on 30 cheese samples. **(A)** Relative abundance of bacteria genus is shown on heat map with dendrogram. Only bacteria with relative abundance > 0.5% in at least one sample are shown. Purple indicates high occupancy and light blue indicates low or non-existent occupancy. **(B)** Hierarchically clustered map. Cheese sample clusters are represented by I, II, and III. Bacteria clusters are represented by α, β, γ, and δ. Each bacteria cluster is further divided into sub1 and sub2. B, Brie de Meaux; BM, Brie de Melun; C, Coulommiers; L, Langres; M, Maroilles.

of surface mold-ripened cheese samples. Within each cluster, the rind and core microbiota coexist. Similarly, bacterial communities were distributed into four major clusters (α, β, γ, and δ). Furthermore, each cluster was distributed into two sub-clusters (sub1 and sub2). Cluster α sub1 consisted of *Enterococcus* and *Carnobacterium*, which are known to be NSLAB in addition to *Staphylococcus*, *Brachybacterium*, and *Hafnia-Obesumbacterium*. Cluster α sub2 consisted of *Brevibacterium* and *Glutamicibacter*, which belong to the phylum *Actinobacteria*, and *Psychrobacter*, which is considered a marine-originated *Proteobacteria* designated as marine bacteria (MB). Cluster β sub1 consisted of *Marinilactibacillus*, *Alkalibacterium*, and *Vagococcus* (HALAB), *Corynebacterium* (phylum *Actinobacteria*), *Psychroflexus* (phylum *Bacteroidetes*), and *Arcobacter* (phylum *Epsilonbacteraeota*). Cluster β sub2 consisted of *Halomonas*, *Pseudoalteromonas*, and *Vibrio*, which belong to the MB. In cluster γ, sub1 consisted of *Pediococcus*, which is known to be NSLAB and *Leucobacter* of the phylum *Actinobacteria*, while sub2 consisted of *Marinomonas* and *Pseudomonas*, which were considered as MB. Cluster δ sub1 consisted of *Lactobacillus* and *Leuconostoc*, which are generally used as SLAB, such as *Lactobacillus delbrueckii* and *Leuconostoc mesenteroides*. Cluster δ sub2 was assigned to *Lactococcus*, which is known as SLAB.

Cluster I, composed of Langres samples, formed a characteristic bacterial microbiota consisting of lactic acid bacteria (LAB) (I-δ) and MB (I-β sub2 and I-γ sub2). Cluster II, composed of Maroilles samples, showed a low abundance of cluster δ bacteria, especially δ sub1 (II-δ), compared with the abundance in other cheeses. Additionally, this cluster was dominated by the bacteria belonging to the cluster α sub2 and cluster β bacteria characterized by the existence of MB, HALAB, and *Actinobacteria* (II-α sub2 and II-β). Cluster III, composed of surface mold-ripened cheese samples, was widely occupied by the bacteria belonging to the cluster α and δ (III-α and III-δ). Furthermore, *Leucobacter* was detected in Brie de Meaux and Coulommiers, while *Pediococcus* was detected in Brie de Melun (III-γ).

Thus, surface mold-ripened cheese formed large clusters regardless of cheese varieties, whereas bacterial smear-ripened cheese also formed large clusters, which were further divided by cheese variety. Moreover, it was indicated that MB and HALAB are characteristic constituents, especially in bacterial smear-ripened cheese microbiota.

Identification of Fungi Dominating Ripened Cheeses

Fungi were also identified using the ITS2 region based on the same cheese samples. ITS amplicon sequencing analysis on 30 cheese samples yielded 9,039,163 reads; of these, 2,385,721 reads remained after denoising. Among the 29 fungi that were

identified at the genus level from 20,000 reads per sample, 10 fungi were present with relative abundance > 0.5% in at least one sample (**Supplementary Figure 1**).

Cheese samples were distributed into four major clusters (I, II, III, and IV) by hierarchical cluster analysis based on relative abundance at the genus level. Cluster I consisted of the Maroille samples. Cluster II consisted of Langres samples. Cluster III mainly consisted of Brie de Melun samples. Cluster IV consisted of Brie de Meaux and Coulommiers samples. Similarly, fungal communities were distributed into four major clusters (α, β, γ, and δ). Furthermore, cluster α was distributed into two sub-clusters (sub1 and sub2). Cluster α sub1 and cluster α sub2 were assigned to *Dipodascus* and *Penicillium*, respectively. Cluster β was assigned to *Debaryomyces*. Cluster γ consisted of *Kluyveromyces*, *Candida*, *Saturnispora*, *Pichia*, and *Cyberlindnera*. Cluster δ was assigned to *Scopulariopsis*.

Cluster I consisted of Maroilles samples that were strongly dominated by *Debaryomyces* alone (I-β). Cluster II, consisting of Langres samples, was dominated by *Dipodascus* in addition to *Debaryomyces* (II-α sub1 and II-β). Further, clusters III and IV were dominated by *Dipodascus* and *Penicillium* (III-α and IV-α). Additionally, *Scopulariopsis* was detected in cluster III and *Candida* and *Kluyveromyces* in cluster IV (III-δ and IV-γ). In general, *Geotrichum candidum* is found in cheese. This species is synonymous with *Dipodascus geotrichum*. Although our results have limitations in identification at the species level, it can be considered that ASVs identified as *Dipodascus* were likely to be *G. candidum*. Thus, *Penicillium*, *Debaryomyces*, and *Dipodascus* were the predominant fungal microbiota in the cheese samples. This result suggests that the fungal microbiota is dominated by a small number of species compared with the bacterial microbiota.

Relationship Between Cheese Variety and Components

Multivariate analysis of metabolites (organic acids, free amino acids, and volatile compounds) was performed to reveal the characteristic components of each ripened cheese. Organic acids were quantified using HPLC (**Supplementary Table 1**), and the amounts are summarized in **Figure 3**. The tendency of acetate accumulation in Brie de Meaux and Brie de Melun, as well as that of lactate accumulation in Coulommiers was observed. However, overall, the amount of each organic acid was variable for each cheese sample. Therefore, PCA was performed in an attempted to reveal the characteristics of organic acid contents in each cheese variety. The PCA score and loading plots of the first two principal components of the organic acids are shown in **Figure 4**. The loading plots showed that lactic acid was distributed in the positive direction on the PC1 (accounting for 41% of the total variance), and vice versa, while the others were distributed in the negative direction (**Figure 4B**). These implied that lactic

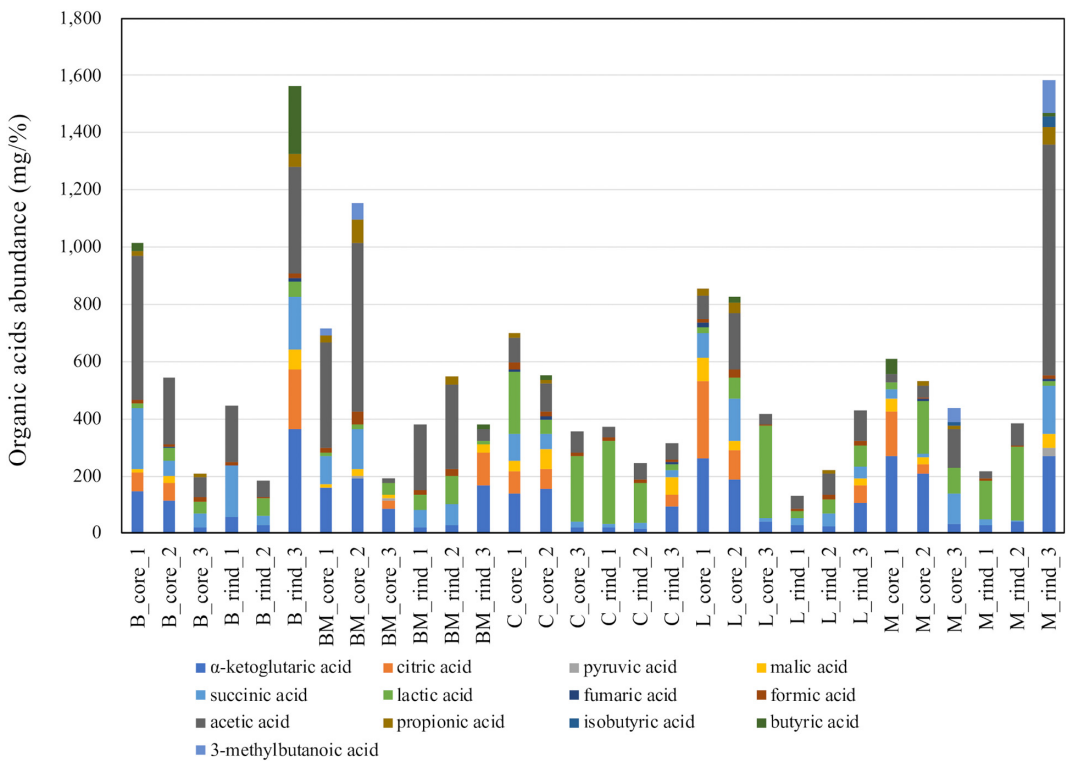

FIGURE 3 | Abundances of organic acids in 30 cheese samples. The total amount of organic acid in each sample is shown in the barplot. B, Brie de Meaux; BM, Brie de Melun; C, Coulommiers; L, Langres; M, Maroilles.

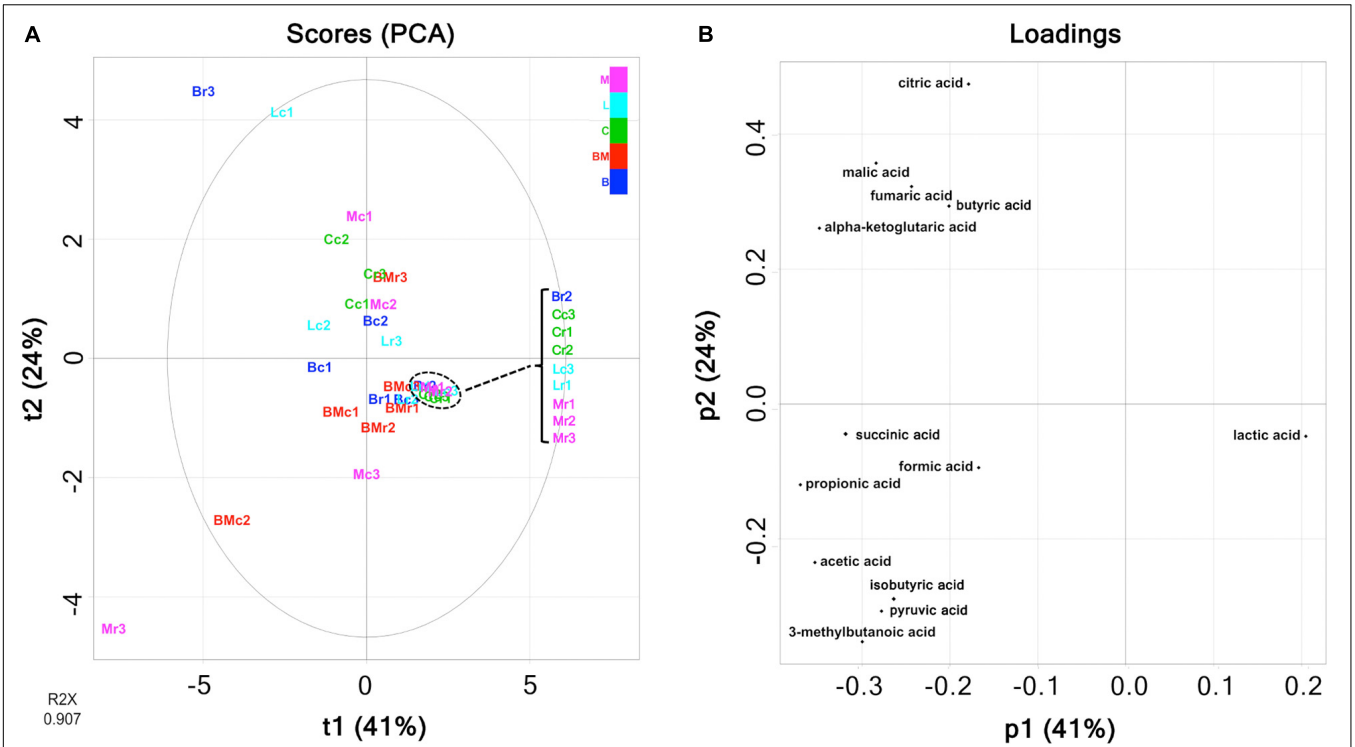

FIGURE 4 | Principal component analysis (PCA) on the abundance of organic acids in 30 cheese samples. **(A)** Score plot. **(B)** Loading plot. Bc, Brie de Meaux core; Br, Brie de Meaux rind; BMc, Brie de Melun core; BMr, Brie de Melun rind; Cc, Coulommiers core; Cr, Coulommiers rind; Lc, Langres core; Lr, Langres rind; Mc, Maroilles core; Mr, Maroilles rind.

acid content in cheeses was negatively correlated with other organic acids in cheeses. However, because the score plots were not separated depending on the cheese variety, the organic acid content did not reflect the characteristics of the cheese variety (**Figure 4A**).

Free amino acids were quantified using HPLC (**Supplementary Table 2**), and the amounts are summarized in **Figure 5**. Comparing each cheese variety, the amounts of amino acids in rind seemed to be higher than those in the core. However, it was difficult to infer the cheese characteristics because the amount of each free amino acid was variable for each cheese sample. As with organic acids, PCA was performed in an attempted to reveal the characteristics of free amino acid contents in each cheese variety. The PCA score and loading plots of the first two principal components of the free amino acids are shown in **Figure 6**. In the loading plots, all free amino acids were distributed in the positive direction on the PC1 (accounting for 62% of the total variance) (**Figure 6B**). This result implied that the total content of free amino acids was indicated by the PC1 and varied widely in each cheese sample. However, similar to organic acids, the score plots were scattered regardless of cheese type (**Figure 6A**). Thus, the free amino acid content in each cheese did not reflect the characteristics of the cheese variety.

To detect volatile compounds in ripened cheese, HS-GC/MS was performed. By comparing the mass spectra and retention time, 58 volatile compounds were detected (**Supplementary Tables 3, 4**). These detected compounds were classified into eight categories (alcohols, aldehydes, carboxylic acids, esters, hydrocarbons, ketones, pyrazines, and sulfur compounds; **Supplementary Table 5**), and these categorical data were counted and are summarized in **Figure 7**. Total counts of detected volatile compounds in each cheese sample were generally approximately 20. Further, these categorical data were used for CA. The principal coordinate and the standard coordinate plot in the first dimension (explained 47.7% of the variance) and the second dimension (explained 18.9% of the variance) are shown in **Figure 8**. Surface mold-ripened cheese were clustered in the positive direction on the second dimension and tended to contain various ketones and alcohols. In bacterial smear-ripened cheese, Langres and Maroilles were found in the positive and negative directions on the first and second dimensions, respectively, and associated with ester and pyrazine. These results imply that Langres and Maroilles contain various types of esters and pyrazines as characteristic components, respectively.

Therefore, the characteristics of each cheese type is determined by the presence or absence of volatile compounds, and not by differences in the organic acid and free amino acid contents. Since this study used cheeses sold on the market, it was considered that the amounts of organic acids and free amino acids caused variation due to the difference in production lots and period until purchase, even though they had the same variety of cheeses. In contrast, qualitative analysis by

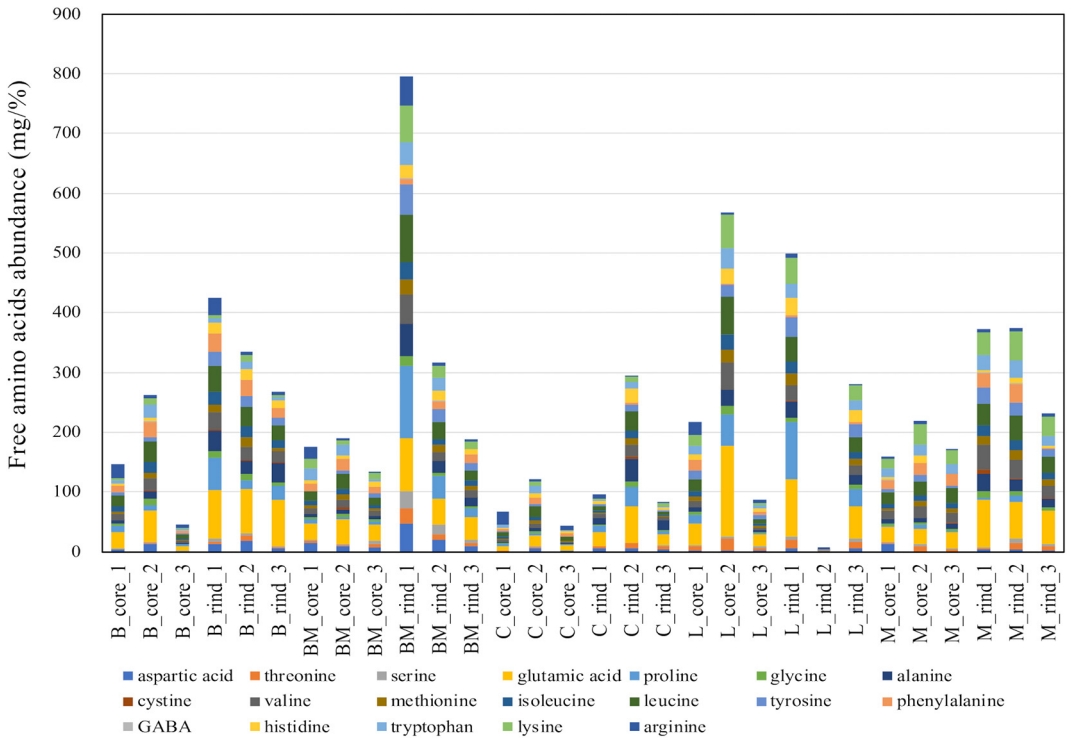

FIGURE 5 | Abundances of free amino acids in 30 cheese samples. The total amount of free amino acids in each sample are shown in the barplot. B, Brie de Meaux; BM, Brie de Melun; C, Coulommiers; L, Langres; M, Maroilles.

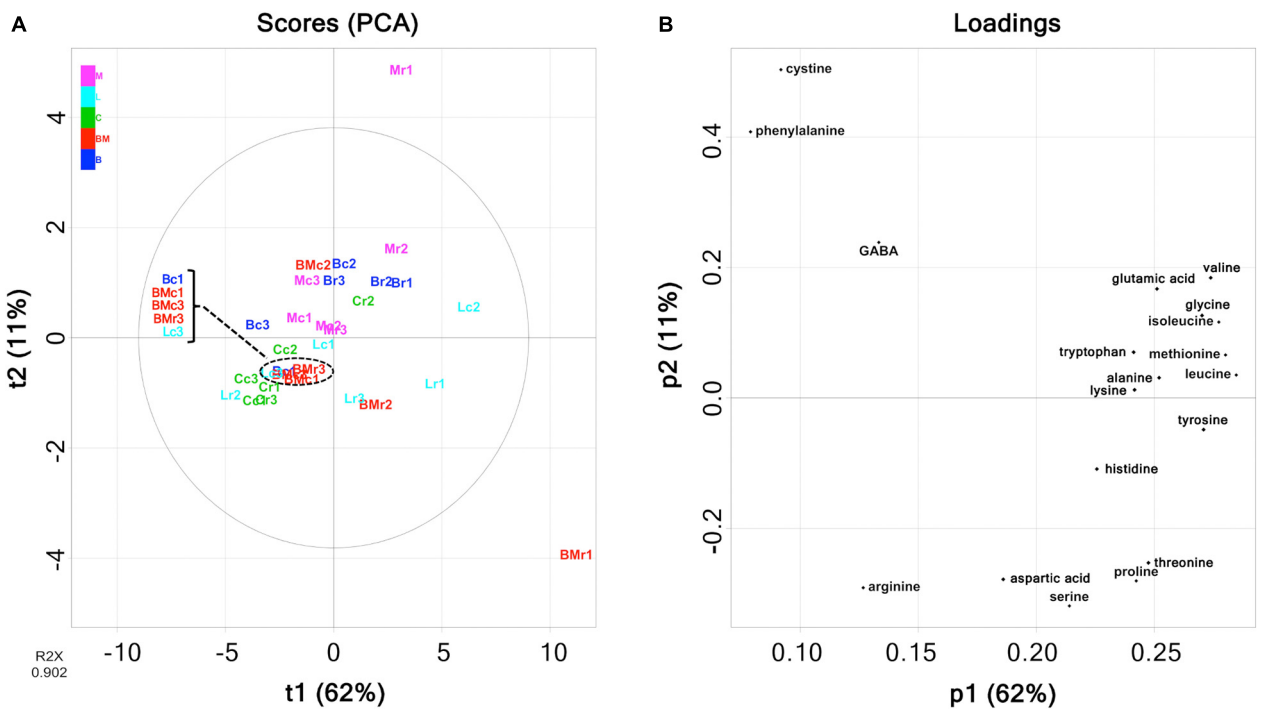

FIGURE 6 | Principal component analysis (PCA) on the abundance of free amino acids in 30 cheese samples. **(A)** Score plot. **(B)** Loading plot. Bc, Brie de Meaux core; Br, Brie de Meaux rind; BMc, Brie de Melun core; BMr, Brie de Melun rind; Cc, Coulommiers core; Cr, Coulommiers rind; Lc, Langres core; Lr, Langres rind; Mc, Maroilles core; Mr, Maroilles rind.

