Food Research International 192 (2024) 114798

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Microbiome signatures associated with flavor development differentiate Protected Designation of origin water Buffalo Mozzarella cheese from different production areas

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ARTICLE INFO

Keywords:
Cheese microbiome
Cheese metagenome
Cheese volatilome
Protected Designation of Origin
PDO cheese
Microbial *terroir*
Microbiome markers
Cheese traceability
Fraud protection

ABSTRACT

Water Buffalo Mozzarella (BM) is a typical cheese from Southern Italy with unique flavor profile and texture. It is produced following a traditional back-slopping procedure and received the Protected Designation of Origin (PDO) label. To better understand the link between the production area, the microbiome composition and the flavor profile of the products, we performed a multiomic characterization of PDO BM collected from 57 different dairies located in the two main PDO production area, i.e. Caserta (n = 35) and Salerno (n = 22). Thus, we assessed the microbiome by high-throughput shotgun metagenomic sequencing and the Volatile Organic Compounds (VOCs) by gas chromatography/mass spectrometry (GC/MS). *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subsp. *delbrueckii* were identified as the core microbiome present in all samples. However, the microbiome taxonomic profiles resulted in a clustering of the samples based on their geographical origin, also showing that BM from Caserta had a greater microbial diversity. Consistently, Caserta and Salerno samples also showed different VOC profiles. These results suggest that the microbiome and its specific metabolic activity are part of the *terroir* that shape BM specific features, linking this traditional product with the area of production, thus opening new clues for improving traceability and fraud protection of traditional products.

1. Introduction

Many foods produced in Europe have far historical roots and obtained the Protected Designation of Origin (PDO) EU label because of the strong link with their geographical area of production (EU Regulation 2024/1143). The PDO regulation is rooted in the concept of *terroir*, a French term encompassing the physical, chemical, and biological properties, as well as the traditional craftsmanship, involved in the manufacture of a product (Brunschwig et al., 1999). Even though the term *terroir* has mostly been associated with the wine industry, the scenario of cheese has been catching the interest of the researchers over the last 20 years due to the social heritage related to *terroir* (Barham, 2003). Indeed, the PDO cheeses sector stands as a pivotal component of the EU economic landscape, characterized by a rich diversity of PDO labelled cheeses. These cheeses significantly contribute to the economic prosperity of local communities and of the broader European market, thanks to the presence of trade agreements and consumers awareness on product authenticity (Galli, 2024). This economic prominence makes PDO cheeses susceptible to fraudulent practices, including mislabeling (Cunha et al., 2016). Water Buffalo Mozzarella (BM) PDO cheese is a typical dairy product made from the river buffalo (*Bubalus bubalis bubalis*) milk (Minervino et al., 2020) and produced in Central and Southern Italy. Briefly, PDO BM is a fresh "*pasta-filata*" cheese produced employing a traditional back-slopping procedure. Previous works already focused on the microbiota composition along the manufacturing process (Coppola et al., 1990; De Filippis et al., 2014; Ercolini et al., 2004; Ercolini et al., 2012). According to the disciplinary of production, only whole milk from river Buffalo raised with very specific breeding techniques in the areas reported in Supplementray Table S1 is allowed for PDO BM production. The milk can be used fresh or pasteurized, and

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https://doi.org/10.1016/j.foodres.2024.114798
Received 9 June 2024; Received in revised form 16 July 2024; Accepted 17 July 2024
Available online 25 July 2024