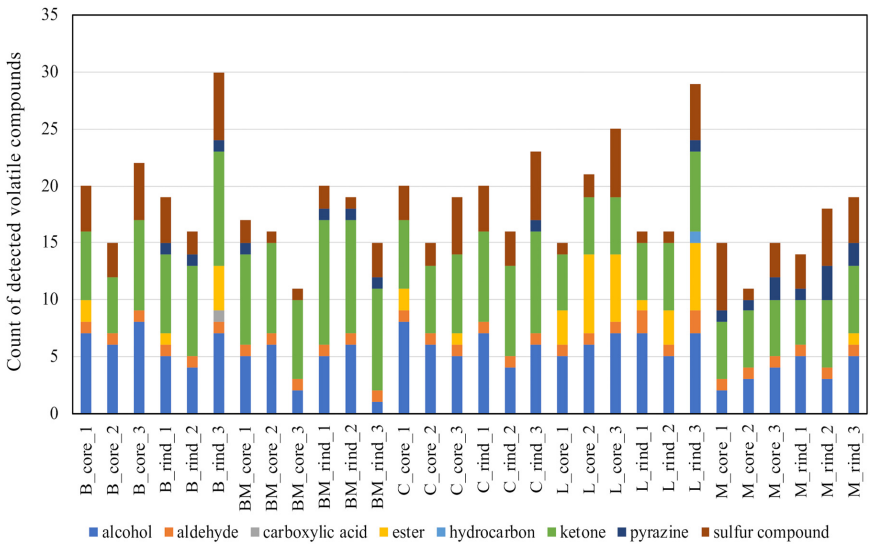

FIGURE 7 | Count data of the detected volatile compounds in 30 cheese samples. Detected compounds were classified into eight categories and aggregated. Total counts of detected volatile compounds in each sample are shown in the barplot. B, Brie de Meaux; BM, Brie de Melun; C, Coulommiers; L, Langres; M, Maroilles.

classifying volatile compounds into eight categories revealed the tendency for cheese types, indicating the difference in the types of volatile compounds.

Correlations Between Appearance of Bacteria and Constituent Metabolites in Ripened Cheeses

Thus far, although microbiota and volatile compounds suggested differences in cheese types, it was insufficient to determine the difference in cheese types by comparing organic acids and free amino acids only. Therefore, whether the characteristics of cheeses can be revealed by analyzing the relationship between the microbiota and metabolites in the cheese samples was examined.

The relationship between bacterial communities and metabolites in ripened cheeses was verified by hierarchical cluster analysis based on Spearman's correlation coefficient. As a result, the correlation tendency of the metabolites differed depending on the frequency of appearance of each bacterium. The degree of correlation between the appearance of bacteria and organic acids, free amino acids, and volatile compounds is shown in **Figures 9**–**11**, respectively.

Hierarchical cluster analysis divided organic acids and bacteria into three clusters (I, II, and III) and four clusters (α, β, γ, and δ), respectively (**Figures 9A,B**). Cluster I contained six types of organic acids, such as citric acid and propionic acid. Cluster II contained formic acid, acetic acid, and succinic acid, and cluster III grouped pyruvic acid, 3-methylbutanoic acid, lactic acid, and isobutyric acid. In bacterial clusters, cluster α was mainly composed of LAB, such as *Lactococcus*. Cluster β mainly grouped NSLAB, such as *Pediococcus*. Cluster γ was mainly composed of MB, HALAB, and *Corynebacterium* of the phylum *Actinobacteria*. Cluster δ was mainly composed of *Actinobacteria*, except for *Corynebacterium*. Cluster α bacteria were negatively correlated with some components constituting cluster III (α-III). Cluster β bacteria, especially *Pediococcus*, positively correlated with the cluster II component (β-II: *Pediococcus* - acetic acid and formic acid) and negatively correlated with the cluster I component (β-I: *Pediococcus* - citric acid). Cluster γ bacteria such as HALAB and *Psychrobacter* were positively correlated with the cluster III component, especially in isobutyric acid (γ-III), and negatively correlated with the cluster II component, especially in formic acid (γ-II). Cluster δ bacteria tended to show negative correlations with cluster I and II components (δ-I and δ-II). Significant correlations between the appearance of bacteria and organic acids are shown in **Supplementary Tables 6, 7**.

Free amino acids and bacteria were divided into two clusters (I and II) and five clusters (α, β, γ, δ, and ε), respectively (**Figures 10A,B**). Cluster I contained four free amino acids, such as aspartic acid, and cluster II contained the other free amino acids. In bacterial clusters, cluster α was composed of *Psychrobacter*, *Halomonas*, and *Marinobacter*, which belong to MB, *Marinilactibacillus*, *Alkalibacterium*, and *Vagococcus*, which belong to HALAB and *Brevibacterium*, *Glutamicibacter*, and *Corynebacterium* of the phylum *Actinobacteria*. Cluster β was composed of *Pseudoalteromonas*, *Marinomonas*, *Vibrio*, and *Pseudomonas*, which belong to MB. Cluster γ was composed of *Brachybacterium* and *Leucobacter* of the phylum *Actinobacteria* and *Pediococcus* is known as NSLAB. Clusters δ and ε were mainly composed of NSLAB, such as *Enterococcus* and *Carnobacterium*, and LAB such as *Lactococcus*, respectively. Cluster α bacteria negatively correlated with the cluster I component (α-I: *Alkalibacterium* – proline), but positively correlated with the cluster II component (α-II: *Brevibacterium* – cystine, *Psychrobacter* – phenylalanine, and *Halomonas* – lysine). Cluster β bacteria positively correlated with cluster II components, except for cystine and phenylalanine (β-II: *Pseudoalteromonas* - methionine and *Vibrio* – lysine). Clusters

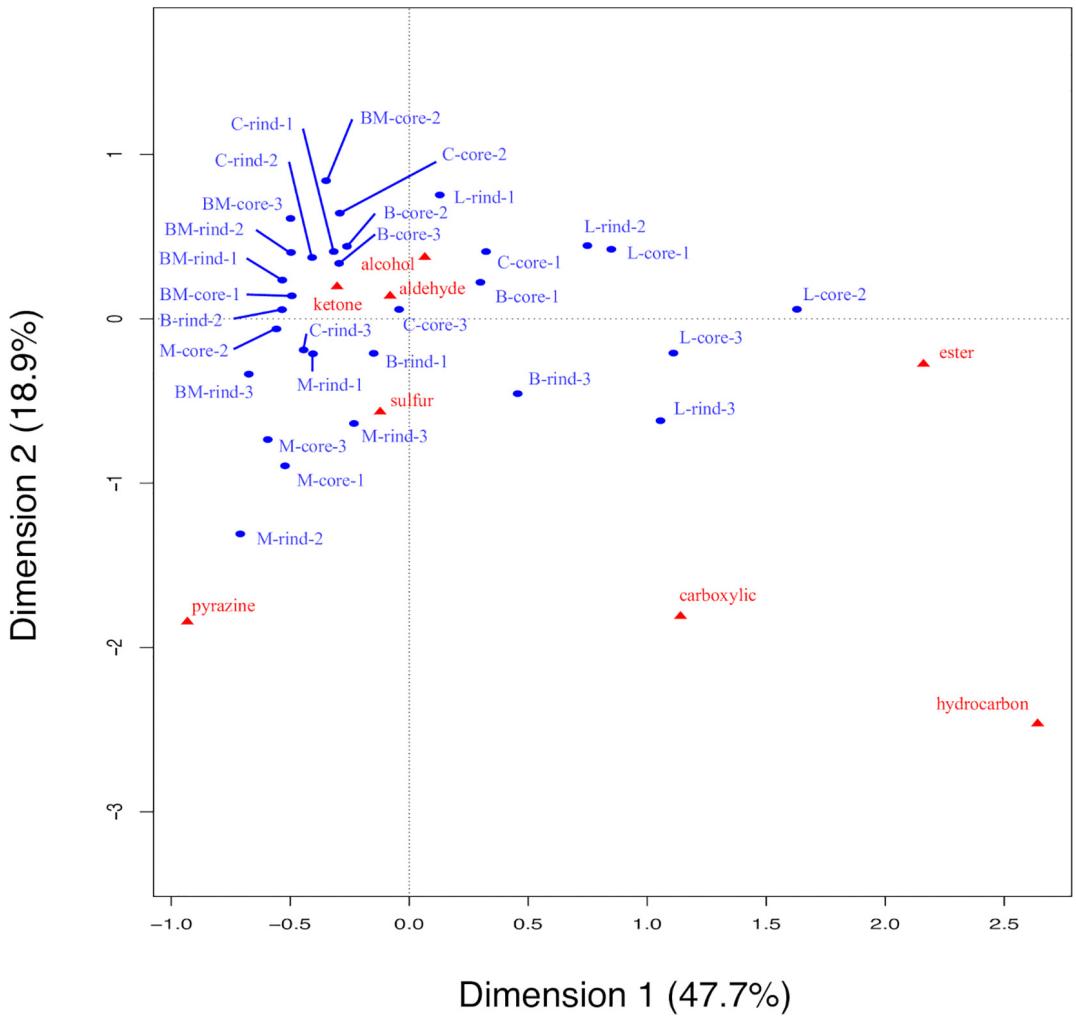

FIGURE 8 | Biplot of principal coordinates and standard coordinates obtained by correspondence analysis (CA) for volatile compounds in 30 cheese samples. Detected compounds were classified into eight categories (alcohols, aldehydes, carboxylic acids, esters, hydrocarbons, ketones, pyrazines, and sulfur compounds). Blue plot indicates principal coordinates and red plot indicates standard coordinates. B, Brie de Meaux; BM, Brie de Melun; C, Coulommiers; L, Langres; M, Maroilles.

γ and δ positively correlated with the cluster I component, especially aspartic acid (γ-I: *Pediococcus* - aspartic acid and δ-I: *Carnobacterium* - aspartic acid). In addition, these clusters were positively correlated with cystine (γ-II: *Brachybacterium* – cystine and δ-II: *Carnobacterium* – cystine). Cluster ε bacteria was negatively correlated with the cluster II component (ε-II). Significant correlations between the appearance of bacteria and free amino acids are shown in **Supplementary Tables 8, 9**.

Volatile compounds and bacteria were divided into four clusters based on their correlations (I, II, III, and IV and α, β, γ, and δ) (**Figures 11A,B**). Cluster I contained various kinds of esters such as ethyl acetate and ethyl hexanoate. Cluster II contained 2-pentanol, 2-heptanol, 2-propanol, 2-heptanone, and acetoin. Cluster III contained various kinds of alcohols and ketones, such as 2-nonanol and acetone, in addition to sulfur components such as dimethyl disulfide. Cluster IV mainly contained 2-butanone, pyrazines such as 2,5-dimethylpyrazine, and sulfur compounds, such as methanethiol. In bacterial clusters, cluster α was composed of MB, such as *Marinomonas* and *Pseudoalteromonas*. Cluster β was composed of *Carnobacterium*, *Enterococcus*, and *Pediococcus*, which are known as NSLAB; and *Brachybacterium* and *Leucobacter* of the phylum *Actinobacteria*. Cluster γ was composed of *Lactococcus*, *Lactobacillus*, and *Leuconostoc*, which are known as LAB. Cluster δ was composed of *Psychrobacter*, *Halomonas*, and *Marinobacter*, which belong to MB; *Marinilactibacillus*, *Alkalibacterium*, and *Vagococcus*, which belong to HALAB; and *Brevibacterium*, *Glutamicibacter*, and *Corynebacterium,* of the phylum *Actinobacteria*. Cluster α bacteria positively correlated with the cluster I component (α-I: *Marinomonas* – ethyl acetate) and negatively correlated with cluster II and cluster III components (α-II: *Pseudoalteromonas* – 2-pentanol and α-III: *Marinomonas* – acetone). In contrast, cluster β bacteria negatively correlated with the cluster I

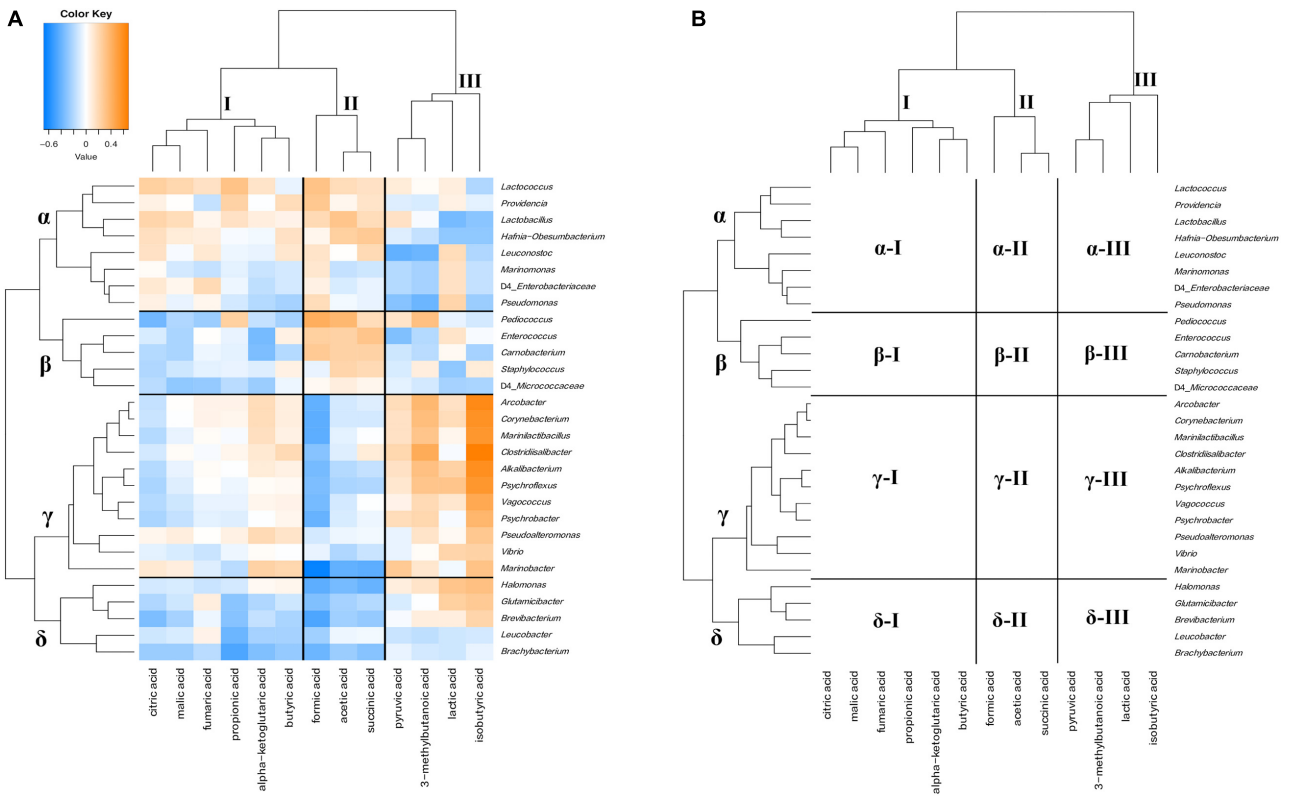

FIGURE 9 | Hierarchical clustered map showing the Spearman correlation between bacteria genus and organic acids detected on 30 cheese samples. **(A)** Spearman correlation heat map. Orange indicates positive correlation and blue indicates negative correlation. **(B)** Hierarchically clustered map. Organic acids clusters are represented by I, II, and III. Bacteria clusters are represented by α, β, γ, and δ.

component (β-I: *Carnobacterium* – ethyl hexanoate) and positively correlated with cluster II and cluster III components (β-II: *Pediococcus* – 2-heptanol and β-III: *Brachybacterium* - acetone). Cluster γ bacteria were positively correlated with cluster II and cluster III components (γ-II: *Lactococcus* – 2-pentanol and γ-III: *Lactobacillus* – 2-pentanone). Moreover, cluster γ bacteria were negatively correlated with the cluster IV component (γ-IV: *Lactococcus* - 2,5-dimethylpyrazine). Cluster δ bacteria negatively correlated with cluster I, cluster II, and cluster III components (δ-I: *Glutamicibacter* – ethanol, δ-II: *Marinilactibacillus* – 2-pentanol, and δ-III: *Halomonas* – 2-butanol) and positively correlated with the cluster IV component (δ-IV: *Glutamicibacter* - methanethiol and *Marinilactibacillus* – 2,5-dimethylpyrazine). Among them, *Glutamicibacter* and *Brevibacterium* tended to show positive correlations with dimethyl disulfide, dimethyl trisulfide, and acetone in the cluster III component (δ-III). Significant correlations between the appearance of bacteria and volatile compounds are shown in **Supplementary Tables 10, 11**.

As mentioned above, PCA based on the organic acid and free amino acid contents in each cheese did not reflect the characteristics of the cheese variety. However, Spearman correlation analysis revealed that the variation in cheese containing metabolites was associated with the bacterial abundance and genus differences. Furthermore, hierarchical clustering based on Spearman correlation showed that the volatile compounds characterized by CA were also strongly associated with the bacterial abundance and differences in genera. In particular, the difference between LAB and MB was remarkable with regard to all of the tested metabolites. Thus, the presence of LAB and MB was considered to be crucial for ripened cheese components.

Correlations Between the Appearance of Fungi and Constituent Metabolites in Ripened Cheeses

Similar to bacteria, the relationship between fungal communities and the metabolites in the ripened cheeses was verified using hierarchical cluster analysis based on Spearman's correlation coefficient (**Supplementary Figures 2–4**). The correlations between organic acids and fungi were separated into different clusters by hierarchical analysis, although the effective size was weak overall (**Supplementary Figure 2**). Organic acids were divided into three clusters (I, II, and III). Cluster I contained isobutyric acid and lactic acid. Cluster II contained 10 kinds of organic acids, such as 3-methylbutanoic acid. Cluster III was assigned to formic acid. Fungi were divided into four clusters (α, β, γ, and δ). Cluster α was composed of *Scopulariopsis* and *Cyberlindnera*. Cluster β was composed of *Debaryomyces*. Cluster γ was composed of *Penicillium*, *Kluyveromyces*, and

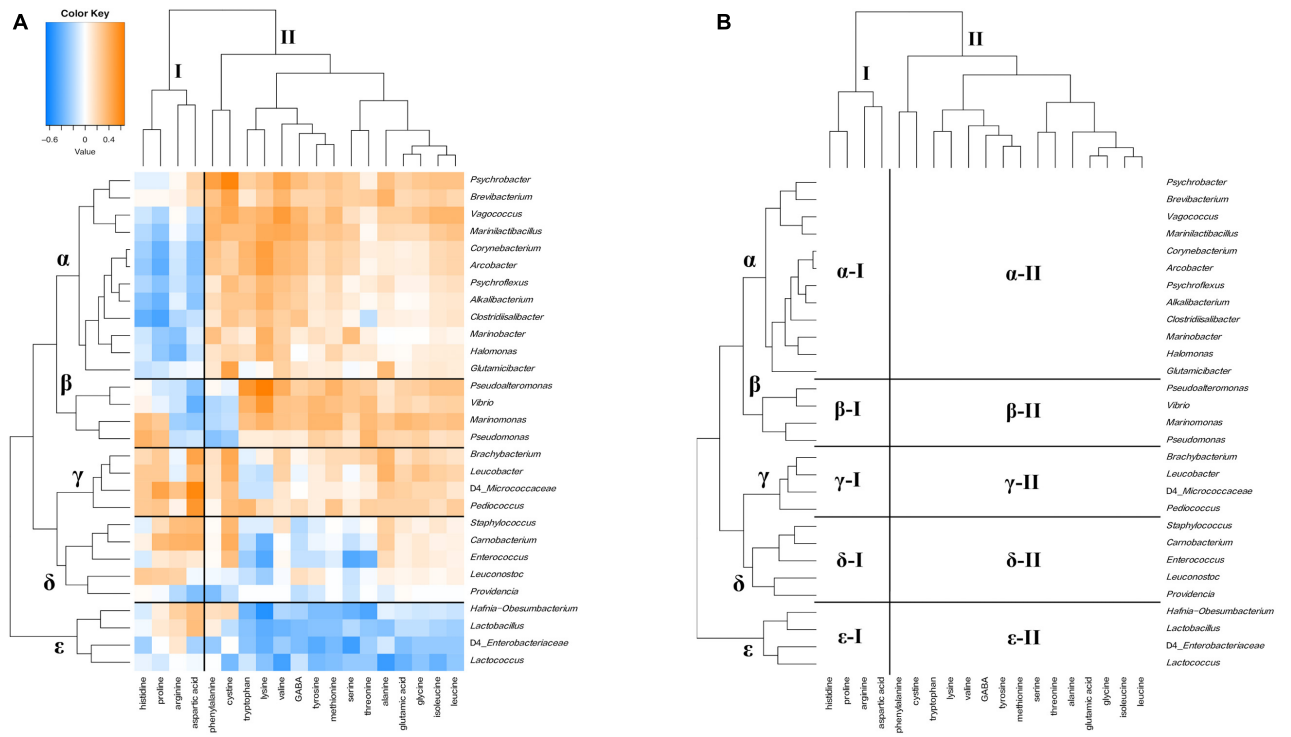

FIGURE 10 | Hierarchical clustered map showing the Spearman correlation between bacteria genus and free amino acids detected on 30 cheese samples. **(A)** Spearman correlation heat map. Orange indicates positive correlation and blue indicates negative correlation. **(B)** Hierarchically clustered map. Free amino acids clusters are represented by I, II, and III. Bacteria clusters were represented by α, β, γ, δ, and ε.

other two genera. Cluster δ was composed of *Dipodascus* and *Saturnispora*. Cluster α fungi were negatively correlated with the cluster I components (α-I). *Debaryomyces* was positively correlated with isobutyric acid (β-I) and negatively correlated with formic acid (β-III). In contrast, cluster γ and cluster δ fungi, such as *Penicillium* and *Dipodascus*, were negatively correlated with isobutyric acid (γ-I and δ-I) and positively correlated with formic acid (γ-III and δ-III). In addition, cluster δ fungi were negatively correlated with the cluster II component (δ-II). Significant correlations between the appearance of fungi and organic acids are shown in **Supplementary Tables 12, 13**.

The correlation between free amino acids and fungi was mostly negative (**Supplementary Figure 3**). The free amino acids were divided into two clusters (I and II). Cluster I contained 13 free amino acids, such as lysine, serine, and threonine. Cluster II contained other free amino acids, such as aspartic acid and proline. Fungi were divided into three clusters (α, β, and γ). Cluster α was assigned to *Debaryomyces*. Cluster β was composed of *Penicillium*, *Dipodascus*, and other two genera. Cluster γ was composed of *Kluyveromyces*, *Saturnispora*, and other three genera. *Debaryomyces* was positively correlated with lysine in the cluster I component (α-I) and negatively correlated with the cluster II component (α-II). In cluster β fungi, *Penicillium* and *Scopulariopsis* were positively correlated with aspartic acid (β-II). Additionally, cluster β fungi, except *Scopulariopsis*, tended to show negative correlations with the cluster II component (β-II: *Penicillium* – tryptophan and *Pichia* – lysine). Cluster γ fungi were negatively correlated with the overall cluster I component (γ-I: *Candida* – serine and *Kluyveromyces* - lysine). Significant correlations between the appearance of fungi and free amino acids are shown in **Supplementary Tables 14, 15**.

In the hierarchical analysis of the correlation between volatile compounds and fungi, volatile compounds were divided into three clusters (I, II, and III) (**Supplementary Figure 4**). Cluster I contained various esters such as ethyl hexanoate and also contained 3-methylbutanal, dimethyl disulfide, and pyrazines. Cluster II contained 2-pentanol, 2-heptanol, 2-pentanone, and 2-heptanone. Cluster III contained ketones such as 2-nonanoe, alcohols such as 2-butanol, esters such as 3-methylbutyl acetate, and sulfur compounds such as methyl thioacetate. Fungi were divided into three clusters (α, β, and γ). Cluster α was composed of *Dipodascus*, *Kluyveromyces*, and other four genera. Cluster β was composed of *Penicillium* and *Scopulariopsis*. Cluster γ was composed of *Debaryomyces*. Cluster α and cluster β fungi showed strong positive correlations with the cluster II component (α-II: *Candida* – 2-pentanol and β-II: *Penicillium* – 2-pentanone). Cluster α fungi were also positively correlated with some compounds in cluster III (α-III: *Dipodascus* – acetoin). *Penicillium* and *Scopulariopsis* were positively correlated with ketones in cluster III (β-III: *Penicillium* – 2-nonanone and *Scopulariopsis* – 8-nonen-2-one). Moreover, cluster α and cluster β fungi negatively correlated with cluster I components (α-I: *Dipodascus* – 2,5-dimethylpyrazine, and β-I: *Penicillium* – ethyl acetate). *Debaryomyces* was negatively correlated with cluster II

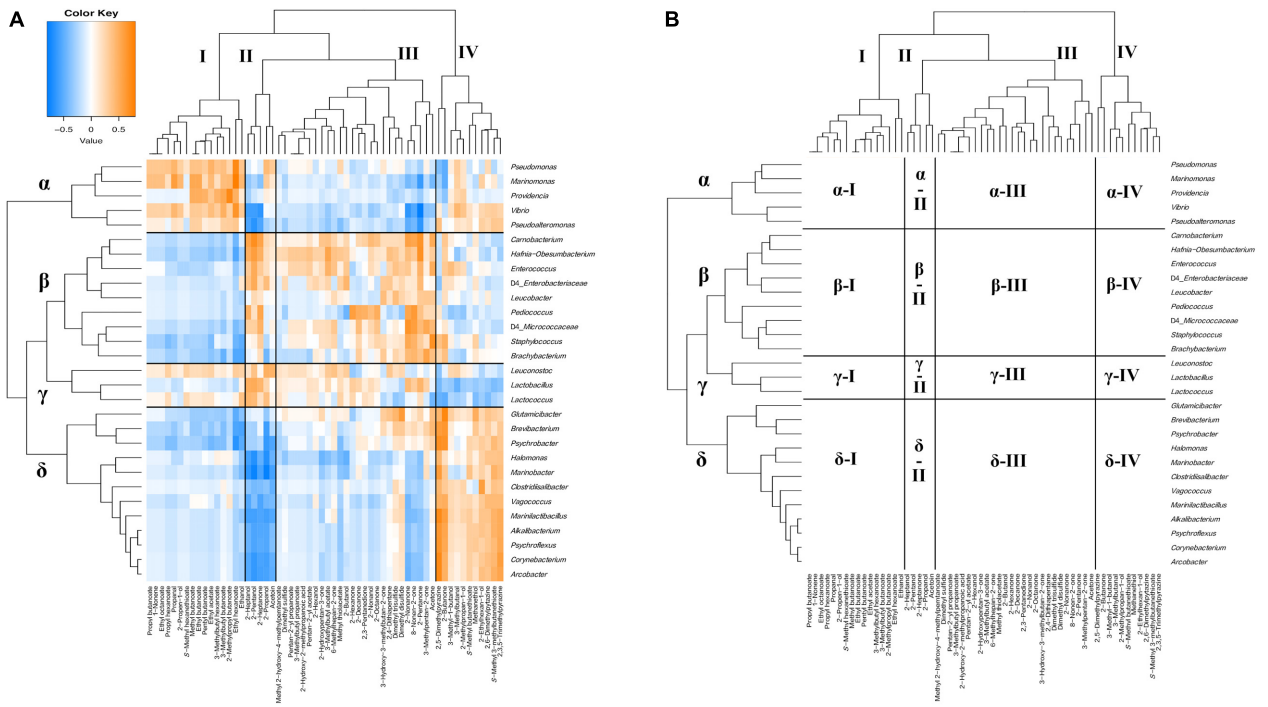

FIGURE 11 | Hierarchical clustered map showing the Spearman correlation between bacteria genus and volatile compounds detected on 30 cheese samples. **(A)** Spearman correlation heat map. Orange indicates positive correlation and blue indicates negative correlation. **(B)** Hierarchically clustered map. Volatile compounds clusters are represented by I, II, III, and IV. Bacteria clusters are represented by α, β, γ, and δ.

and cluster III components (γ-II: *Debaryomyces* – 2-pentanol and γ-III: *Debaryomyces* – 2-nonanone) and positively correlated with 2,5-dimethylpyrazine and 2,3,5-trimethylpyrazine (γ-I). Significant correlations between the appearance of fungi and volatile compounds are shown in **Supplementary Tables 16, 17**.

These results showed that the fungi were particularly correlated with secondary alcohols such as 2-pentanol and 2-heptanol and methyl ketones such as 2-pentanone and 2-heptanone. Hierarchical cluster analysis based on the correlation between fungi and metabolites in ripened cheeses often showed that clusters were assigned to a single fungal genus. This may be a reflection of the fact that only a few fungi were found to dominate the ripened cheese. However, the number and strength of correlation suggest that the fungi strongly affected to the accumulation of limited kinds of components in ripened cheeses.

DISCUSSION

This study was conducted to verify which microorganisms and/or metabolites are affected by the difference in ripening method and to clarify a correlation between the microbiota and metabolites of surface mold-ripened cheese and bacterial smear-ripened cheese. The NaCl concentration (1.0–2.0%) and pH (5.0–7.4) in the core of cheeses tested could stimulate the growth of non-halophilic, halophilic, acidophilic, and/or neutrophilic microorganisms. On the other hand, the NaCl concentration (2.0–3.0) and pH (7.5–8.3) in the rind of cheeses could stimulate the growth of halotolerant, halophilic, neutrophilic, and/or alkaliphilic microorganisms, except for the pH of Langres (pH 6.7–6.8). Considering core and rind together, cheese samples can be regarded as a suitable environment in which various microorganisms grow, in terms of salinity and pH. Thus, although the salinity and pH of the cheese samples were generally similar, metagenomic analysis showed the specific microbiota that reflect different types of cheese. Surface mold-ripened cheese and bacterial smear-ripened cheeses were characterized by LAB and *Penicillium* and MB, HALAB, and *Debaryomyces*, respectively. However, the profile of metabolites in each cheese did not reflect the difference in cheese type, except for the qualitative analysis of volatile compounds. In contrast, Spearman correlation analysis revealed the relationships between microbiota and metabolites in ripened cheese. This result obtained by combining metagenomics and metabolomics shows the superiority of the integrated approach.

In general, fungi are known to play an important role in the deacidification and the development of flavor in ripened cheese (McSweeney, 2004; Gori et al., 2007; Monnet et al., 2015; Fröhlich-Wyder et al., 2019). In this study, *Penicillium*, *Kluyveromyces*, and *Candida*, which dominated surface mold-ripened cheese, showed positive correlations with secondary alcohols and ketones, especially 2-pentanol, 2-heptanol, 2-pentanone, and 2-heptanone, while *Debaryomyces*, which dominated smear-ripened cheese, showed a negative correlation. These results reflected the differences in cheese type derived from the fungal community. Previous studies have

shown that secondary alcohols and methyl ketones were detected in surface mold-ripened cheese (Sablé and Cottenceau, 1999; Spinnler, 2017), and *P. camemberti* and *G. candidum* may produce methyl ketones (Thierry et al., 2017). In addition, *Penicillium* spp. possess the pathway that alkan-2-ones may be reduced to the corresponding secondary alcohol by fatty acid metabolism (McSweeney and Sousa, 2000). These findings are consistent with those of our study. Therefore, the correlation analysis between fungi and metabolites in ripened cheeses was able to show the relationship between fungi and secondary alcohols and methyl ketones.

Further, hierarchical cluster analysis of the correlations between bacterial communities and metabolites in ripened cheeses revealed that the appearance of specific bacteria was associated with the presence of organic acids, free amino acids, and volatile compounds. Our study showed that LAB groups, such as *Lactococcus*, were positively correlated with alcohols and ketones. Moreover, *Pediococcus*, *Enterococcus*, and *Carnobacterium*, which are known as NSLAB, also correlated with formic acid, acetic acid, and aspartic acid. Among the components that showed positive correlation with LAB in our study, ethanol, 2-pentanol, 2-nonanol, 3-methyl-1-butanol, acetone, 2-pentanone, 2-heptanone, 2-octanone, 2-nonanone, and acetoin are known to contribute to the flavors of cheeses such as mold-ripened cheese and surface-ripened cheese (Sablé and Cottenceau, 1999). Additionally, acetate, formate, and ethanol are generated from lactate by NSLAB, especially pediococci (McSweeney, 2004). A previous study investigating the influence of *Pediococcus acidilactici*, which was isolated from hard-type Swiss cheese, in model cheeses showed that higher acetate, 2-butanone, and 2-butanol levels were present in cheese with *P. acidilactici* than in the control cheese (Eugster et al., 2019). In the research focused on SLAB, Ruggirello et al. (2018) revealed that early ripened semi-hard Toma-like miniature cheese samples, that were produced using commercial starter cultures including *Lactococcus lactis* subsp. *lactis*, were characterized by high concentration of ketones such as 1-hydroxy-2-propanone, acetoin, acetone, and diacetyl; and alcohols such as 2-ethylhexan-1-ol, 2,3-butanediol, 3-methyl-2-buten-1-ol, and ethanol. This finding showed a similar relevance to our study in that LAB correlate with ketones and alcohols, although it is not a perfect match for the types of volatile compounds. Moreover, Sgarbi et al. (2013) evaluated the ability of two *Lactobacillus casei* and two *Lactobacillus rhamnosus* strains isolated from hard-type Parmigiano Reggiano cheese to produce volatile flavor compounds on cheese-based medium (CBM) and on starter LAB lysed cell medium (LCM), and found that the volatile compounds after bacterial growth on CBM were characterized by the presence of compounds such as acetoin, diacetyl, acetone, and other ketones, as well as benzaldehyde and acetic acid. In our study, diacetyl and benzaldehyde were not detected; however, the association between LAB and ketones and acetic acid agreed with the findings of previous studies, regardless of the type of cheese or the origin of bacterial isolates.

Actinobacteria are commonly found in ripened cheeses, especially *Brevibacterium linens*, which are important microorganisms in smear-ripened cheese (Rattray and Fox, 1999; Bertuzzi et al., 2018). Furthermore, *Corynebacterium*, *Glutamicibacter* (*Arthrobacter*), *Brachybacterium*, and *Leucobacter* were also detected in various cheeses (Irlinger et al., 2015). Among the five varieties of ripened cheese in this study, Langres hardly harbored *Actinobacteria*. These bacteria, such as *Brevibacterium* sp. and *Corynebacterium* sp., are acid-sensitive and begin to grow when the pH is approximately 5.5–6.0 in cheese (Monnet et al., 2015). The pH of Langres was 5.0–5.2 and 6.7–6.8 in the core and rind, respectively, lower than that in the other samples. Therefore, this might be one of the reasons for the lower proportion of *Actinobacteria* in Langres.

In previous studies, *B. linens* and *Micrococcaceae* (to which *Glutamicibacter* belongs) found in red smear cheese are considered to be the main producers of sulfur compounds (Rattray and Fox, 1999; Bertuzzi et al., 2018). It has been reported that *B. linens* produces fatty acids, alcohols, methyl ketones, pyrazines, sulfur compounds, and cyclic compounds in culture media (Rattray and Fox, 1999). In addition, *Brevibacterium* spp. isolated from hard-type Beaufort cheese produced *S*-methyl thioesters using short-chain fatty acids or branched-chain amino acids as precursors (Rattray and Fox, 1999; Sourabié et al., 2012). Deetae et al. (2007) validated that the *Brachybacterium* strain isolated from surface-ripened cheese produced high amounts of ketones on casamino acid medium.

Actinobacteria were mostly divided into two tendencies based on correlation analysis with metabolites in our study. *Brevibacterium*, *Glutamicibacter*, and *Corynebacterium* were similar to MB and HALAB, while *Brachybacterium* and *Leucobacter* were similarly correlated with LAB. The former showed a positive correlation with sulfur compounds such as methanethiol, dimethyl disulfide, dimethyl trisulfide, *S*-methyl butanethioate, and *S*-methyl 3-methylbutanethioate and pyrazines, which agrees with previous findings. The latter showed a positive correlation with aspartic acid, cystine, alanine, and ketones. The correlation of *Brachybacterium* is consistent with the study of Deetae et al. (2007), indicating that it may also contribute to cheese flavor along with LAB regardless of the type of cheese or the origin of bacterial isolates.

Marine bacteria have been reported to be widespread in cheese microbial communities using high-throughput sequencing technologies (Irlinger et al., 2015). These microorganisms, which are known to be halophilic and psychrotolerant, have previously been detected in brine as well as marine environments (Holmström and Kjelleberg, 1999; Reen et al., 2006; Marino et al., 2017; Haastrup et al., 2018; Vermote et al., 2018). Considering from these findings, it is reasonable to assume that MB are introduced from the sea salts used for the brine and are adapted to the cheese-making environment through the salting step (Irlinger et al., 2015). All of the samples in this study had a slightly saline environment (1.0–3.0%) in which MB can grow. Moreover, our study confirmed the dominance of MB in cheese samples, especially in bacterial smear-ripened cheeses. This indicates that washing with brine in the ripening process is an important factor to encourage MB to dominate cheese microbiota. However, Langres and Maroilles used in our study were dominated by different MB in

spite of being the same type of cheese. As shown in this study, cheese is occupied with adventitious microorganisms that are considered to be transferred from milk and cheese-making environments as well as deliberately added microorganisms (Montel et al., 2014; Irlinger et al., 2015; Gobbetti et al., 2018). Interestingly, a study that investigated the microbial ecosystems of two artisan cheesemaking facilities that produce a similar range of products consisting of fresh, bloomy-rind, and smear-ripened cheese, has shown that cheese and aging-rooms in facility A were dominated by *Brevibacterium*, *Staphylococcus*, *Psychrobacter*, and *Corynebacterium*, whereas those in facility B were dominated by *Pseudoalteromonas*, *Vibrio*, and *Vibrionaceae* (Bokulich and Mills, 2013). This evidence implies that the difference in microbiota between Langres and Maroilles in this study reflects the effects of environmental microbiota.

Although studies on MB in cheeses are limited, some findings have recently demonstrated its contribution to cheese flavor. *Psychrobacter* sp., isolated from surface-ripened French cheese, produced branched aldehydes, alcohols, and esters on casamino acid medium (Deetae et al., 2007) and smear soft cheese experimentally inoculated with *Psychrobacter celer*, which increases throughout the ripening, showed higher concentrations of aldehydes, ketones, and sulfur compounds (Irlinger et al., 2012). *Pseudoalteromonas* is known to possess cold-adapted enzymes, which may contribute to the development of cheese flavor during ripening, storage, and transportation at low temperatures (De Pascale et al., 2008, 2010; Wolfe et al., 2014). Furthermore, Wolfe et al. (2014) reported the *mgl* sequences (coding methionine-gamma-lyase [EC:4.4.1.11]) with high sequence similarity to various *gamma-Proteobacteria* in naturally aged cheeses from both Europe and North America by shotgun metagenomics.

In our study, MB were strongly associated with cheese metabolites, especially volatile compounds. *Pseudoalteromonas*, *Vibrio*, and *Marinomonas* were positively correlated with esters, while *Psychrobacter*, *Marinobacter*, and *Halomonas* were positively correlated with pyrazines along with HALAB. Previous studies have shown that esters are synthesized by the reaction of free fatty acids and alcohols, and this reaction is considered to be caused by LAB, *G. candidum*, and *Pseudomonas fragi* (Bertuzzi et al., 2018; Khattab et al., 2019). Our study indicated that MB, such as *Pseudoalteromonas*, *Vibrio*, and *Marinomonas*, were also associated with esters as well as *Pseudomonas*. Although pyrazines have been found in ripened cheeses, biochemical mechanisms are poorly understood (McSweeney and Sousa, 2000). Therefore, the existence of MB and HALAB is considered very important for the elucidation of the pyrazine production mechanism.