then acidified by adding Natural Whey Culture (NWC) resulting from the previous production as starter (i.e., back-slopping) in a 5 h-long curd fermentation. The Consortium for the Protection of PDO Buffalo Mozzarella was born in 1981 and acknowledged as the only organization for the safeguarding of the PDO BM by the Italian Department for Agriculture, Food and Forestry. Although BM is now produced worldwide, only BM produced within a specific area in Southern Italy can boast of the PDO label (https://www.mozzarelladop.it/consorzio-tutela). The full list of provinces and cities within Campania, Lazio, Puglia, and Molise Italian regions recognized by the official PDO disciplinary (https://www.mozzarelladop.it/storage/pdf/disciplinare_mozzarella_2008.pdf) are summarized in Supplementray Table S1. Notably, approximately 86 % of the production occurs in the area covered by the provinces of Caserta and Salerno in Campania region, as depicted in Fig. 1, which summarizes the location of PDO producers from the available online list (https://www.mozzarelladop.it/members). Even though the microbial consortia involved in PDO BM fermentation have been widely characterized (Ercolini et al., 2004; Ercolini et al., 2012; Levante et al., 2023), only few studies attempted to explore the microbial diversity associated with the different area of production (Mauriello et al., 2003). BM microbiome has a key role in developing several volatile compounds responsible for aroma (Afshari et al., 2020; Coppola et al., 1990). Therefore, exploring the correlations between microbiome and volatilome that define the *terroir* of PDO BM is a crucial point to understand how these interactions can shape cheese quality, as well as if specific microbiome and/or volatilome traits can be used to differentiate the production area. Indeed, VOCs profiles were successfully used to discriminate the country of origin of PDO and non-PDO Emmental cheeses (Pillonel et al., 2003), as well as PDO and non-PDO BM cheeses (Salzano et al., 2020). Also, microbiome has been demonstrated to be an integral part of the *terroir* of fermented foods, such as PDO wines (Nanetti et al., 2023) and kimchi (D.-Y. Lee et al., 2023). As we delve into the intricate world of EU PDO cheeses and their susceptibility to fraud or mislabeling, it becomes necessary to exploit novel scientific approaches to develop tools for geographical origin tracing. This aligns not only with the economic needs but also with the preservation of food cultural heritage and the maintenance of consumers' trust. In this context, the main objective of our work was to understand the roles of microbiome as part of the *terroir* that links PDO BM with the specific area of production and its volatilome. In order to investigate molecular signatures that can be used to track PDO BM geographical origin, we carried out a multiomic approach combining shotgun metagenomic sequencing and volatilomics.

2. Material and methods

2.1. Samples collection

All BM samples were provided by producers registered in the PDO consortium, and thus produced following the official disciplinary (https://www.mozzarelladop.it/members). We focused on producers located in Campania Region (Southern Italy), in the provinces of Caserta (n = 35) and Salerno (n = 22), covering 86 % of producers registered in the PDO BM consortium (Fig. 1). Every dairy was visited once during the period from January to February 2020. For each producer, two replicate cheese samples were collected. Thus, the samples were transported refrigerated to the laboratory, where they were promptly pre-processed as described below.

2.2. Microbial DNA extraction and metagenome sequencing

Replicate samples collected from the same producer were pooled, diluted 1:10 in Phosphate Buffered Saline (PBS, pH 7.4) buffer, and homogenized in a stomacher (Stomacher400 circulator; Seward Medical, London, United Kingdom) for 1 min at 230 g. The resulting cheese homogenate was transferred in a 50 mL conical sterile tube and centrifuged for 1 min at 117 g to pellet debris. The superficial fat layer was removed and the middle phase containing microbial cells was transferred into a new tube and centrifuged for 15 min at 4,960 g to obtain the cell pellet. Thus, the supernatant was discarded, while the pellet was kept frozen at −80 °C until DNA extraction. Cell pellets were resuspended in 400 μL of PBS and vortexed. Then DNA extraction was carried out using the DNeasy PowerFood Microbial Kit (QIAGEN) according to manufacturer's instructions. The purified DNA was then quantified using the Qubit fluorometer (dsDNA HS Assay Kit, Invitrogen). Shotgun metagenome sequencing was performed on an Illumina NovaSeq platform, leading to 2 × 150 bp reads.

2.3. Extraction and analysis of volatile organic compounds

The extraction of volatile organic compounds (VOCs) was performed from 48 samples (Caserta n = 29; Salerno n = 19) by Solid Phase Micro Extraction (SPME, (J.-H. Lee et al., 2003). Briefly, BM sample (stored at −80 °C) was finely grated, and 25 g were placed in a 100 mL glass bottle with a magnetic stirring bar. Then, 6.25 g of sodium phosphate (NaH_2PO_4; Sigma-Aldrich), 25 mL of distilled water and 50 μL of 2-methyl-3-heptanone (purity 99 %, Sigma-Aldrich, St. Louis, MO, USA), used as an internal standard (220 ppm, in water solution), were added.