As with MB, the existence of HALAB in smear-ripened cheeses has been reported previously (Mounier et al., 2017). Previous experiments have shown that *Marinilactibacillus psychrotolerans* B-7-9-5 produces acetate from lactate during ripening in Brie-type model cheeses, and *Vagococcus lutrae* 9A8 produces acetate from lactose under aerobic conditions (Unno et al., 2020; Suzuki et al., 2021). Nevertheless, HALAB were less associated with acetic acid and showed a positive correlation with isobutyric acid in this study. In this way, while the correlations between bacteria and metabolites in our study were broadly in agreement with previous studies, some correlations were not. The flavor and texture characteristics of cheeses occur through microbiological and biochemical events, such as lipolysis, proteolysis, and metabolism of residual lactose, lactate, and citrate during ripening (McSweeney and Sousa, 2000; McSweeney, 2004; Khattab et al., 2019). Moreover, cheese can be regarded as a biocomplex ecosystem colonized by a diverse group of microorganisms that interact with each other (Irlinger and Mounier, 2009; Irlinger et al., 2015; Khattab et al., 2019). These effects also occurred in our study, and it is considered that some correlations showed discrepancies with previous studies, which were verified with a single strain.

Considering the findings in this study along with the previous findings, hierarchical cluster analysis based on the correlation clarified that the presence or absence and abundance of specific bacteria such as LAB, MB, HALAB, and *Actinobacteria* are related to the formation of specific cheese components. This indicates that the relationship between the existing bacteria and components of cheeses is generally similar, even if the type of cheese and the origin of the bacteria are different. Furthermore, even though this study used cheeses after ripening sold in food markets and did not monitor the ripening process of microorganisms and metabolites, the specific relationships between microorganisms and metabolites could be clearly shown. Thus, the correlation analysis method applied in this study can be considered to be appropriate for linking the microbiota and components of various cheeses. Based on this perspective, the role of MB in cheese has not been revealed previously; however, this study suggested that MB may play an important role in inducing cheese characteristics in flavor formation. Although there are many non-starter microorganisms for which their features have not been revealed in cheese, it can be regarded that accumulating data showing the relationship between microorganisms and metabolites in various cheese types will improve our systematic understanding of cheese consortia including adventitious microbiota. In this study, our results showed correlations between microorganisms and metabolites in ripened cheeses, but not causal relations. In addition, the mechanism of the competitive and symbiotic effects among the microorganisms appeared in ripened cheeses remains unclear. Thus, further studies, such as demonstration experiments using cheese isolate strains or metatranscriptomic analysis in the cheese ripening process, are needed to confirm the relationships shown in this study. However, methodologies and results obtained in this study will provide the basis for elucidating cheese consortia widely and help in the selection of cheese adjunct cultures for the accumulation of specific flavors in the future.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ddbj.nig.ac.jp/, DRA011532.

AUTHOR CONTRIBUTIONS

RU and MI wrote the manuscript. RU performed the experiments. RU, TS, and MM contributed to the data analysis. MI coordinated the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Massive Survey on Bacterial–Bacteriophages Biodiversity and Quality of Natural Whey Starter Cultures in Trentingrana Cheese Production

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This study focused on the microbial and bacteriophages identification and characterization in cheese-production facilities that use natural whey starter (NWS) cultures for Trentingrana production. Bacterial and phage screening was carried out on cooked not acidified whey and NWS samples isolated from six dairy factories, for 4 consecutive days in four different months. By means of a combined approach, using plate counts, bacterial isolation, and metataxonomic analysis *Lactobacillus helveticus* was found occurring as the dominant species in NWS cultures and *Levilactobacillus brevis* as codominant in the cheese factories where the temperature of NWS production was mainly lower than 40°C, suggesting that the variability in the parameters of the NWS culture preparation could differently modulate the bacterial species in NWS cultures. Using turbidity test approach on 303 bacterial isolates from the NWS cultures, 120 distinct phages were identified. *L. helveticus* phage contamination of NWS cultures was revealed in most of the analyzed samples, but despite the great recovery of bacteriophage contamination cases, the microbial quality of NWS cultures was high. Our results support the presence of natural bacteriophage resistance mechanisms in *L. helveticus*. The use of NWS cultures probably creates an ideal environment for the proliferation of different *L. helveticus* strains balanced with their phages without a clear dominance. It is evident, from this study, that the presence of a high biodiversity of NWS bacterial strains is relevant to avoid phages dominance in NWS cultures and consequently to keep a good acidification ability.

Keywords: bacteriophages, natural whey starter cultures, *Lactobacillus helveticus*, Grana-like cheese, *Levilactobacillus brevis*

INTRODUCTION

Traditional and artisanal cheese productions are often based on fermentation processes carried on by defined starter cultures used to achieve defined and typical flavors and/or textures (Gatti et al., 2014; Gobbetti et al., 2018b). Natural whey starters (NWSs) are traditionally used in Italian long ripened hard cheese production as for Parmigiano Reggiano, Grana Padano, and Trentingrana

(Bertani et al., 2020). Refreshed daily in the dairy factory from whey collected at the end of the cheese-making process (Bertani et al., 2020), these starters are mainly characterized by thermophilic lactic acid bacteria (LABs) such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *lactis* (Rossetti et al., 2008).

Together with raw milk quality, bacteriophages (or phages) may represent a dealing factor able to negatively affect fitness and performance of dairy starter cultures (Carminati et al., 2011). Phages are viruses able to infect bacteria; they are present in all the environments, including niches related to human activities as in the case of dairy factories (Carminati et al., 2011). Despite intense efforts as adapted factory design, sanitations, adequate ventilation, and culture rotation, a complete phage eradication in the dairy industry remains a utopian goal, and phage infection of starter LABs is still the most common cause of slow and/or incomplete fermentation (Guglielmotti et al., 2012). Consequently, high pH values and residual lactose may promote growth of pathogenic or spoilage bacteria, negatively affecting the quality or the yield of the final product (Josephsen and Neve, 1998). The most permanent source of phage in dairy is the raw milk, and considering that Trentingrana dairy factories use raw milk in cheese production, they can rapidly grow up to high concentration in NWS cultures (Guglielmotti et al., 2012).

Previous works showed the coexistence in NWS for Grana Padano of phage ecologically related to bacterial strains belonging to species such as *L. helveticus* and *L. delbrueckii* (Zago et al., 2006, 2008). As in other ecosystems, bacteriophages play an ecological role within NWS culture, acting as a biological pressure agent following the "kill-the-winner" hypothesis. NWS culture bacteriophages lead the natural selection of phage-resistant bacterial strains, where the fastest-growing bacteria are inhibited in dominating the NWS community, thus preserving the overall technological performances of the NWS cultures (Zago et al., 2008; Gobbetti et al., 2018a). Therefore, the comprehension of bacteria and phage biodiversity dynamic in NWS cultures is of prominent importance, and a good management of NWS processing for the Grana-like cheese production should consider the overall players in this ecological niche.

It has been demonstrated that small changes in the technological parameters, such as curd cooking temperature, titratable acidity, and pH, could affect the bacterial consortium present in NWS cultures (Rossetti et al., 2008). Despite the findings provided by these studies, the microbial and bacteriophage biodiversity of NWS culture is not fully known. To the best of our knowledge, this is the first study reporting the dynamics of bacteria and phage biodiversity in NWS by means of a massive sampling system in cheese factories following different NWS technologies of production. Therefore, in this study, we focused on NWS, collected just before addition to the vat milk, and cooked not acidified whey (cNAW), collected at the end of curdle cooking.

The samples were collected from six selected Trentingrana dairies, over 1 year of production, for 144 samples: 72 cNAW and 72 NWS samples.

This study was performed to understand the dynamics of bacteria and phages of NWS cultures over time, so in this work, we (i) characterized the dynamic of bacterial and phage community of the NWS collected to increase the existing knowledge and (ii) investigated if and how the cheese factory technology of NWS production affects the bacterial and phage community in the NWS cultures. The final goal is to maintain the high quality of Trentingrana production, avoiding economic loss associated with failure in milk fermentation and low cheese yield.

MATERIALS AND METHODS

Collection of cNAW and NWS Samples, and Determination of Temperatures and pH

cNAW and NWS culture samples used in Trentingrana manufacturing were collected for four consecutive days in four different months (February, May, August, and November 2018) from six dairy factories (A–F) located in the province of Trento (north eastern of Italy) and operating into the Trentingrana Protected Designation of Origin cheese area of production (total of 144 samples).

The Trentingrana production had stopped at cheese factory C for July and August months, so NWS and cNAW samples were collected in September only for cheese factory C.

NWS titratable acidity was measured and expressed as Soxhlet–Henkel degrees (°SH); the pH was determined using a pH electrode (Crison Instruments, Barcelona, Spain). Samples were shipped to the laboratory under liquid nitrogen and stored at −80°C before the analysis.

The temperature of the room and of the NWS tank has been determined using Testo 175 T2 (Testo Ltd., Alton, United Kingdom). Temperature was recorded each minute for the overnight fermentation of the NWS.

Microbiological Counts and Isolation

All the samples were diluted in sterile peptone water and plated onto whey agar medium (WAM) according to Gatti et al. (2003) for cultivating thermophilic lactobacilli in anaerobic conditions for 72 h at 45°C. All culture media and anaerobic system were purchased from Oxoid (ThermoFisher Scientific, Milan, Italy).

Eight colonies grown onto WAM plates were randomly isolated from NWS samples (plates with a number of colonies in the range of 10–300). Each isolate was purified by subsequent culturing in whey broth (the broth version of the WAM used for plate counting). Pure cultures were stored at −80°C in glycerol (40% vol/vol) stocks. Cell morphology was determined by microscopic observation, Gram characterization was performed applying the KOH method (Gregersen, 1978), and catalase activity was tested by adding 5% H_2O_2 drops on the colonies.

DNA Extraction and Genotypic Identification of the NWS Bacteria

All bacterial isolates were grown 48 h in the whey broth culture at 45°C before DNA extraction. The bacterial DNA

was isolated using Quick-gDNA™ MicroPrep (Zymo Research, Italy) following the manufacturer's instructions.

Randomly amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) was carried out in a total volume of 25 μL using primer M13 (Rossetti and Giraffa, 2005). Cluster analysis of DNA patterns was carried out using GelCompar II-BioNumerics software (package version 6.0; Applied Maths, bioMérieux, Belgium), exploiting the unweighted pair group method arithmetic averages. Similarity of PCR fingerprint profiles was calculated based on Pearson product–moment correlation coefficient. The threshold breakpoint value was fixed to 80%; isolates with similarity coefficient higher than 80% were classified into the same cluster, according to Gatti et al. (2003).

One isolate representative of each biotype was genotypically identified by 16S rRNA gene analysis. A fragment of the 16S rRNA gene was amplified using the primers 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′), designed by Grifoni et al. (1995). The PCR products were purified using the Exo-SAP-IT™ kit (USB Co., Cleveland, OH, United States) and sequenced in an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, United States), using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). The obtained sequences were compared using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI, United States). All amplifications were run in a T100™ ThermalCycler (Bio-Rad Laboratories, Hercules, CA, United States).

DNA Extraction, MiSeq Library Preparation, and Illumina Sequencing

Illumina analysis was performed on 24 cNAW and 24 NWS samples (one cNAW and NWS sample for each month and cheese factory). Three milliliters of sample was centrifuged at 3,200 × *g* for 15 min at 4°C. The genomic DNA was extracted from the pellet using the Power Food™ Microbial DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNA samples were purified by PowerClean DNA Clean-up Kit (Qiagen). The DNA quality and concentrations were determined by NanoDrop™ 8000 Microvolume UV-Vis spectrophotometer (ThermoFisher Scientific).

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, United States) were carried out at the Sequencing Platform in Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy). Briefly, for each sample, a 464-nucleotide sequence of the V3–V4 region (Baker et al., 2003; Claesson et al., 2010), of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Brea, CA, United States) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen, ThermoFisher Scientific) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, United States) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Illumina Data Analysis and Sequences Identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp[1] and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2020.8). Sequences were quality filtered, trimmed, denoised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences had been identified and removed *via* the consensus method in DADA2. Representative bacterial sequences had been aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (K. Price et al., 2009; Katoh and Standley, 2013). α- and β-diversity metrics had been calculated using the core-diversity plugin within QIIME2 and visualized by emperor (Vazquez-Baeza et al., 2013). Bacterial taxonomic and compositional analyses were carried on by using plugins feature-classifier[2]. A pretrained naive Bayes classifier based on the Greengenes 13_8 99% operational taxonomic unit (OTU) database, which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive and are available under Ac. Number PRJNA695135[3] from sample SAMN17602423 to SAMN17602468.

Enrichment, Isolation, and Purification of Phages

All the 72 samples of NWS cultures were screened for the presence of bacteriophages. The related cNAW samples were used to isolate the virulent phages, which used the NWS isolated bacteria as host. Phage enrichment was performed as previously reported by Zago et al. (2005). Briefly 1 g of $CaCO_3$ was included in tubes of 40-mL cNAW sample, acidified with 0.5 mL of sodium maleate buffer (maleic acid 0.05 M titrated with 0.2 N NaOH until pH 5.15 is reached), and then inoculated with 1 mL of NWS from the same sampling day and dairy. After anaerobic incubation at 45°C for 72 h, the tubes were centrifuged at 4,000 revolutions/min for 15 min at 4°C. Supernatants obtained from the enrichment step were filtered with 45-μm membrane filter pore size (Merck–Millipore, Darmstadt, Germany) and checked for the presence of phages by means of turbidity test.

Tubes of WAM-Ca broth (WAM supplemented with 10 mM $CaCl_2$) were added with 5% of the previously filtered

[1]https://github.com/yhwu/idemp/blob/master/idemp.cpp

[2]https://github.com/qiime2/q2-feature-classifier

[3]https://www.ncbi.nlm.nih.gov/bioproject/695135

supernatants and inoculated with 2% mL of host culture in the logarithmic growth phase. The tubes were incubated at 45°C and observed each 6 h until cell lysis occurred. The lysis was detected by the higher clearness of the broth, compared to controls (tubes without addition of the supernatants), which were more turbid due to bacteria growth. After 24 h, where no lysis was detected, the bacterial cultures were used for 2% inoculation of new tubes of WAM-Ca broth. After four rounds of inoculations without lysis, the bacterial isolate was considered as not suitable for the phage isolation. Where lysis was observed, the tubes were then filtered through 0.45-μm membrane filter pore size (Merck–Millipore), set to pH 7, and stored at 4°C until use, for no more than 7 days. Phage stocks were stored at −80°C with 40% vol/vol of glycerol.

Titer Determination of Phage

Phage titer was expressed as the number of plaque-forming units per mL of sample (pfu/mL). The suspension of the phage was diluted sevenfold by using phosphate-buffered saline added with $CaCl_2$ 10 mM. One hundred thirty microliters of the host bacteria in the logarithmic growth phase was added to 5 mL of semisolid WAM-Ca (WAM-Ca, 4% wt/vol agar). The mixture was poured on the surface of WAM-Ca plates and then inoculated with 10 μL of phage dilutions in seven plate sectors. After anaerobic incubation at 45°C, clear plaques were checked. The phage titer was calculated using the formula titer (pfu/mL) = plaque number × dilution factor × 100.

Detection of the Lysogenic State

Each isolated bacterial biotype, characterized as a phage host, was tested for the detection of prophage, adapting the method of Cochran and Paul (1998). Overnight WAM cell cultures were split into two aliquots: the first aliquot was added with mitomycin C ($MytC^+$, 1 μg/mL); the second aliquot was not added with $MytC^+$ and used as control. After 24 h of incubation at room temperature, in the dark, induced phages were detected as follows: 100 μL of each cell suspension was used to inoculate 7 mL of soft WAM (WAM, 0.7% wt/vol agar) plated on a layer of 1.8% (wt/vol) agar and incubated for 24 h at the optimal conditions for each strain. The growth reduction onto $MytC^+$ plates, after comparison with control plates, indicated the presence of phage.

If not specified, all chemical compounds were purchased from Sigma–Aldrich (St. Louis, MO, United States).

Statistical Analysis

A normality test (Shapiro-Wilk *W*) was performed, as well as a nonparametric test (Kruskal–Wallis) analyzing the day of collection as the independent variable and the microbial plate counts as the dependent variable. All the tests on plate counts were performed using the STATISTICA data analysis software system, version 9.1 (StatSoft, Inc, 2010[4]).

Differences in diversity indices (OTUs number and Shannon diversity index) of different samples were tested by Kruskal–Wallis test by a plug-in implemented in QIIME2. The overall structural changes of bacterial community were visualized by principal coordinate analysis (PCoA) based on both weighted and unweighted UniFrac distance matrices. The statistical significance was assessed *via* the nonparametric PERMANOVA (permutational multivariate analysis of variance) by means of plug-in implemented in QIIME2. For the differential abundance test, the taxonomy information for each OTU sequence was provided using ANCOM method (Mandal et al., 2015) by means of plug-in implemented in QIIME2.

[4]www.statsoft.com

RESULTS

Thermophilic Bacteria Counts in Trentingrana NWS and cNAW Samples and Technological Process of NWS Production

The WAM counts of thermophilic bacteria in NWS and cNAW samples during the monitoring from February to December are shown in **Table 1**. NWS thermophiles varied without significant differences in the same and among the different dairy factories, ranging between 7.3 and 8.8 log colony-forming units (cfu)/mL. Only in November, at dairy factory D, NWS cultures' averaged value was lower than 7.3 (6.7 log cfu/mL). The dairy factory C was the only one showing significant differences in the NWS thermophilic counts that were lower in February (7.5 log cfu/mL) and higher in August (8.6 log cfu/mL). More variability was observed in the cNAW thermophilic counts that ranged between 4.8 and 7.1 log cfu/mL. With the exceptions of dairy factories B and F, cNAW thermophilic counts were showing significant differences during the year without a well-defined trend.

The NWS pH mean values ranged between 3.3 and 3.6 and the titratable acidity between 28°SH and 32°SH / 50 mL without any significant difference for month or cheese factory (data not shown).

As the final acidification activity in NWS cultures is directly associated with a slow temperature decrease from 55°C to 40°C during the overnight process of NWS production (Di Cagno et al., 2006), the NWS production cycle was split into two stages: a first thermophilic stage, when the cNAW is cooled from cooking temperature (55°C) to 40°C, and a second mesophilic stage, after the first cNAW cooling, when the temperature was in the range between 20°C and 40°C (**Table 2**).

At dairy plants A, B, and C, the first thermophilic was at least 2 h 25 min shorter than the second mesophilic stage, conversely at dairy plant D, the first was approximately 7 h longer than the second stage. At cheese factories E and F, there was no relevant difference between the first and second stage of NWS culture production cycle.

Identification of Bacteria From NWS Cultures

A total of 576 isolates were picked up from WAM agar plates. A RAPD-PCR fingerprinting had been performed (i)

TABLE 1 | Thermophilic lactic acid bacteria (TLAB) counts onto WAM of Trentingrana NWS and cNAW.

	A	B	C	D	E	F	TOT ($n = 18$)
NWS TLAB							
February	7.4 ± 0.39	7.8 ± 0.35	7.5 ± 0.38^{A}	7.3 ± 0.39	7.3 ± 0.63	7.6 ± 0.31	7.5 ± 0.41
May	7.7 ± 0.29	8.0 ± 1.13	7.9 ± 0.35^{AB}	7.6 ± 0.39	7.8 ± 0.30	8.0 ± 0.77	7.8 ± 0.50
August	8.3 ± 0.57	8.3 ± 0.84	$*8.6 \pm 0.33^{B}$	7.3 ± 0.98	8.1 ± 0.61	8.7 ± 0.48	8.2 ± 0.76
November	7.6 ± 0.82	8.8 ± 0.60	8.3 ± 0.81^{AB}	6.7 ± 0.44	7.4 ± 0.57	8.2 ± 0.59	7.8 ± 0.90
TOT ($n = 12$)	7.8 ± 0.73	8.3 ± 0.74	8.0 ± 0.61	7.2 ± 0.65	7.7 ± 0.61	8.1 ± 0.62	
cNAW TLAB							
February	5.3 ± 0.30^{A}	6.4 ± 0.50	7.1 ± 0.07^{C}	4.8 ± 0.01^{A}	6.5 ± 0.73^{B}	5.7 ± 0.25	6.0 ± 0.92
May	5.5 ± 0.14^{A}	6.1 ± 0.84	5.3 ± 0.41^{A}	5.8 ± 0.71^{B}	4.9 ± 0.14^{A}	6.2 ± 0.98	5.7 ± 0.65
August	6.8 ± 0.54^{B}	6.2 ± 0.07	$*5.8 \pm 1.4^{AB}$	6.4 ± 0.64^{B}	6.1 ± 0.06^{B}	5.8 ± 0.10	6.2 ± 0.31
November	6.6 ± 0.09^{B}	6.7 ± 0.47	6.4 ± 0.26^{BC}	5.7 ± 0.47^{AB}	6.5 ± 0.43^{B}	6.9 ± 0.17	6.5 ± 0.47
TOT ($n = 12$)	6.1 ± 0.68	6.4 ± 0.49	6.2 ± 0.77	5.6 ± 0.75	6.2 ± 0.63	6.2 ± 0.62	

All the samples were collected from six dairy factories labeled from A to F, four times a year, from February to November 2018. The plate count data were expressed as means ± standard deviation of bacterial number of cfu/mL transformed to log10.
For a given column and dairy of sampling, microbial count, pH, and temperature values with A, B, and C superscripts are significantly different ($P < 0.05$).
**At cheese factory C, the samples were collected at the end of September because in August the Trentingrana production stopped.*

TABLE 2 | Length in minutes of the first thermophilic and second mesophilic stage of the overnight NWS culture production cycle.

	A	B	C	D	E	F
First thermophilic stage (T° over 40°C)						
February	537 ± 58^{A}	372 ± 27^{A}	447 ± 5^{AB}	722 ± 74^{A}	598 ± 9^{A}	595 ± 32^{A}
May	549 ± 53^{A}	344 ± 34^{A}	525 ± 45^{B}	705 ± 52^{A}	635 ± 43^{AB}	782 ± 14^{C}
August	555 ± 67^{A}	552 ± 45^{B}	$*388 \pm 36^{A}$	739 ± 6^{A}	629 ± 74^{AB}	683 ± 25^{B}
November	512 ± 62^{A}	510 ± 38^{B}	438 ± 14^{AB}	773 ± 63^{A}	652 ± 37^{B}	519 ± 49^{A}
TOT ($n = 12$)	538 ± 59	444 ± 42	560 ± 24	735 ± 62	629 ± 59	645 ± 37
Second mesophilic stage (T° between 20 and 40°C)						
February	757 ± 58^{B}	$1{,}020 \pm 48^{D}$	891 ± 12^{C}	318 ± 74^{B}	644 ± 5^{AB}	705 ± 3^{BC}
May	744 ± 53^{B}	$1{,}098 \pm 44^{D}$	801 ± 45^{C}	319 ± 52^{B}	565 ± 15^{A}	610 ± 24^{A}
August	721 ± 67^{B}	804 ± 59^{C}	$*893 \pm 37^{C}$	361 ± 65^{B}	578 ± 74^{A}	670 ± 25^{B}
November	763 ± 62^{B}	786 ± 52^{C}	843 ± 14^{C}	327 ± 63^{B}	655 ± 37^{B}	751 ± 49^{C}
TOT ($n = 12$)	746 ± 61	927 ± 52	857 ± 38	321 ± 64	611 ± 39	684 ± 37

All the times were recorded from six dairy factories, labeled from A to F, four times a year, from February to November 2018.
For a given column and dairy of sampling, the values with A, B, and C superscripts are significantly different ($P < 0.05$).
**At cheese factory C, the samples were collected at the end of September because in August the Trentingrana production stopped.*

to preliminarily recognize the bacterial isolates purified from colonies onto the same plate originated from genetically alike single mother cells and (ii) to investigate the microbial diversity beyond the species level, establishing the number of different biotypes inside cheese factory and over time. One isolate for each biotype was selected for further identification by 16S rRNA gene sequencing.

Of the 576 isolates, 45 did not grow after plate isolation and were discarded, 383 had been identified as *L. helveticus*, 120 as *Levilactobacillus brevis*, 13 as *Lacticaseibacillus paracasei*, eight as *Lactiplantibacillus plantarum*, and the other seven as *Limosilactobacillus fermentum* (**Figure 1**).

Cluster analysis of RAPD-PCR profiles showed that, at a similarity level of 85%, *L. helveticus* and *L. brevis*, accounting for the 95% of all the isolates, grouped, respectively, into 243 and 51 different biotypes. *L. paracasei*, *L. plantarum*, and *L. fermentum* grouped into two, three, and five different biotypes, respectively (**Table 3**).

Lactobacillus helveticus was always dominant in NWS samples of all cheese factories, with the exception of cheese factory A. In particular, its relative presence ranged between 58 and 96% and was higher than 90% in NWS samples at cheese factories E and F. Conversely, *L. brevis* presence ranged between 0 and 42% with the exception of factory A where its relative presence was 51% and dominated NWS samples from May to November (**Figure 1**). *L. paracasei* and *L. plantarum* isolates were found only in NWS samples collected in February, and the higher presence was at cheese factories D and C, respectively. *L. fermentum* isolates were found only in NWS samples collected in May and more frequently at cheese factory D.

The distribution of the isolates into the NWS cultures was mainly related with the dairy plant rather than with the month of production. In fact, considering only the month of NWS production, there was no great difference in the distribution of *L. helveticus* and *L. brevis* isolates whose relative presence ranged between 62 and 89% and 11 and 34%, respectively.

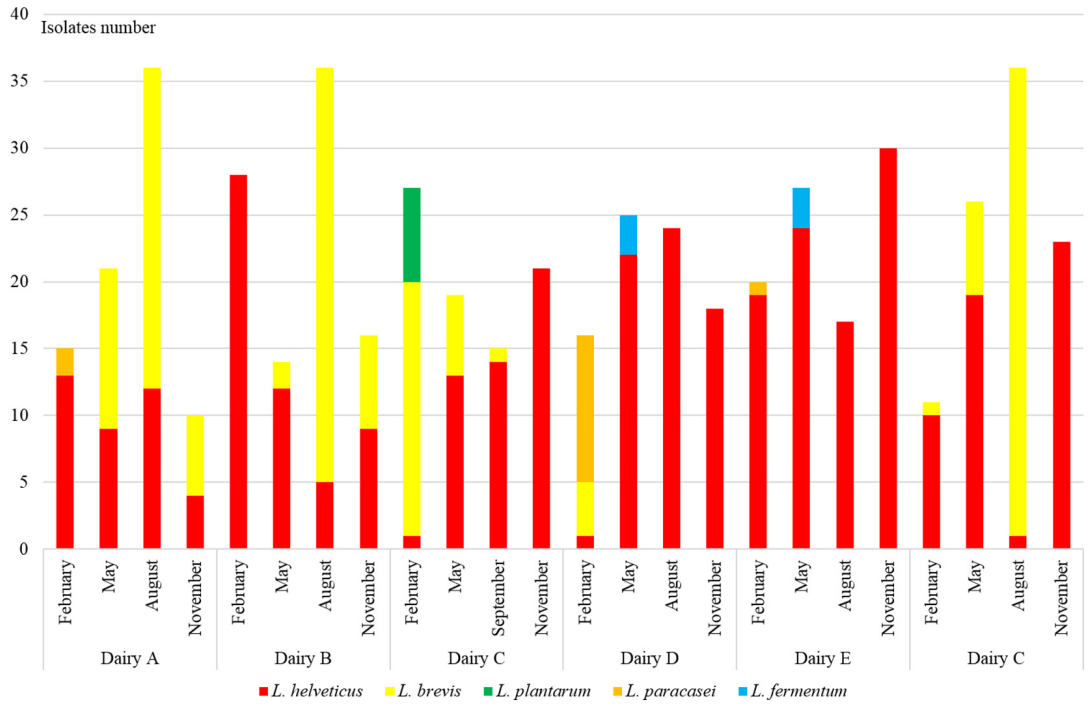

FIGURE 1 | Distribution of *L. helveticus*, *L. brevis*, *L. paracasei*, *L. plantarum*, and *L. fermentum* isolates in NWS samples collected from six dairy factories labeled from A to F four times in 2018 year: February, May, August, and November. At cheese factory C, the samples were collected at the end of September because in August the Trentingrana production stopped.

Characteristics of the Sequencing Data, Diversity Analysis, and Differential Abundance Analysis of the Bacterial Community in cNAW and NWS Samples

With the exception of two NWS samples, the extracted DNA was always successfully amplified in the bacterial V3–V4 16S rRNA gene region. A total of 2,082,145 paired-end sequences were obtained.

To address the hypothesis that species richness and biodiversity vary with sample source (cNAW or NWS sample), month (February, May, August, or November), and cheese factory (A, B, C, D, E, and F), the intragroup diversity estimation (α diversity) was calculated, using both the number of observed OTUs and Shannon diversity indexes. Both α-diversity indexes were not significantly different between cNAW and NWS samples (Kruskal–Wallis, $P > 0.1$), and no significant difference was found for month or dairy of sampling (data not shown).

In order to assess the amount of variation in bacteria composition among the samples, we calculated the phylogenetic β diversity based on both weighted and unweighted UniFrac distances. The PCoA plot onto weighted UniFrac distance matrix (**Figure 2A**) shows a little separation of the bacterial populations between cNAW (rings) and NWS samples (squares), visible on axis 2 explaining 18.3% of total variation. Conversely, the PCoA plot onto unweighted UniFrac distance matrix (**Figure 2B**) showed, with few exceptions, all cNAW and NWS samples overlapped in the same cloud on the left side of the graph. As the unweighted UniFrac distance accounts for the presence/absence of OTUs, so does the weighted UniFrac distance for abundance, although this result suggests that the individual microbial abundance more than the composition in species drives the distance among cNAW and NSW samples.

A PERMANOVA was performed to explore the effects and significance of the variables (**Table 4**): sample source (cNAW and NWS), cheese factory (A, B, C, D, E, and F), and month of sampling (February, May, August, and November). The test revealed that sample source affected microbial communities ($P < 0.05$); the month of sampling was slightly significant in cNAW and NSW samples, and in particular, the samples collected in November showed a significantly different bacterial community. Cheese factory was the main factor affecting microbial population diversity in all the samples, and the cheese factory D showed a significantly higher effect on cNAW and NSW samples (**Table 4**).

Considering only the OTUs whose relative abundance in each sample was greater than 0.01% (**Table 5**), *Firmicutes* was the dominant phylum, able to describe always more than 99% of the bacterial microbiota in all the samples. *L. helveticus* was always the dominant species over *Lactobacillus* spp. and *Streptococcus* spp., constituting more than 58% of the bacterial population in all the samples (**Table 5**). OTUs identified as *Chryseobacterium, Staphylococcus equorum, Lactococcus, Acinetobacter guillouiae*, and *Acinetobacter johnsonii* phylotypes were present in traces (<0.01%) and not in all the samples.

TABLE 3 | Distribution of *L. helveticus*, *L. brevis*, *L. paracasei*, *L. plantarum*, and *L. fermentum* biotypes (n° BioT) in NWS samples collected from six dairy factories labeled from A to F four times a year from February to November 2018.

	A	B	C	D	E	F	TOT n° BioT
L. helveticus							
February n° BioT	8	23	1	1	12	5	50
May n° BioT	8	11	10	17	18	11	73
August n° BioT	7	4	*8	14	11	16	60
November n° BioT	4	4	14	14	11	15	61
TOT n° BioT	27	42	33	46	52	47	243
Biodiversity (%)	0.71	0.78	0.67	0.72	0.58	0.54	0.63
L. brevis							
February n° BioT	—	—	14	3	—	1	18
May n° BioT	7	1	4	—	—	2	14
August n° BioT	7	8	*1	—	—	—	16
November n° BioT	1	2	—	—	—	—	3
TOT n° BioT	15	11	19	3	—	3	51
Biodiversity (%)	0.36	0.28	0.73	0.75	—	0.38	0.43
L. paracasei							
February n° BioT	1			1			2
Biodiversity (%)	0.50	—	—	0.09	—	—	0.15
L. plantarum							
February n° BioT			2		1		3
Biodiversity (%)	—	—	0.29	—	1	—	0.38
L. fermentum							
May n° BioT	—	—	—	2	2	1	5
Biodiversity (%)	—	—	—	0.67	0.67	1	0.71
TOT n° BioT	43	49	52	52	55	51	303

The similarity level was calculated by using the Pearson product–moment correlation coefficient: isolates having 85% of similarity were grouped into the same biotype. The biodiversity index was calculated as ratio between number of biotypes and total number of isolates.

**At cheese factory C, the samples were collected at the end of September because in August the Trentingrana production stopped.*

To identify taxonomic groups driving differences among the bacterial community, a differential abundance test by ANCOM method had been performed. No difference was found considering the month of sampling. Some taxa had been found differentially abundant among the cheese factories; in particular, both *Lactobacillus* and *Streptococcus* spp. were informative for characterizing the microbial community of cheese factories A and B.

Dissemination and Titer of Phages in NWS Samples and Lysogenic State Analysis

All the 72 NWS samples collected from the six local cheese factories had been phage screened. The bacterial biotypes isolated from the different NWS cultures (**Table 3**) represented a reliable set of isolates suitable as hosts for phage detection in NWS samples; consequently, for each NWS sample screening, a set of putative bacterial hosts was selected, grouping all the isolates representative for each different biotype found in each NWS sample.

One hundred twenty phages were readily detected in 41 of the 72 NWS samples (**Table 6**) and were all lytic for their *L. helveticus* host isolate, with the exception of two phages lytic for *L. fermentum*. To detect the presence of prophage in their genomes, the 120 host bacterial isolates were treated with sublethal concentrations of $MytC^{+}$. In none of the isolates, the $MytC^{+}$ induced a sharp decrease in plate counts, confirming the lytic nature of all the phages.

Phage titers were determined in all the 41 positive NWS samples. For *L. helveticus* phages, titers ranged between 2×10^6 and 9×10^8 pfu/mL with the exception of all the 12 phages from dairy factory C and six phages from dairy factory F, whose titers were in a range between 2×10^2 and 4×10^5 pfu/mL.

DISCUSSION

The NWS cultures used in Grana-like cheese production originate from artisanal back-slopping practices and face several selective pressures during the production process such as heat treatments and strong lactic acid fermentation. The bacterial consortia are characterized by a lower species complexity but high degree of strains diversity (Erkus et al., 2013), and bacteriophages have a regulatory role in the bacterial population dynamics through density-dependent predation (Rodriguez-Valera et al., 2009).

This study was carried out to investigate the bacterial and phage dynamics of cNAW and NWS production analyzing samples collected in 72 different days of Trentingrana production in six dairy plants, over a year period, by means of a combined approach, using plate counts, bacterial isolation, metataxonomic analysis, and phage isolation.

The NWS culture thermophilic counts, acidity, and pH were not significantly different for month (with exception of cheese factory C) or cheese factory, and their values were according to previously studied NWS cultures for Trentingrana (Franciosi et al., 2012) and Parmigiano Reggiano (Coppola et al., 2000). The homogeneity of the microbial counts throughout the overall sampling year could be the consequence of the consolidated process of NWS production from the cNAW, which guarantees for each dairy plant a microbial homogeneity of the NWS cultures. At cheese factory C, the thermophilic counts of NWS cultures were significantly higher in September and November. This cheese factory was the only one to stop the Trentingrana production in July and August. In September, the production of NWS cultures started again with the cheese. Therefore, this difference in thermophilic counts at dairy plant C could be a consequence of the total change in the NWS cultures that in September were no more linked to the NWS cultures of the previous months.

The sequencing of the 16S ribosomal gene of the isolates dominating the thermophilic microbial population in NWS cultures revealed the presence of five species, of which *L. helveticus* and *L. brevis* were the two dominant ones (95% of the thermophilic analyzed isolates). *L. paracasei*, *L. plantarum*, and *L. fermentum* were present in few NWS samples collected only in February and May. *L. helveticus* is well known as

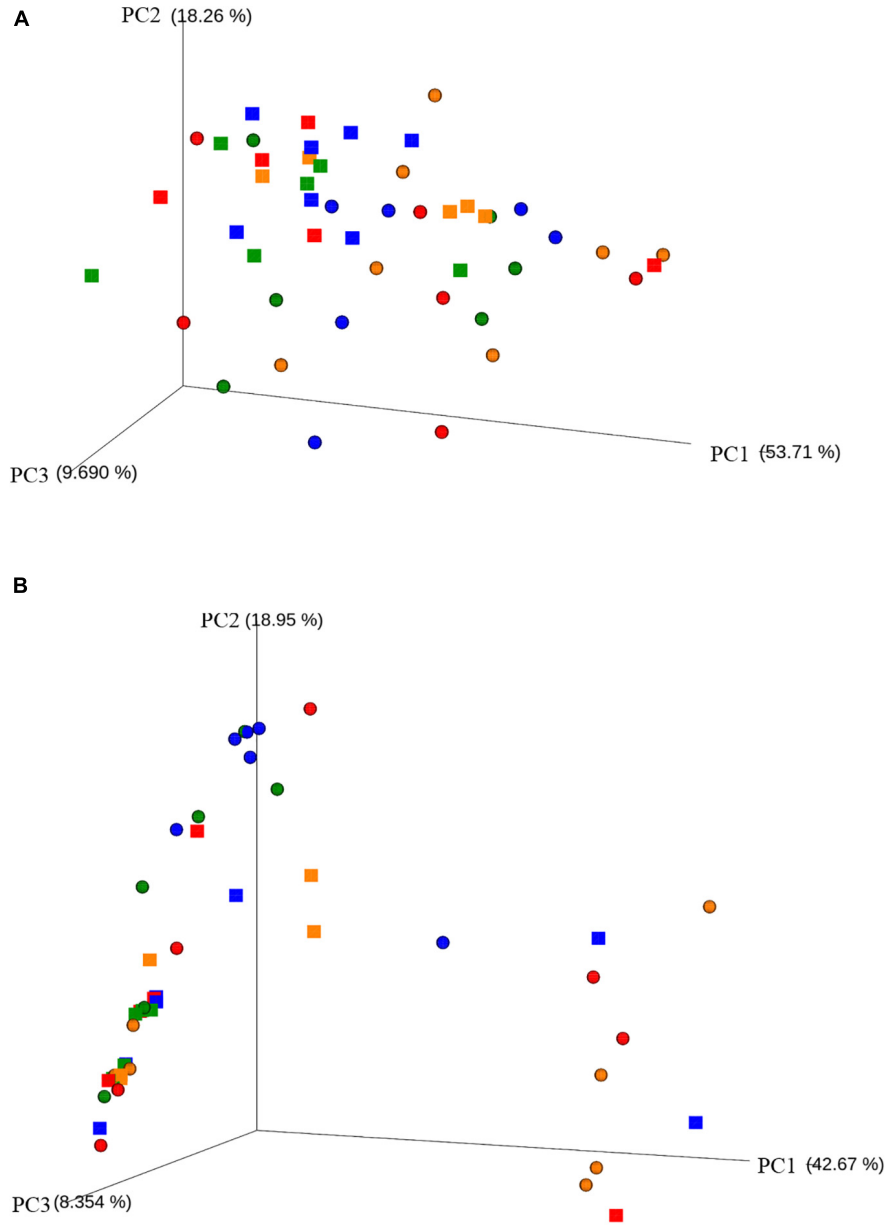

FIGURE 2 | Principal coordinate analysis (PCoA) based on weighted **(A)** and unweighted **(B)** UniFrac distances of bacterial OTUs. **(A)** The first, second, and third axes explained 81.66% of the total variance. **(B)** The first, second, and third axes explained 69.97% of the total variance. Different symbols represent the different sampling sources: rings for cNAW and squares for NWS samples. Different colors represent the different sampling months: February = red, May = blue, August = orange and November = green.

dominant in NWS cultures for Grana-like cheeses (Rossetti et al., 2008; Morandi et al., 2019; Bertani et al., 2020). *L. fermentum* had already been isolated in low abundance in NWS cultures of Grana-like cheeses (Rossetti et al., 2008; Morandi et al., 2019; Bertani et al., 2020) and the presence of two biotypes of *L. paracasei* and three of *L. plantarum* could be explained as an occasionally NWS culture contamination that occurred only in the February month of sampling. By contrast, the dominance of *L. brevis* together with *L. helveticus* in the thermophilic community of the NWS cultures in three cheese factories (*L. brevis* constituted > 30% of the isolates in A, B, and C) and during the overall year was surprising. The presence of 51 biotypes of *L. brevis* could not be explained as a contamination. *L. brevis* is one of the most common nonstarter LAB species found in the dairy industry (Settanni and Moschetti, 2010) and was already found in traces among the mesophilic bacteria of NWS cultures used for Parmigiano Reggiano (Coppola et al., 2000). To our knowledge, this is the first time this species had been isolated as one of the codominant thermophilic bacteria in NWS for Grana-like cheeses. *L. brevis* was in higher

TABLE 4 | PERMANOVA analysis (999 permutations) results for bacterial communities based on weighted UniFrac distances, respectively.