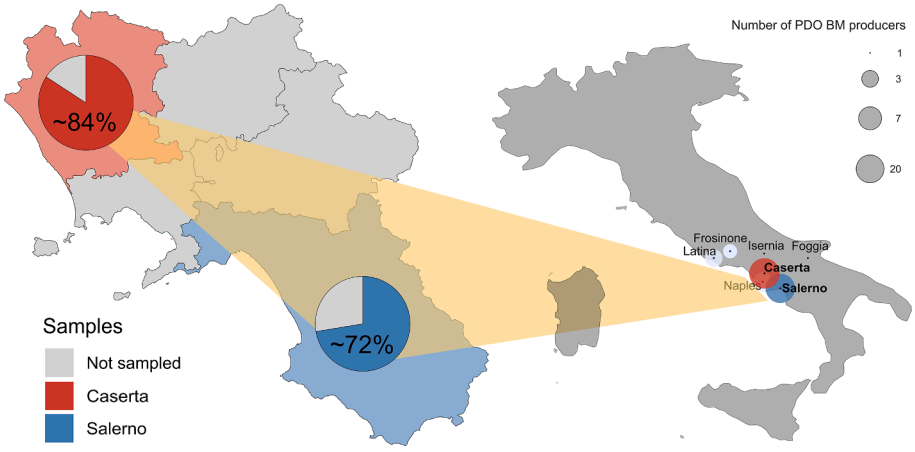

Fig. 1. Distribution of PDO BM producers in Italy. Bubble map plot showing the number of PDO Buffalo Mozzarella (BM) producers. Bubble size is proportional to the number of producers for each province. Most of the dairies are located in Campania region, although the PDO consortium also includes producers from Lazio, Puglia, and Molise. In the zoom on Campania region, the proportion of PDO BM producers sampled in this study is shown as percentage on total PDO producers in those provinces.

The sample was initially conditioned at 50 °C for 10 min without stirring, followed by magnetic stirring (3 g) for 20 min at the same temperature. The VOCs adsorption was performed by introducing a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 2 cm fibre (Supelco, Bellefonte, PA) into the headspace of the bottle and exposing the polymer for 30 min at 40 °C while stirring. The VOCs were desorbed directly into the GC inlet port kept at 250 °C, 4:1 split ratio, for 10 min. Volatile compound analysis was performed on an Agilent 7890A GC System gas chromatograph coupled to an Agilent 5975C VL MSD with Triple-Axis-Detector mass spectrometer (Agilent Technologies, Inc., Palo Alto, CA, USA). GC was equipped with a Zebron ZB-WAX capillary column (60 m × 0.25 mm i.d. × 0.25; Phenomenex, USA). Helium served as carrier gas at a flow rate of 1 mL/min. The temperature program started at 40 °C for 10 min, followed by an increase of 5 °C/min until 240 °C, where it remained constant for 11 min (Balivo et al., 2023). Mass spectra were recorded at 70 eV. The source, quadrupole, and interface temperatures were set at 230 °C, 150 °C, and 250 °C, respectively. The identification of VOCs was performed by comparing retention times and mass spectra obtained by analysing pure reference compounds in the same conditions. All chemical standards were supplied by Sigma-Aldrich (St. Louis, MO, USA). The identification was confirmed by comparing mass spectra with those of the National Institute of Standards and Technology (NIST) database. The fibre was conditioned at 270 °C for 1.5 h before the analysis. A blank test was performed before each analysis. The quantitative data of the volatile compounds of the sample were obtained by normalising the peak areas of each compound with respect to the peak area of the internal standard. Peak area data were processed by MSD ChemStation 5975 TAD Data Analysis software (Agilent Technologies, Palo Alto, CA, USA). FlavorDB (Garg et al., 2018) was queried with the VOCs outcoming from the volatilome analysis in order to explore their flavor.