Main effects	Pseudo-F	*p* value
Source of sample	4.25	0.012*
Month	1.553	0.111
Cheese factory	2.017	0.015*
Pairwise comparisons for month		
Feb vs. May	0.743	0.547
Feb vs. Aug	0.388	0.873
Feb vs. Nov	2.279	0.068*
May vs. Aug	1.430	0.211
May vs. Nov	2.083	0.065*
Aug vs. Nov	3.237	0.017*
Pairwise comparisons for cheese factory		
A vs. B	0.740	0.701
A vs. C	0.858	0.509
A vs. D	2.155	0.058*
A vs. E	2.983	0.169
A vs. F	2.006	0.128
B vs. C	1.200	0.276
B vs. D	2.112	0.044*
B vs. E	2.821	0.050*
B vs. F	2.259	0.090
C vs. D	3.089	0.006*
C vs. E	2.225	0.083
C vs. F	2.170	0.090
D vs. E	2.502	0.037*
D vs. F	1.504	0.066*
E vs. F	1.024	0.366

*The pairwise comparisons were calculated for different sampling sources collected from six dairy factories labeled from A to F four times a year from February to November 2018 (Feb = February, Aug = August, and Nov = November). Significance levels: *P < 0.07, **P < 0.01.*

abundance in NWS from cheese factories A, B, and C where the overnight NWS production was dominated by a mesophilic stage with temperature of less than 40°C that is more selective for *L. brevis* than *L. helveticus*. As *L. brevis* growth could be strongly inhibited by the presence of homofermentative bacteria such as *L. helveticus* (Laleye et al., 1989), we speculate that a longer thermophilic stage greater than 40°C could be desirable because it is more selective for the growth of *L. helveticus* over *L. brevis* during the NWS culture production. *L. brevis* is an obligate heterofermentative bacteria producing CO_2 from lactose and known to be a possible cause of early gas production in cheeses (Michael and Mullan, 2000); therefore, it is better to limit its abundance in NWS cultures.

Cluster analysis of RAPD-PCR bacterial isolates profiles showed a higher genotypic diversity within *L. helveticus* than *L. brevis* isolates; in fact, the 383 isolates of *L. helveticus* grouped into 243 biotypes, whereas the 120 *L. brevis* into 51 biotypes. The better adaptability of the *L. helveticus* species to the stressing conditions caused by the strongly acidic environment of the NWS culture may explain its higher strain diversity and consequently the lower *L. brevis* diversity at strain level. Many *L. helveticus* strains were probably able to adapt to acidity and high temperature of NWS cultures, and conversely, fewer *L. brevis* biotypes were resistant to these same stress conditions. The qualitative distribution of the biotypes into the different cultures was mainly dairy plant specific rather than correlated with the month of production. This is confirming our previous speculation—that the dominant LAB species of the NWS cultures are modulated by the variability in the parameters of the culture preparation that are different in each dairy plant. NWS cultures characterized by a low species-level complexity but a higher strain-level diversity were already described in other studies performed on dairy starter cultures strains (Frantzen et al., 2018; Schmid et al., 2018; Somerville et al., 2019). The differences in NWS culture production associated with the dairy plant characteristics (farmers, dairy factory operators, and environment) may affect the selection of NWS culture biotypes. Previous studies have also highlighted that NWS species and biotypes show different ability to adapt to dairy ecosystem (Giraffa and Neviani, 1999; Coppola et al., 2000; Moser et al., 2018).

Regarding the relative composition of the bacterial community, the α indices did not show significant differences in the biodiversity of the microbial communities of cNAW and NWS samples. The β-diversity analysis showed that the same bacterial species were characterizing both NWS and cNAW samples but with different relative abundances, and the

TABLE 5 | Relative abundances of *L. helveticus*, *Lactobacillus*, and *Streptococcus* spp. of bacterial sequences from cNAW and NWS samples using Illumina MiSeq.

Cheese factory	Sample	*Lactobacillus* spp.	*L. helveticus*	*Streptococcus* spp.
A	cNAW	21.1 ± 13.9	75.3 ± 14.3	3.5 ± 1.3
	NWS	37.3 ± 6.7	60.5 ± 6.6	2.2 ± 0.3
B	cNAW	29.8 ± 4.4	62.6 ± 4.4	7.6 ± 1.0
	NWS	35.8 ± 5.7	61.7 ± 5.6	2.5 ± 0.9
C	cNAW	30.9 ± 11.6	68.1 ± 12.1	0.9 ± 0.8
	NWS	32.9 ± 10.9	65.1 ± 13.8	2.0 ± 1.3
D	cNAW	26.9 ± 3.6	71.7 ± 4.1	1.1 ± 0.8
	NWS	29.2 ± 8.2	69.8 ± 8.6	1.0 ± 0.5
E	cNAW	23.6 ± 8.5	74.2 ± 10.5	2.1 ± 1.9
	NWS	33.9 ± 10.0	64.9 ± 10.5	1.2 ± 0.6
F	cNAW	17.5 ± 6.2	80.1 ± 6.7	2.2 ± 1.0
	NWS	38.7 ± 6.7	58.6 ± 7.5	2.6 ± 1.0
Month				
February	cNAW	27.2 ± 13.4	69.1 ± 13.9	3.5 ± 2.2
	NWS	31.6 ± 5.4	66.6 ± 5.9	1.8 ± 1.1
May	cNAW	24.3 ± 6.8	72.4 ± 6.9	2.9 ± 2.7
	NWS	34.4 ± 5.0	64.1 ± 5.1	1.4 ± 1.1
August	cNAW	25.5 ± 9.6	75.4 ± 12.4	2.3 ± 3.5
	NWS	35.1 ± 4.9	62.1 ± 5.9	2.8 ± 1.8
November	cNAW	22.2 ± 8.8	75.4 ± 9.8	2.3 ± 2.6
	NWS	28.8 ± 8.1	70.2 ± 9.0	1.0 ± 0.9

Samples were collected from six different cheese factories labeled from A to F for four months (February, May, August, and November). The data were expressed as means ± standard deviation of relative abundances transformed into a percentage.

TABLE 6 | Distribution of phage isolates (n° Is.) from NWS samples collected from six dairy factories labeled from A to F, four times a year from February to November 2018.

	A	B	C	D	E	F	TOT
February	0	2 (1)	0	0	6 (6)	4 (1)	12 (8)
May	8 (2)	4 (0)	8 (1)	6 (3)	17 (8)	10 (5)	53 (19)
August	3 (2)	4 (1)	*3 (2)	3 (1)	4 (0)	12 (8)	29 (14)
November	3 (0)	1 (1)	1 (1)	5 (2)	2 (0)	14 (9)	29 (15)
Cheese Factory TOT	14 (4)	11 (3)	12 (4)	14 (6)	29 (14)	40 (23)	120 (54)
Presence (%) in the cheese factory	52%	26%	36%	30%	56%	85%	49%

In the bracket, the number of phages able to form lysis plaques by agar layer technique on MRS-Ca.
The presence in the cheese factory was calculated as ratio between number of isolated phages and number of tested bacterial host.
**At cheese factory C, the samples were collected at the end of September because in August the Trentingrana production stopped.*

dairy factory had a higher significant effect than the month of sampling on the abundance of the different species inside the microbial community. Dairy factory was already considered the most important source of variation of the Trentingrana cheese quality index (Bittante et al., 2011), and our results are in agreement with this previous study. The pairwise comparison showed that the species and relative abundances of cNAW and NWS cultures were very similar in all the dairy plants with the exception of the cheese factory D, whose NWS samples showed the highest relative abundance of *L. helveticus* and the lowest of *Lactobacillus* spp. and *Streptococcus thermophilus*. The high abundance of *L. helveticus* could be due to the NWS cycle of production: dairy plant D was the only cheese factory where the NWS cultures were produced with a thermophilic stage significantly longer than the mesophilic one (approximately 7 h longer), confirming the importance of NWS technology of production in modulating the microbial community of the NWS cultures. The analysis of both metataxonomic and quantitative data obtained by plate counts and isolation suggested that *L. helveticus* decreased in relative abundance but increased in amount from cNAW to NWS. This was in agreement with Bertani et al. (2020), showing that *L. helveticus* growth was mainly favored, in the acidic conditions occurring during the overnight fermentation of NWS. In Trentingrana dairy plants A, B, and C, the NWS cultures showed a high abundance of *L. brevis* isolates adapted to survive in thermophilic environment. Their dominance was also confirmed by metataxonomic data. *Lactobacillus* spp. relative abundance was very high (29–39%) when compared to previous metataxonomic studies on NWS for Grana-like cheeses (Bertani et al., 2020) where *Lactobacillus* spp. was never higher than 1%. *L. delbrueckii* was neither isolated nor sequenced in our study, and the abundance of *S. thermophilus* was never higher than 2.6% in NWS cultures. Both results were in agreement with a previous study by Morandi et al. (2019) on NWS cultures for Trentingrana production collected from 2014 to 2016, when a drastic reduction of *L. delbrueckii* and *S. thermophilus* isolates had been observed.

Ubiquitously distributed among all ecosystems, bacteriophages are commonly present in dairy plants (Pujato et al., 2019). It has been widely shown that all the most common starter bacteria species used in dairy industry, such as *S. thermophilus*, *Lactococcus*, and *Lactobacillus* spp. (Zago et al., 2006, 2015; Oliveira et al., 2018), are suitable hosts for phages, with consequent possible impairment in fermentations performance.

Almost all the 120 investigated phages were infecting the *L. helveticus* isolates, and 54 were able to form lysis plaque. This low ability in plaques forming was already observed in *L. helveticus* phages (Carminati et al., 2011; Zago et al., 2015). All the 120 phages were isolated over 1 year from NWS samples suggesting a simultaneous presence of phages and different sensitive bacteria within the cultures, as shown in previous studies (Zago et al., 2005, 2015). Although the widespread presence of lytic phages, all the NWS cultures were successful in their acidification activity, suggesting that the phages virulence could be counteracted by the presence of many bacteria, with different phage sensitivity, representing a natural way to control phage overwhelm. The individual resistance of the NWS isolates to phage predation could be one of the factors influencing NWS culture diversity as previously confirmed by Spus et al. (2015). In this study, the phage presence was not equally distributed among dairy plants; in fact, approximately 58% of the phages have been isolated from NWS cultures produced in only two dairy plants (E and F). It is remarkable that the same dairy plants E and F showed the lower biodiversity in the number of *L. helveticus* biotypes, confirming that the lower the bacterial biodiversity, the higher could be the phage presence. Few bacterial biotypes have been already observed in dairy starter cultures, where the phage predation operated mainly at strain rather than at species level (Erkus et al., 2013). The high biodiversity of dairy bacteria biotypes may prevent massive phage multiplication in NWS cultures and explain the recovery of a lower number of phages from the dairy plants with the higher biodiversity in *L. helveticus* biotypes. We speculate that, if phage activity changed in NWS culture, a population of few bacteria biotypes could be more susceptible to a phage predation with the consequent loss of NWS activity and generation of a defective cheese (Madera et al., 2004). Conversely, NWS cultures rich in different biotypes have a better chance to include phage-resistant bacteria able to counteract changes in phages community.

These considerations highlight the importance in assessing the strain biodiversity in NWS cultures: the higher the biodiversity, the higher the probability of a natural selection of bacterial strains resistant to phage predation and thus higher preservation of the overall activity of the NWS cultures, as also stated by Carminati et al. (2011).

In conclusion, this study added further knowledge on the microbial composition and diversity of NWS cultures for Grana-like cheeses in relation with the phage isolation. We speculate the importance of the technological process of NWS production and in particular of the thermophilic and mesophilic stages as drivers of the bacterial species dominant in NWS cultures. In addition, we found a possible correlation among bacterial starter biodiversity and the number of recovered lytic phages.

The abundance of phages in NWS cultures underlines again the importance of phage control strategies in the dairy industry. Ongoing studies are in progress to evaluate the roles of bacterial biodiversity, phages, and NWS production technology on the species composition of Trentingrana NWS cultures.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA695135.

AUTHOR CONTRIBUTIONS

EF and AMe devised the study. EF and AMa drafted the manuscript. EF, AMa, NC, AG, IC, and MR performed sampling plane and the experiments. MZ contributed to the study with her know-how on phages. EF, AMe, and KT provided resources and intellectual input that supported the study. All authors contributed to the article and approved the submitted version.

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Whole Genome-Based Characterization of *Listeria monocytogenes* Isolates Recovered From the Food Chain in South Africa

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Listeria monocytogenes is an important foodborne pathogen which has the ability to adapt and survive in food and food processing facilities where it can persist for years. In this study, a total of 143 *L. monocytogenes* isolates in South Africa (SA) were characterized for their strain's genetic relatedness, virulence profiles, stress tolerance and resistance genes associated with *L. monocytogenes*. The Core Genome Multilocus Sequence Typing (cgMLST) analysis revealed that the most frequent serogroups were IVb and IIa; Sequence Types (ST) were ST204, ST2, and ST1; and Clonal Complexes (CC) were CC204, CC1, and CC2. Examination of genes involved in adaptation and survival of *L. monocytogenes* in SA showed that ST1, ST2, ST121, ST204, and ST321 are well adapted in food processing environments due to the significant over-representation of Benzalkonium chloride (BC) resistance genes (*bcrABC* cassette, *ermC, mdrL* and *lde*), stress tolerance genes (SSI-1 and SSI-2), Prophage (φ) profiles (LP_101, vB LmoS 188, vB_LmoS_293, and B054 phage), plasmids profiles (N1-011A, J1776, and pLM5578) and biofilm formation associated genes. Furthermore, the *L. monocytogenes* strains that showed hyper-virulent potential were ST1, ST2 and ST204, and hypo-virulent were ST121 and ST321 because of the presence and absence of major virulence factors such as LIPI-1, LIPI-3, LIPI-4 and the internalin gene family members including *inlABCEFJ*. The information provided in this study revealed that hyper-virulent strains ST1, ST2, and ST204 could present a major public health risk due to their association with meat products and food processing environments in SA.

Keywords: cgSNP, cgMLST, AMR, virulence profiles, Benzalkonium chloride resistance, stress tolerance, plasmids, prophages

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INTRODUCTION

Listeria monocytogenes remains a considerable public health concern due to its complex ecology and ability to survive in various harsh environmental conditions posed in the food processing facilitates (Ferreira et al., 2014; Hurley et al., 2019; Chen et al., 2020). Assessing the genetic diversity of *L. monocytogenes* is critical in understanding the epidemiology, ecology, and pathogenicity of this

pathogen. *Listeria monocytogenes* consists of three major evolutionary lineages including lineages I, II, and III, as well as a rare lineage IV (Chen et al., 2020). These lineages represent 13 recognized serotypes of *L. monocytogenes* which are further grouped into four PCR-serogroups: IIa (1/2a and 3a), IIc (1/2c and 3c), IIb (1/2b and 3b), and IVb (4b, 4d, and 4e) (Doumith et al., 2004; Chen et al., 2020). Molecular typing of *L. monocytogenes* strains can also be done using Multilocus Sequence Typing (MLST), which is based on the sequence variants of seven housekeeping genes to determine their ST and CC. Recently, the cgMLST typing method that takes into account the sequence variation of 1,748 *L. monocytogenes* core genes, has been used to improve isolates discrimination and allowing a standardized comparison with isolate databases for outbreak investigations and surveillance of listeriosis (Moura et al., 2016, 2017).

The adaptation and survival of *L. monocytogenes* in the food processing facilities occur mainly through their ability to proliferate in low temperature, pH and osmotic stress (Takahashi et al., 2014), as well as resistance to sanitation agents and formation of biofilm (Hurley et al., 2019). The control of *L. monocytogenes* in the food processing facilities is mostly based on application of quaternary ammonium compounds (QACs) biocides, such as BCs (Zacharski et al., 2018; Maury et al., 2019). However, the evolution of *L. monocytogenes* resistant to the BCs has been reported in several studies and has become a serious global concern (Zacharski et al., 2018; Korsak et al., 2019). These BC resistances are associated with several efflux resistance genes including *bcrABC* cassette, *Ide, mdrL, qacH, qacA*, *qacEΔ1-sul*, and *emrE* which have been reported in various serotypes, ST and CC of *L. monocytogenes* isolated from diverse sources (Kovacevic et al., 2016; Korsak et al., 2019). Furthermore, another key adaptation of *L. monocytogenes* in the environment is the ability to tolerate toxic metals such as arsenic and cadmium (Jesse et al., 2014; Nunes et al., 2016). As result, the co-occurrence of toxic metals and biocide resistance genes in *L. monocytogenes* contribute to the selection of different resistance genotypes and phenotypes that can cause human listeriosis (Angelo et al., 2017; Parsons et al., 2018).

However, despite antibiotic treatment including β-lactam antibiotic such as amoxicillin, penicillin, or ampicillin, and aminoglycosides, such as gentamycin, listeriosis is responsible for mortality rate of 20–30% world-wide (Wang et al., 2015; Wilson et al., 2018). There are reports on *L. monocytogenes* isolates resistant to one or more antibiotics primarily cephalosporins, oxacillin and fosfomycin, particularly in Southern and Western regions of Asia (Sugiri et al., 2014; Wang et al., 2015). The genetic basis of antibiotic resistance in *L. monocytogenes* is associated with different genes such as genes encoding for efflux pumps, particularly for the major facilitator superfamily (*Ide*); erythromycin ribosome methylase (erm) genes (*ermA, ermB*, and *ermC*); tetracycline resistance genes (*tetA, tetK,* and *tetL); fosX,* and *lmrB* (Wilson et al., 2018). The role of mutations in DNA gyrase topoisomerase II (*gyrA* and *gyrB*), topoisomerase IV (*parC* and *parE*) in the development of antibiotic resistance by *L. monocytogenes* was also pointed out by Moreno et al. (2014) and Wilson et al. (2018). The virulence potential of this bacteria is mainly contributed by virulence genes such as *prfA, plcA, hly, mpl, actA, plcB, inlA, inlB*, and *lspA* (Chen et al., 2019).

Several studies in SA have reported the presence of *L. monocytogenes* in food products (Matle et al., 2019; Smith et al., 2019; Thomas et al., 2020). Matle et al. (2019, 2020) conducted a national survey to determine the occurrence and population structure of *L. monocytogenes* strains in meat and meat products isolated from retail, meat processing facilities and abattoirs in SA. Although, this study provides crucial information on meat contamination with *L. monocytogenes*, further investigations are still required to determine the hyper-virulent strains, antibacterial resistance genes, stress tolerance capabilities of *L. monocytogenes* in SA food products. Thus, the objectives of this study were to: (1) use core genome-SNP analysis to determine the genetic relatedness of the most common *L. monocytogenes* strains in SA; (2) assess the genetic basis of the resistance, stress tolerance, genomic localization of the resistance genes in *L. monocytogenes* isolated from food products in SA; and (3) identify key genomic features contributing to virulence potential of *L. monocytogenes* strains in the host.

MATERIALS AND METHODS

Isolates Selection, Genome Assembly, and Annotation

A subset of 152 isolates were selected from a total of 217 isolates from our previous study (Matle et al., 2020). The isolates were selected based on quality of the raw reads and *de novo* assembly in order to avoid false prediction of genes of interest in the present study. Briefly, the raw read quality was assessed with FastQC v.0.11.9 (Andrews, 2010) and the adapters and low-quality reads were trimmed using Trimmomatic v.0.39 (Bolger et al., 2014). SPAdes v.3.13.1 program (Bankevich et al., 2012) was used to create *de novo* assembly of each isolate. The resulting genome assembly were further quality assessed with QUAST v.5.0.2 (Gurevich et al., 2013) and annotated using Prokka v.1.13.7 (Seemann, 2014).

About nine isolates showed poor *de novo* assembly statistics and they were only included in the MLST analysis and subsequently removed from further statistical analysis (**Supplementary Table 1**). The large scale MLST analysis of *L. monocytogenes* isolates including the isolates of the present study were published by Matle et al. (2020). The cgMLST analysis was also performed using chewBBACA v.3.0 (Silva et al., 2018) only on the isolates used in the present study (a subset of 217 isolates). The cgMLST typing was run with an external schema adapted from BIGSdb-*Lm* platformhttps://bigsdb.pasteur.fr/listeria[1] (Jolley and Maiden, 2010; Moura et al., 2016). The allele calling on the target genomes were performed with chewBBACA Allele Calling algorithm using the *Listeria_monocytogenes*.trn training file based on the reference strain *L. monocytogenes* EGD-e (acc. No. NC003210). The cgMLST results of these isolates were included as **Supplementary Figures 1**, **2**.

[1]https://bigsdb.pasteur.fr/listeria

Core Genome Single-Nucleotide Polymorphism

A reference-based variant calling analysis was performed using the Snippy v.2.6[2]. The annotated genomes were mapped against the complete reference genome of *L. monocytogenes* EGD-e (acc. No. NC003210) with the Burrows-Wheeler Aligner (BWA) v.0.7.12 using default settings (Li and Durbin, 2009). After mapping, the average depths were determined with SAMtools v.1.3 (Li et al., 2009). The variants were called using Freebayes v.0.9.20 (Garrison and Marth, 2012) with the following parameters: minimum base quality of 20, minimum read coverage of 10X, and 90% read concordance at a locus for a variant to be reported. A calling of core genome single nucleotide polymorphisms (SNPs) was produced in Snippy v2.5 to infer a high-resolution phylogeny using Fasttree v.2.1.10 (Price et al., 2010). The total number of SNPs from both inside and outside recombination events were determined with Gubbins (Croucher et al., 2014) using the core alignment file produced by Snippy v2.5.

Prediction of Virulence Factors, Antimicrobial Resistance, and Stress-Related Genes

Genome assemblies were screened for the presence/absence of genes rendering resistance to antimicrobials, biocides, and heavy metals; and also stress tolerance genes and virulence factors as well as biofilm formation associated genes. ABRicate v0.8.10 was used for this screening with the minimum identity and coverage cut-offs values set by default settings. All alleles for stress tolerance, virulence factors and resistance genes were retrieved from the *Listeria* database hosted by the Pasteur Institute, Paris, France[1]. The biofilm formation associated genes were also retrieved from NCBI (**Supplementary Table 3**). Other databases used for analyses of virulence factors and resistance genes with ABRicate v0.8.10 were CARD v2.0.3 (Jia et al., 2017), BacMet database (Pal et al., 2014) and Virulence Factor database (VFDB) (Chen et al., 2016). Virulence factors and resistance genes identified by ABRicate v0.8.10 were validated by blastn v.2.10.0^{+}.

Plasmid Reconstruction

Plasmids of the *L. monocytogenes* strains were *de novo* predicted using MOB-suite software (Robertson and Nash, 2018). The MOB-recon algorithm was used to identify plasmid contigs from the draft genomic assemblies. The BLAST-based MOB-recon tool uses markers from sequence databases of known replicons and relaxases in conjunction with a reference database of clustered plasmids provided by MOB-suit software. Finally, the PLSDB web-resource (Galata et al., 2019), a comprehensive large-scale database comprising 13,789 (November 2018) complete sequences of bacterial plasmid, was used for a large-scale comparative analysis to retrieve plasmid records similar to the herein assembled plasmids. The PLSDB database was interrogated using ABRicate v0.8.10[3] with minimum identity and coverage cut-offs values set by default settings.

Prediction of Prophages

In order to identify putative prophages, genome assemblies were searched by the PHASTER (PHAge Search Tool–Enhanced Release) server (Arndt et al., 2016). This application scores prophage regions as "intact," "questionable," or "incomplete" based on several criteria such as the number of CDSs homologous to certain phages and the percentages of CDSs that match a certain phage. Intact and questionable regions with sequence lengths over 20 kbp were used for the prophage profiling.

Statistical Validation

Statistical validation of the results was performed using R v.3.6.0[4] Distribution and association testing were done using Chi-Square tests and over-representation was indicated by a Pearson residual value larger than 2. Additional analysis was done using in-house python scripts.

RESULTS

The Core-SNP Phylogenetic Clustering of the Most Common *L. monocytogenes* STs in SA

To investigate the genetic relatedness of the most common *L. monocytogenes* strains in SA, the isolates were mapped against the *L. monocytogenes* EGD-e reference genome and aligned, generating an alignment with core SNPS and a phylogenetic tree. The core-SNP analysis showed that the most frequent ST204 was grouped in three distinct clusters with SNP difference ranging up to 41 SNPs in the core parts of the genomes of these strains (**Figure 1**). Moreover, the ST1 and ST2 were grouped in two distinct clusters with SNP difference ranging up to 27 and 34 SNPs, respectively (**Figure 1** and **Supplementary Table 2**). These results indicate that SA *L. monocytogenes* isolates belonging to ST1, ST2, and ST204 were generally paraphyletic mixes of diverse genetic variants. Contrary, the strains belonging to ST321 were highly monophyletic and showed maximum two SNPs core genome difference between these isolates (**Figure 1** and **Supplementary Table 2**). Another observation from these results was that ST clustering did not follow the specific isolation sources. In general, the core-SNP phylogenetic tree displayed a good congruence to the cgMLST phylogenetic tree as it is demonstrated in **Figure 2**. Discrepancies between trees in many cases could be resolved by reordering of the clusters without influencing topologies of the trees.

[2]https://github.com/tseemann/snippy

[3]https://github.com/tseemann/ABRicate

[4]https://www.R-project.org/

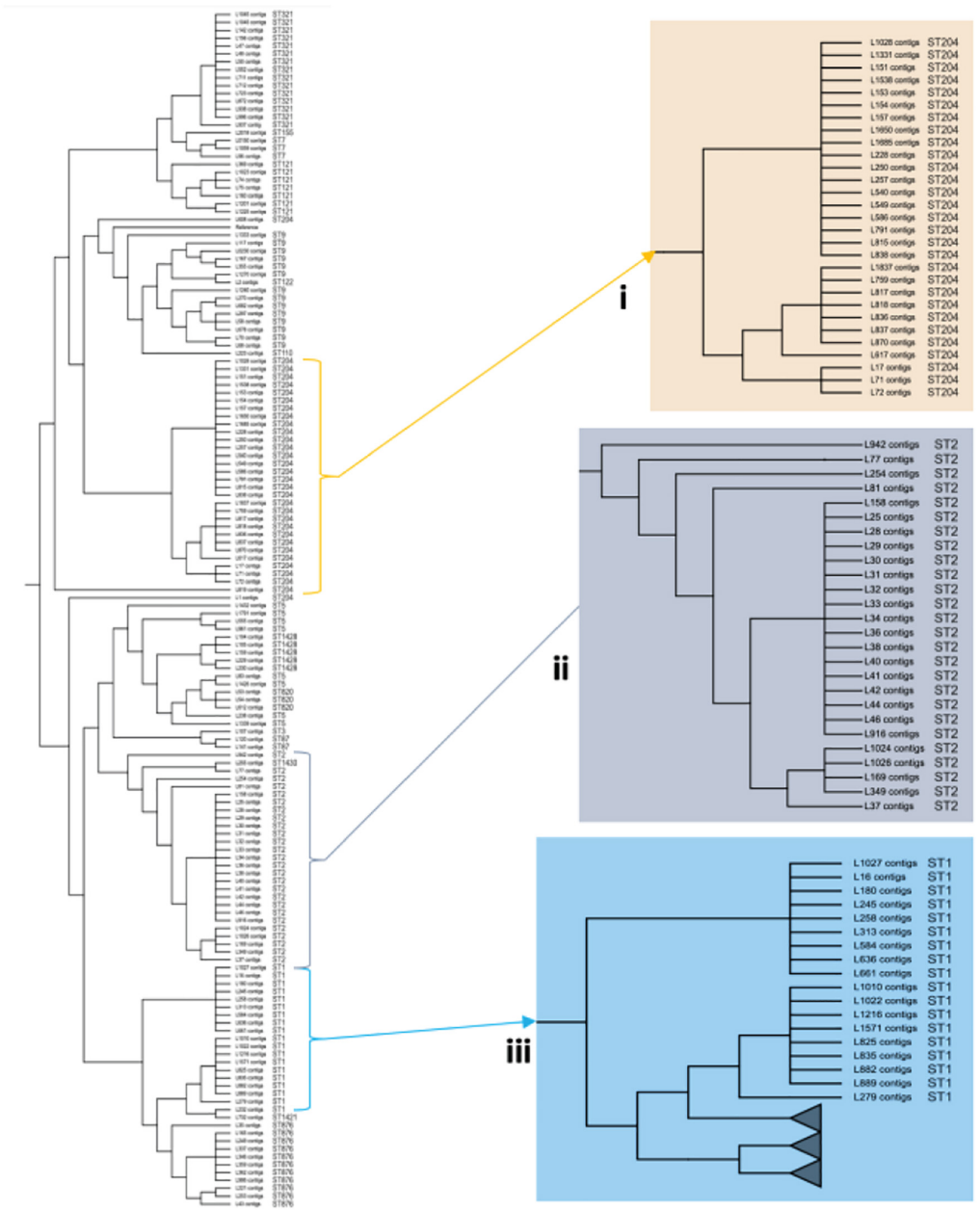

FIGURE 1 | Core-SNP phylogeny showing genetic relatedness of the *L. monocytogenes* strains in SA. **(i)** A section pruned from the original tree showing the South African genetically related ST204 strains. **(ii)** A section pruned from the original tree showing the South African genetically related ST2 strains. **(iii)** A section pruned from the original tree showing the South African genetically related ST1 strains.

Antimicrobial Resistance and Biofilm Formation Genes

The antimicrobial resistance genes were identified in all the isolates of *L. monocytogenes*. These genes include *fosX*, *lin*, *norB*, and *mprF* which confer resistance, respectively, to fosfomycin, lincosamides, quinolones and cationic peptides that disrupt the cell membrane such as defensins (**Figure 3**). Genes *tetM* and *tetS* that confer resistance to tetracycline were infrequent among isolates. The *tetM* was found only in ST2 and ST9 belonging to serogroups IIb and IVb of lineage I. The *tetS* was observed only in one isolate belonging to ST2 from serogroup IVb of lineage II. Tetracycline resistance genes *tetM* and *tetS* were detected in isolates originated from beef and poultry meat samples obtained from retail and butchery (**Figure 3**).

Biofilm formation associated genes including *inlL, prfA, actA, lmo0673, bapL, recO, lmo2504,* and *luxS* which play a significant role in survival and persistence of *L. monocytogenes* were analyzed and detected in ($n = 72$, 47%; $n = 149$, 98%; $n = 72$,

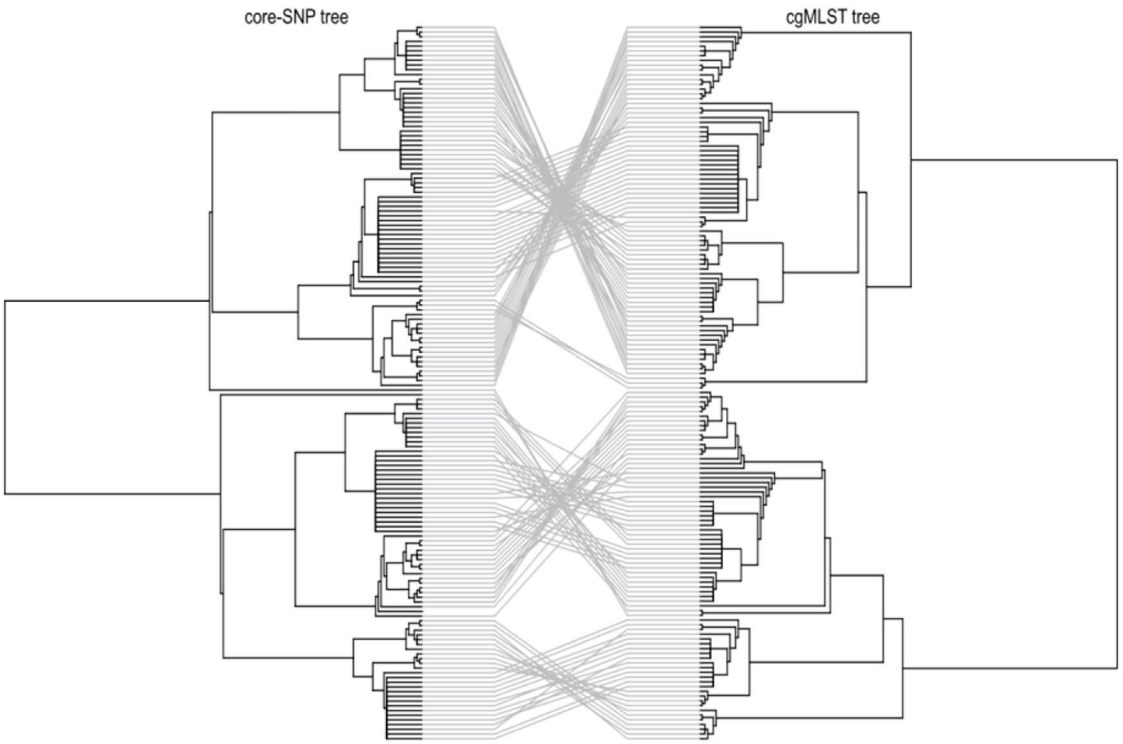

FIGURE 2 | Tanglegram comparison between core-genome SNP (Left) and cgMLST (Right) linking tips with the same label to each of the 152 *L. monocytogenes* isolates.

47%, n = 78, 51%; n = 6, 3.9%; n = 82, 53%; n = 130, 86%; and n = 145, 95%) of the isolates, respectively (**Supplementary Table 3**). The *L. monocytogenes* strains which harbored majority of these genes except for *lmo0673* and *bapL* genes were ST204 and ST321 both belonging to serogroup lla. The well-known ST1 and ST2 isolates which are associated with clinical human listeriosis appear to have less overall biofilm formation associated genes and were also missing the *actA* gene, an important biofilm formation gene. More than 90% of these isolates harbored *prfA, lmo2504,* and *luxS genes.* However, none of the isolates harbored all 8 genes associated with biofilm formation (**Supplementary Table 3**). Interestingly, *bapL* gene was only specific for ST121 which also harbored most of these genes, but also lacked *actA* gene in the sequenced genomes.

Benzalkonium Chloride Resistance and Stress Tolerance Genes

The chromosome-borne BC resistance genes including *mdrL* and *Ide*, which are the major facilitator superfamily efflux pumps of *L. monocytogenes* conferring resistance to BC were present in (n = 143, 100%) and (n = 124, 86.7%) of the isolates, respectively (**Figure 3**). In many cases, these genes were found in chromosomal inserts of the plasmid-borne BC resistance *bcrABC* cassette (n = 55, 38%). The presence of the *bcrABC* cassette was characteristic for ST204 and ST321 all belonging to serogroup IIa of lineage II ($p < 0.05$). Another plasmid-borne BC resistance *ermC* gene was present in (n = 58, 40%) of the isolates and was over-represented in ST321 belonging to serogroup IIa of lineage II ($p < 0.05$; **Figure 3**). No specific over-representation of *Ide*, *bcrABC* cassette and *ermC* was observed in isolates from beef or poultry meat samples ($p > 0.05$). However, the *bcrABC* cassette and *ermC* were significantly over-represented in the isolates from butchery and retail samples ($p < 0.05$; **Figure 3**).

The stress survival islets (SSI-1 and SSI2), which are known to be responsible for proliferation of *L. monocytogenes* under stressful conditions in food processing facilities, were present in (n = 86, 55%) and (n = 11, 7.7%) of the isolates, respectively. The SSI-1 was found to be significantly over-represented in ST9, ST204 and ST321 belonging to serogroups IIa and IIc of lineage II ($p < 0.05$; **Figure 3**). The SSI-2 was found to be significantly over-represented in ST121 belonging to serogroup IIa of lineage II ($p < 0.05$; **Figure 3**). Islets SSI-2 were over-represented with $p < 0.05$ in the isolates obtained from meat samples from meat processing plants and cold stores in contrast to the distribution of islets SSI-1 showing nor statistically reliable preferences regarding different sources of isolation of *L. monocytogenes* (**Figure 3**).

The Assessment of Virulence Factor Genotypes Across Different Serogroups, STs and Isolation Sources

A total of 68 putative virulence factors were present across the *L. monocytogenes* isolates. The presence and integrity of *Listeria* pathogenicity islands LIPI-1, LIPI-2, LIPI-3, and LIPI-4 were

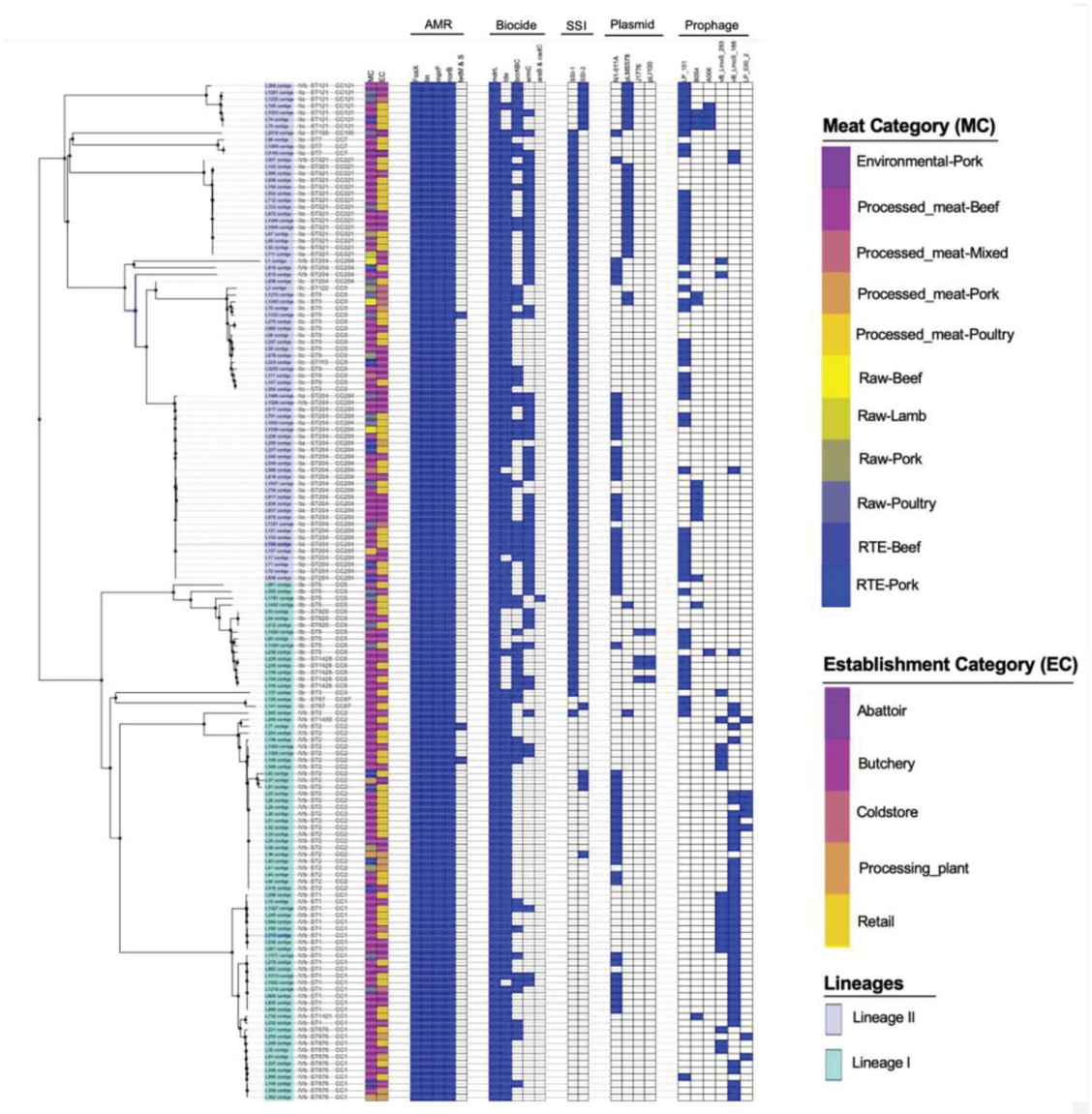

FIGURE 3 | Core-genome MLST phylogenetic grouping of AMR, stress tolerance, biocide resistance genes, plasmids and prophages across the 152 *L. monocytogenes* isolates. The heat map shows the presence (blue) or absence (white) of genes involved in *L. monocytogenes* resistance and virulence. The isolation source MC and EC labeling on the heat map indicate the Meat category and Establishment category, respectively.

investigated in our previous published study (Matle et al., 2020) and the *Listeria* pathogenicity islands results for the present isolates were included as **Supplementary Figure 3**. The internalin gene family members including *inlABCEFJK* were present in more than 90% of the isolates. The *inlD* and *inlG* were present in 88 and 47% of the isolates, but absent in ST9 and ST1 which were part of the most abundant ST identified (**Figure 4**). Other important virulence factors detected in genomes of *L. monocytogenes* isolates include adherence virulence factors such as *ami, fbpA, lap,* and *lapB*, which were present in 54.6, 98.68, 91, and 98% of the isolates; invasion virulence factors *aut*, *gtcA*, *lpeA* and *vip*, which were present in 97, 43, 95, and 72% of the isolates; as well as intracellular survival factors *lplA1*, *prsA2* and *svpA*, which were present in 98, 98.6, and 98.6%, respectively. The *ami*, *gtcA* and *vip* genes were over-represented, respectively in ST204, and ST321; ST1, ST2, ST876; ST1, ST2, ST9, and ST876 ($p < 0.05$; **Figure 4**).