2.4. Metagenome bioinformatic data analysis

Raw reads were quality filtered using PRINSEQ 0.20.4 (Schmieder & Edwards, 2011). Nucleobases with a Phred quality score < 15 were trimmed and reads were discarded if length < 75 bp. Microbiome taxonomic profiling at species-level was obtained using the MetaPhlAn 3.0 tool with default options (Beghini et al., 2021). In addition, high-quality reads from the metagenomes were independently assembled using MEGAHIT v1.2.2 (Li et al., 2015) with the option "–k-list 21,33,55,71,81,91". All the contigs < 1000 bp were discarded from further analyses. The contigs were then processed with MetaGeneMark v3.26 (Zhu et al., 2010) to identify the coding sequences (CDS). Metagenomes' gene richness was estimated by mapping reads against the centroids of all the CDS through BowTie2 v2.2.9 (with the option "–very-sensitive-local"; (Langmead & Salzberg, 2012). A gene abundance ≥0.1 RPKM was set as threshold to discriminate between present and absent genes. Furthermore, the predicted genes were annotated against the 2024–04-26 version of the KOfam database (Aramaki et al., 2020) through a hidden Markov model approach using the 'hmmscan' tool from the version 3.1b2 of the HMMER suite (https://hmmer.org/; Eddy, 2011). Strains from the principal Starter Lactic Acid Bacteria (SLAB) species were detected in each metagenome using StrainPhlAn version 4.0.2 with default settings (Blanco-Míguez et al., 2023). The resulting phylogenetic trees were visualized through iTol version 6.5.3 (Letunic & Bork, 2021).

2.5. Statistical analyses and data visualization

Statistical analyses were performed using R version 4.2.3 (https://www.R-project.org/). In particular, the relative abundances of taxa, genes and pathways, as well as the VOCs emission rates values were compared between the two PDO province of production using the Wilcoxon-Mann-Whitney test. The null hypothesis of no differences between the groups was rejected when the p-value was higher than 0.05, unless otherwise stated. p-values were corrected using the False Discovery Rate (FDR) method when needed. Heatmaps showing the unsupervised clustering of the relative abundance of microbial species and VOCs were visualized using the ComplexHeatmap R package, the Euclidean distance and the complete-linkage clustering method (Gu, 2022). Network analysis between taxa and VOCs were computed using the Spearman correlation, and only significant correlations (corrected $p < 0.05$) were considered for the network construction visualized through the Gephi software (https://gephi.org/). Between-Class Discriminant Analysis (BCA) was performed to explore the relationship between taxa, VOCs, and geographical area using the ade4 and adegraphics packages in R (Bougeard & Dray, 2018; Dray & Dufour, 2007; Siberchicot et al., 2017; Thioulouse et al., 2018). The *lefser* R package was used to perform Linear Discriminant Analysis Effect Size (LEfSe) analysis on microbial taxa and VOCs datasets using the geographical area of production as grouping variable (https://github.com/waldronlab/lefser). The output files from HMMER suite containing domain hits were processed to extract KO identifiers and parsed to calculate the KEGG pathway completeness through KEGG-Decoder script (Graham et al., 2018).

2.6. Data availability

Raw reads are available on the Sequence Read Archive database under the accession numbers PRJNA997801 (BioSamples SAMN36690459, SAMN36690461, SAMN36690463, SAMN36690465, SAMN36690471, SAMN36690473, SAMN36690487, SAMN36690493, SAMN36690531, SAMN36690537) and PRJNA1084214.

3. Results

3.1. Characterization of the microbiome in PDO BM cheese

The analysis of microbiome taxonomic profiles allowed for high-resolution taxonomic profiling at the species level in terms of relative abundances. This approach provided a comprehensive view of the microbial communities present in the PDO BM samples. Our results highlighted *Streptococcus thermophilus* (Caserta = 47.49 ± 21.93; Salerno = 45.75 ± 28.57), *Lactobacillus helveticus* (Caserta = 31.49 ± 19.47; Salerno = 41.41 ± 26.10), and *Lactobacillus delbrueckii* (Caserta = 17.82 ± 14.36; Salerno = 11.13 ± 13.23) as common dominant species in all samples from Caserta and Salerno (Fig. 2; Wilcoxon-Mann-Whitney test, $p > 0.05$). However, by employing the Euclidean distance and complete-linkage clustering method, distinct patterns emerged, revealing the relationships within the data (Fig. 2E). This method allowed us to show differences in microbiome profiles and in the occurrence of sub-dominant taxa led to the clustering of BM samples based on their geographical area of production (Fig. 2E). In particular, higher microbial diversity and gene richness – that had been estimated with the purpose of comparing the functional diversity of microbial communities – were observed in PDO BM from Caserta compared to Salerno (Fig. 3; Wilcoxon-Mann-Whitney test, $p < 0.05$). *Lactococcus lactis* was the characteristic LAB of Caserta PDO BM (Table 1), showing a significantly higher abundance in Caserta compared with Salerno samples (Wilcoxon-Mann-Whitney test, $p < 0.05$). In addition, other taxa such as *Moraxella osloensis*, *Streptococcus suis*, *Kaistella haifensis*, *Acinetobacter johnsonii*, *Acinetobacter guillouiae*, *Lactococcus raffinolactis* and *Streptococcus parauberis* were higher in Caserta (Wilcoxon-Mann-Whitney test, $p < 0.05$), as shown in Table 1. Microbial species that were significantly different between the two PDO provinces of Caserta and Salerno are also shown in Supplementary Fig. S1. In addition, we explored the presence of different putative sub-species within the three dominant taxa. Interestingly, the phylogenetic analysis performed on *St. thermophilus* and *Lb. delbrueckii* highlighted that samples from Salerno and Caserta might harbour distinct subgroups within these species (Fig. 4).