The Distribution of *L. monocytogenes* Plasmids Between Different Serogroups, STs and Isolation Sources

A total of four unique plasmids that contribute to the resistance of *L. monocytogenes* to antibiotics were identified in 71% of the tested isolates. Of the four unique plasmids, the most frequent was plasmid N1-011A (n = 52, 36.34%), followed by plasmids

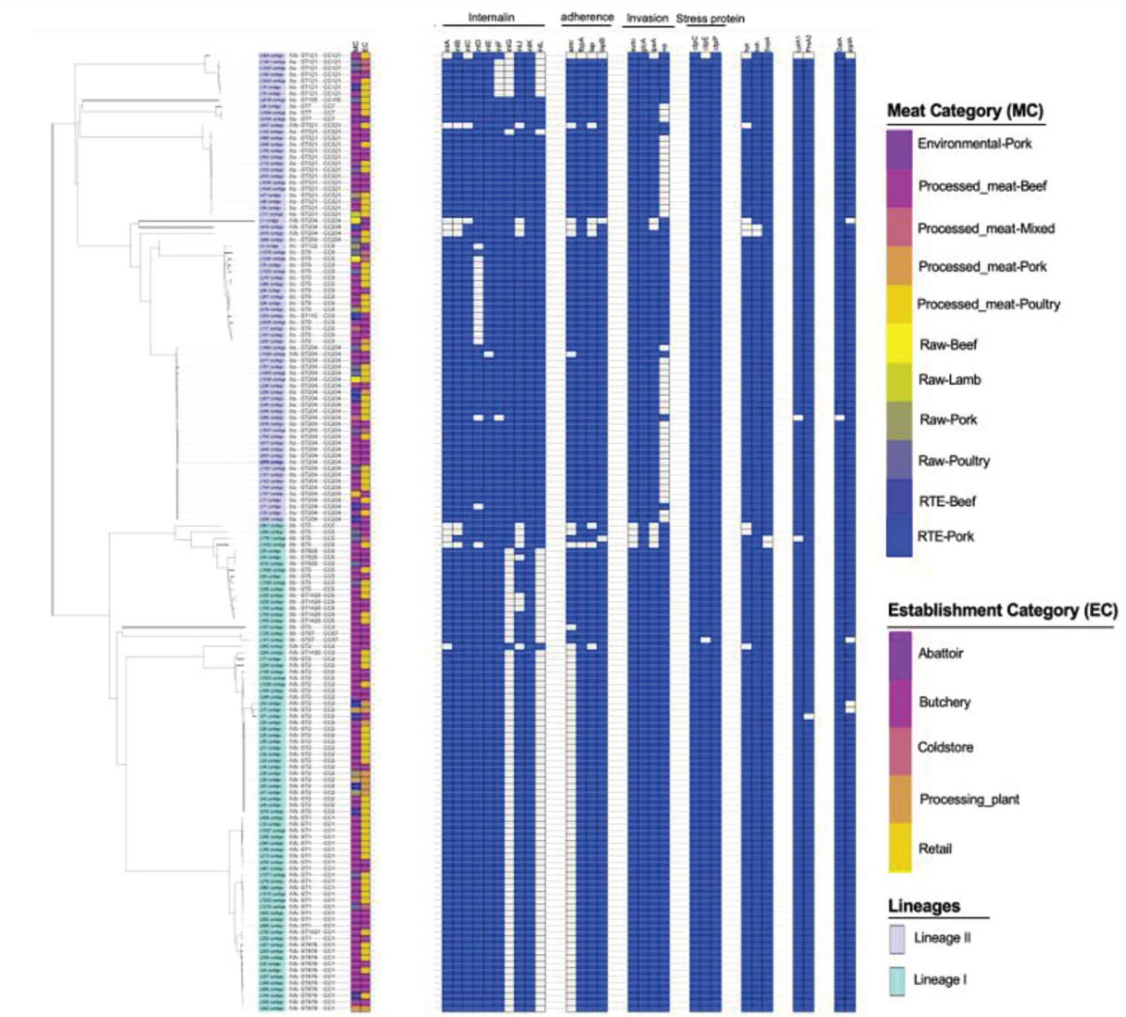

FIGURE 4 | Core-genome MLST phylogenetic grouping of putative virulence factors across the 152 *L. monocytogenes* isolates. The heat map shows the presence (blue) or absence (white) of genes involved in *L. monocytogenes* virulence. The isolation source MC and EC labeling on the heat map indicate the Meat category and Establishment category, respectively.

J1776 (n = 28, 19.6%), pLM5578 (n = 25, 16.8%), and pLI100 (n = 4, 2.6%) across all the study isolates (**Figure 3**). Plasmid N1-011A was significantly over-represented in ST2 belonging to serogroup IVb of lineage I, and was also over-represented in ST204 belonging to serogroup IIa of lineage II ($p < 0.05$). Plasmid J1776 was over-represented in ST2 belonging to serogroup IVb of lineage I, and was also over-represented in ST9 belonging to serogroup IIc of lineage II ($p < 0.05$). Plasmid pLM5578 was over-represented in ST121 and ST321 belonging to serogroup IIa of lineage II ($p < 0.05$). Plasmid pLI100 was observed only in four isolates belonging to ST1428 of serogroup IIb from lineage I, which also contained plasmid J1776 (**Figure 3**). The significant association of the plasmids with different isolation sources showed that for the meat category: plasmids N1-011A, J1776, and pLM5578 showed no statistically reliable association with the source of isolation of the pathogen ($p > 0.05$). Contrary, as to the establishment categories: plasmids J1776 and pLM5578 showed a significant association with the retail and butchery category ($p < 0.05$). However, that was not the case with plasmid N1-011A showing no significant associations with either source or establishment categories ($p > 0.05$; **Figure 3**).

Prophage (φ) Profiles of *L. monocytogenes* Isolates

Prophage (φ) profiles of the *L. monocytogenes* genomes sequenced in this study were determined using the PHASTER tool for identification and annotation of putative prophage sequences. A total of nine different intact, questionable, or incomplete prophages regions were found across different *L. monocytogenes* isolates (**Figure 3**). The intact prophage LP_101 [NC_024387] (n = 53, 37%) was the most prevalent followed by vB LmoS 188 [NC_028871] (n = 45, 31.46%), vB_LmoS_293 [NC_028929] (n = 18, 12.58%), and B054 [NC_009813] (n = 14,

9%). The LP_101 phage was over-represented in ST121, ST204, and ST321 belonging serogroups IIa of lineage II and also in ST9 belonging to serogroup IIc of lineage II ($p < 0.05$). Phage vB LmoS 188 was over-represented in ST1 and ST2 belonging to serogroup IVb of lineage I ($p < 0.05$). Phage vB_LmoS_293 was over-represented in ST1 belonging to serogroup IVb of lineage I ($p < 0.05$). Phage B054 was over-represented in ST204 belonging to serogroup IIa of lineage II ($p < 0.05$). The significant association of the prophages with different isolation sources showed that for the meat category showed no statistically reliable association with the source of isolation of the pathogen or establishment categories ($p > 0.05$).

DISCUSSION

The application of the MLST based approach provided important information on the distribution and grouping of genetically related *L. monocytogenes* strains in the SA food processing environment. In the present study, a total of four serogroups represented by 19 STs belonging to 11 different CCs which are a group of closely related STs were identified and classified to lineage I and lineage II using the cgMLST analysis (**Supplementary Figures 1**, **2**; Matle et al., 2020). The current study revealed that the most prevalent serogroups among SA isolates were IVb and IIa, which have also been found to be over-represented in food sources in other countries and were causative agents of more than 80% of global *L. monocytogenes* infections in human (O'Connor et al., 2010; Jamali et al., 2015; Lee et al., 2018). The most prevalent STs were ST204 and ST321 belonging to lineage II, which were mainly found in foods and food processing environments. Other common isolates were ST1 and ST2 belonging to lineage I, which are highly associated with clinical human listeriosis and demonstrate an enhanced pathogenetic potential (Maury et al., 2019; Matle et al., 2020; Palma et al., 2020). The *L. monocytogenes* strains and variants reported in the present study have been shown to be globally distributed and able to survive and persist for months and even years in food-processing environments and to be kept in contaminating food products in food processing environments for long time (Knudsen et al., 2017; Harrand et al., 2020; Matle et al., 2020).

The current study showed a paraphyletic variability of isolates ST1, ST2, and ST204, which differed by up to 41 SNPs in their core genome sequences contrasting them from ST321 isolates, which showed a significant level of conservation of their core genome with not more than two SNPs difference between them. It shows that ST1, ST2, and ST204 variants potentially are more dangerous in generating unusual genetic variants of the pathogen causing disease outbreaks. A study by Li et al. (2017) also reported a significant genetic variability of different *L. monocytogenes* isolates from foods demonstrated by SNP calling. Grouping of isolates by their core-SNP displayed a good congruence with cgMLST clustering; however, it should be noted that the strains grouped into clusters by these two methods still may show quite different pathogenicity potentials due to absent or present of different resistance and virulence genes located within chromosomes, plasmids and prophages (Li et al., 2017; Blanc et al., 2020).

Recent studies on antimicrobial resistance of *L. monocytogenes* have typically reported low levels of antimicrobial resistance in isolates from the food production environments. These reports were based on several studies performed in SA, Europe and Asia (Li et al., 2016; Matle et al., 2019; Wilson et al., 2018). The present study has reported that various antibiotic resistance genes, including *fosX*, *lin*, *mprF*, *norB*, and *mgrA*, were present in all the isolates including the strains from food processing environments. This global trend to a wider distribution of the antibiotic resistant genes in *L. monocytogenes* population was reported in a recent publication by Wilson et al. (2018). The repertoire of resistance genes typical for *L. monocytogenes* is enriching other genes, particularly by tetracycline resistance genes *tetM* and *tetS* found in a few isolates belonging to ST2 and ST9 of serogroups IVb and llb, which were isolated from butchery and retail. These genes have been detected previously in *L. monocytogenes* strains isolated from food and environmental samples (Escolar et al., 2017; Olaimat et al., 2018). Although, tetracycline is believed to be the most frequent resistance trait in *L. monocytogenes* isolated from human and food processing environments, the present study found tetracycline resistance genes only in few *L. monocytogenes* isolates, which most likely were acquired by *L. monocytogenes* with conjugative plasmids and transposons originating from *Enterococcus* or *Streptococcus* as result of horizontal gene transfer (Olaimat et al., 2018).

The key factors of adaptation and survival of *L. monocytogenes* in the food processing environments is the ability to develop resistance to QACs, such as BC, through the activity of efflux pumps encoded by *qacH* and genes of the *brcABC* cassette (Horlbog et al., 2018) and biofilm formation. The present study identified several chromosome-borne BC resistance genes, *mdrl* and *ide*, that confer tolerance to BCs in all the isolates. A study by Conficoni et al. (2016) also reported the presence of *mdrl* and *ide* in isolates from meat-processing environment that agrees with the present study. Several other genes, such as *ermC emrE, qacH,* and *bcrABC* cassette, also are responsible for tolerance to BC, a very common compound of sanitizers which is used in food industry (Kovacevic et al., 2016; Muhterem-Uyar et al., 2018; Kurpas et al., 2020). The present study identified *bcrABC* cassettes in 38% of isolates and the plasmid-borne *ermC* gene in 40% of the isolates belonging to serogroup IIa (ST121, ST204, and ST321) of lineage II, which suggests that these isolates are well adapted to survival in the food-processing environment where QACs are commonly used as sanitizers. Indeed, it was shown in the current study that these genes were over-represented in retail and butchery. Identification of drug resistance genes performed in this study may not be comprehensive due to inability to complete whole genome sequences of the isolates. Particularly, several well-known *Listeria* resistance genes such as *emrE* (Kovacevic et al., 2016) and *qacH* carried with Tn6188 (Horlbog et al., 2018) were not found when the sequences were searched against the BacMet database. Additionally, nucleotide sequences of these genes were obtained from the database of *Listeria* genes hosted at http://bigsdb.pasteur.fr/listeria/ and

blasted against the assembled contigs of the *Listeria* isolates. This search didn't retrieve any significant matches. Either these genes were absent in the sequenced genomes, or they were fragmented in the contigs sequences. The SSI-1, which has been linked to tolerance toward acidic, bile, gastric, and salt stresses, was present in 55% of the isolates and was found to be significantly over-represented in ST9, ST204, and ST321 belonging to IIa serogroup of lineage II ($p < 0.05$). The SSI-2, which is responsible for survival under alkaline and oxidative stresses (Harter et al., 2017), was found to be significantly over-represented in ST121 from lineage II isolated from processing plant and cold store categories ($p < 0.05$). These results corroborate with a previous study (Hurley et al., 2019) showed that SSI-2 was only found in ST121, whereas SSI-1 was distributed in various STs from both lineages I and II. Co-occurrence of BC resistance genes with the stress response genes revealed by the current study implies a serious hygiene management concern. The only available data with regard to the resistance of *L. monocytogenes* to disinfectants applied in food production environments refer to genotypic resistance to QACs. Dilution in the environment and biodegradation result in QAC concentration gradients and as a result, the microorganisms are frequently exposed to sub inhibitory concentrations of QACs. The low-level resistance to QACs in *L. monocytogenes* may contribute to its environmental adaptation and persistence (Martínez-Suárez et al., 2016). Therefore, a need exists to evaluate the use of QACs disinfectants groups and the occurrences of resistance in food production facilities in SA and worldwide. Moreover, the present study also showed that ST204 and ST321 appear to have high ability of biofilm formation capacity which contribute to *L. monocytogenes* adaptation and survival in food processing environment. These results corroborate with a previous study (Pasquali et al., 2018; Stoller et al., 2019) showed that these strains have high biofilm forming capacity under specific environmental conditions. Pasquali et al. (2018) showed that the biofilm formation associated *actA* gene was truncated in all ST121 isolates. Similar trend was observed in the present study were *actA* gene was not detected in all the ST121, ST1, and ST2 isolates. This *actA* gene is known to be responsible for polymerization of actin which is important for motility of *L. monocytogenes* within the host cell as well as in the first steps of biofilm formation (Travier et al., 2013; Pasquali et al., 2018).

The pathogenic potential of a given *L. monocytogenes* strains is determined by the functionality of a large number of genes known as "virulence factors," all of which have different roles at various stages of the infection cycle. The present study assessed for the presence of 115 putative virulence markers that could be used to predict the level of potential virulence of *L. monocytogenes* isolates. It was suggested to classify isolates of this species as putatively hypo-virulent, with unknown virulence potential, and putatively hyper-virulent (Hurley et al., 2019). A total of 68 virulence markers were identified across the isolates suggesting that most virulence markers are ubiquitous across *L. monocytogenes* strains in SA. Intact LIPI-1, which harbor Prf-A dependent virulence cluster genes that are critical in the infectious cycle of *L. monocytogenes*, was mostly presented in ST1 and ST876 isolates from serogroup IVb belonging to lineage I, and also in ST9, ST204, and ST321 from serogroups IIa and IIc belonging to lineage II (**Supplementary Figure 3**; Matle et al., 2020). LIPI-1 has been reported to be the first identified pathogenicity island in *L. monocytogenes* distributed across different *L. monocytogenes* strains (Chen et al., 2020). In the present study, the LIPI-3, which is associated with enhancing the virulence capabilities of *L. monocytogenes,* was found ubiquitous in ST1 from serogroup IVb belonging to lineage I, but was also present in 2 isolates from lineage II belonging to ST204 (**Supplementary Figure 3**; Matle et al., 2020). The LIPI-3 Island carries a gene encoding the hemolytic and cytotoxic factor known as listeriolysin S, which contributes to the intracellular survival of *L. monocytogenes* in human polymorphonuclear neutrophils (Clayton et al., 2011; Hurley et al., 2019). Painset et al. (2019) and Chen et al. (2020) reported similar findings and revealed that LIPI-3 is ubiquitous to lineage I, which was also observed in the present study. Hyper-virulent strains have also been shown to possess the recently described pathogenicity island LIPI-4 that confers hyper-virulence by enhancing the invasion of the CNS and placenta (Grad and Fortune, 2016; Maury et al., 2016). The LIPI-4 Island was identified in the present study in 3.2% of the isolates belonging to serogroup IIb and IVb (ST2 and ST87) from lineage I (**Supplementary Figure 3**; Matle et al., 2020). While isolates of ST1, ST2, ST204, and ST321 generally were characterized with an abundance of virulence genes. However, the known adhesion and invasion related genes, *aut, inlF, inlJ,* and *vip*, were not found in genomes of these microorganisms which suggests a possible limitation of the invasiveness and virulence of this *L. monocytogenes* strains (Lindén et al., 2008; Martins et al., 2012). The *inlA gene* was found in more than 90% of the isolates in the current study. A recently published work on the same *L. monocytogenes* isolates revealed the truncation of the gene *inlA* due to premature stop codon, which has been associated with reduced invasiveness in some *L. monocytogenes* strains (Matle et al., 2020). This mutation may serve as a marker of hypo-virulence. Analysis of translated *inlA* protein sequence from isolates in this study identified 18 isolates, all from ST121 and ST321 of lineage II having this mutation reported for the first time for SA isolates (Matle et al., 2020).

This study suggested an important role of virulence plasmids of *L. monocytogenes* to confer increased tolerance to multiple stress condition in food processing environments. Blasting of nucleotide sequences of the found plasmids against NCBI database revealed homology of these plasmids at more than 90% similarity with the virulence plasmid N1011A, pLM5578, J1776, and pLI100 common for *L. monocytogenes* isolates (Palma et al., 2020). The majority of plasmids N1011A and pLM5578 isolates also carried *bcrABC* cassette suggesting a high correlation between the presence of these plasmids and BC tolerance in *L. monocytogenes* strains. Plasmid N1011A was associated with the most common isolates of serogroups IVb and IIa (ST204 and ST2), while pLM5578 was associated with serogroup IIa (ST121 and ST321) suggesting an importance of this plasmids in contribution to survival of hyper-virulent *L. monocytogenes* strains in the food processing environments (Kuenne et al., 2010). Furthermore, Kropac et al. (2019) showed that small

plasmid pLMST6 which harbor *emrC* gene confers increased BC tolerance in *L. monocytogenes*. Plasmid PLMST6 was not detected in the present study. In addition to the virulence plasmids, nine prophages were distributed across the *L. monocytogenes* isolates from different sources. Analysis of the genetic repertoire of these prophages suggested their possible involvement in virulence and resistance. ST1, ST2, ST204, and ST321 displayed the highest numbers of prophages per genomes. This shows that adaptation of *L. monocytogenes* to specific environmental niches in food processing industry and short-term evolution of both distantly and closely related *L. monocytogenes* strains have been linked to the diversification of these prophages (Harrand et al., 2020; Palma et al., 2020).

CONCLUSION

The findings of this study that was based on NGS sequencing of *L. monocytogenes* isolates revealed the overall contribution of plasmids, prophages chromosomal genes toward pathogenicity and adaptation to meat processing and storage environment. The study showed that ST1, ST2, ST121, ST204, and ST321 were the most frequent among isolates and well adapted to survive in food processing environments in SA. Several hyper-virulent strains were revealed among isolates belonging to ST1, ST2, and ST204, which could present a major public health risk due to their association with meat products and food processing environments in SA, whereas hypo-virulent isolates from both lineage I and II belonged to ST121 and ST321. The information provided in this study is important for enhancing our understanding of the adaptation and survival of this pathogen in the food-processing environments. Also, the obtained results will aid in developing new approaches to assess the virulence potential of *L. monocytogenes* isolates and the efficacy of using BC disinfectants in food-processing facilities in SA.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository, accession number: PRJNA720786.

ETHICS STATEMENT

Ethical approval was obtained from University of Pretoria, Faculty of Natural and Agricultural Sciences Research Ethics Committee (NAS324/2020). All methods in this study were approved by University of Pretoria, Faculty of Natural and Agricultural Sciences Research Ethics Committee, and performed in accordance with the relevant guidelines and regulations.

AUTHOR CONTRIBUTIONS

RP, IM, KM, and OR: conceptualization. OR and RP: supervision. TM: writing original draft preparation, methodology, bioinformatics, and statistical analysis. RP, OR, and IM: manuscript review and editing. IM and KM: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.669287/full#supplementary-material

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