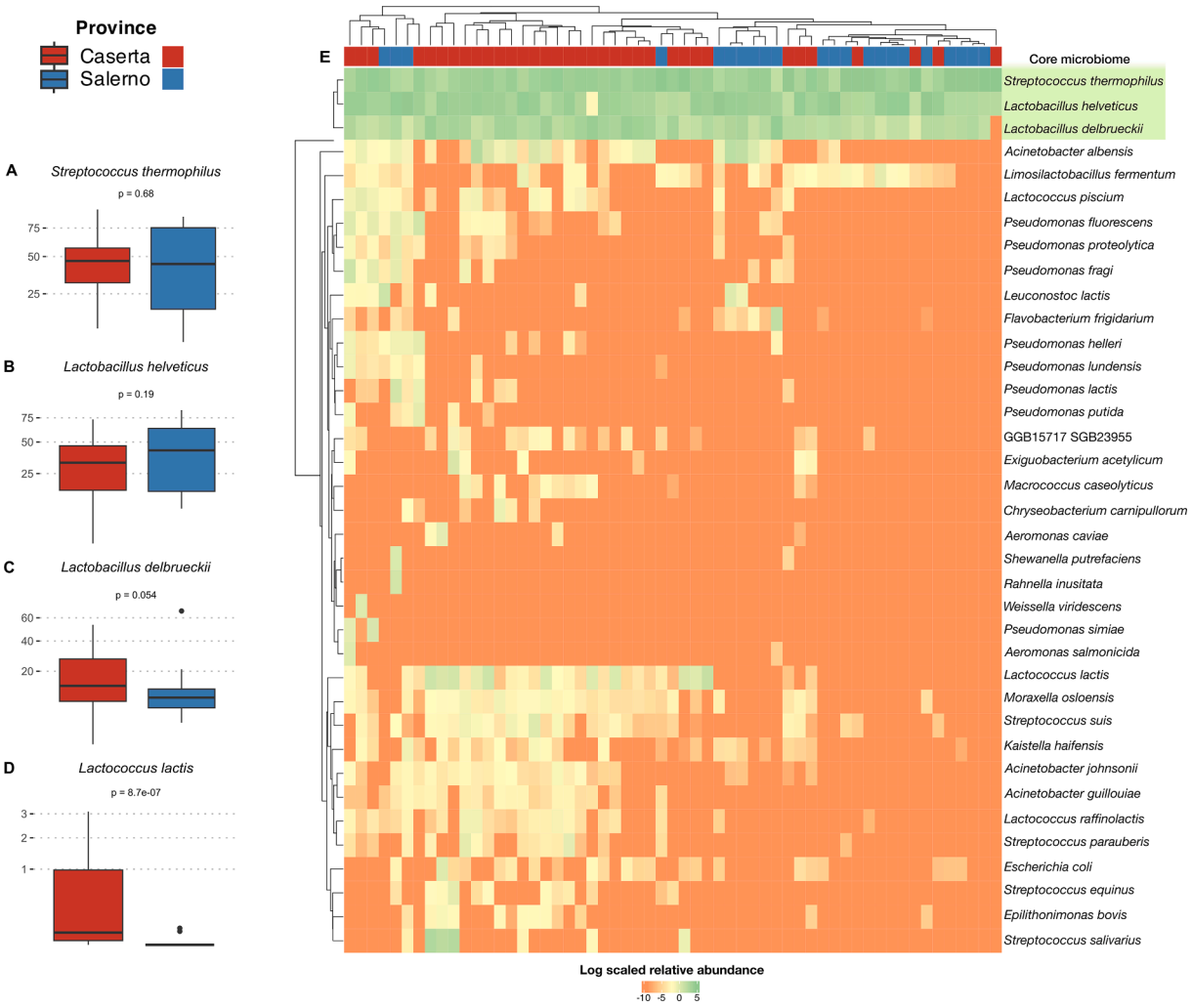

Fig. 2. Microbial taxonomic profiles and key species abundances in PDO BM. (A, B, C) Box plots showing the relative abundances of the three core taxa in PDO BM samples from Caserta and Salerno provinces, that are *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* (highlighted in green). No statistically significant differences in the relative abundances of these microbial species were observed between Caserta and Salerno provinces (Wilcoxon-Mann-Whitney, $p > 0.05$). (D) *Lactococcus lactis* was significantly more abundant in Caserta samples compared to Salerno (Wilcoxon-Mann-Whitney test, $p < 0.05$), identifying it as a characteristic LAB of the Caserta PDO BM. (E) Heatmap representing microbiome taxonomic profiles in Caserta and Salerno samples. Column color bar is colored according to the province of production. The colors represent the Log scaled relative abundances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

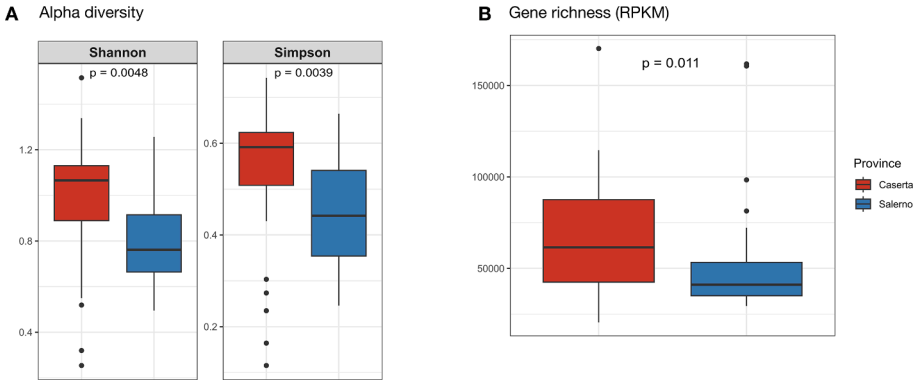

Fig. 3. Diversity indices and gene richness in PDO BM by production area. (A) Box plots illustrating alpha diversity indices of PDO BM grouped by their production area (Caserta and Salerno provinces). The alpha diversity was calculated using both Shannon and Simpson indices. Group comparisons were conducted using the Wilcoxon–Mann–Whitney's test ($p < 0.05$). (B) Box plot illustrating the gene richness of PDO buffalo mozzarella samples, grouped by their production area (Caserta and Salerno provinces). Gene richness was calculated and normalized using the RPKM method, with reads mapped against the centroids of all CDS (gene abundance threshold ≥ 0.1 RPKM) to discern present and absent genes. Statistical comparison between the two groups was performed using the Wilcoxon–Mann–Whitney's test ($p < 0.05$).

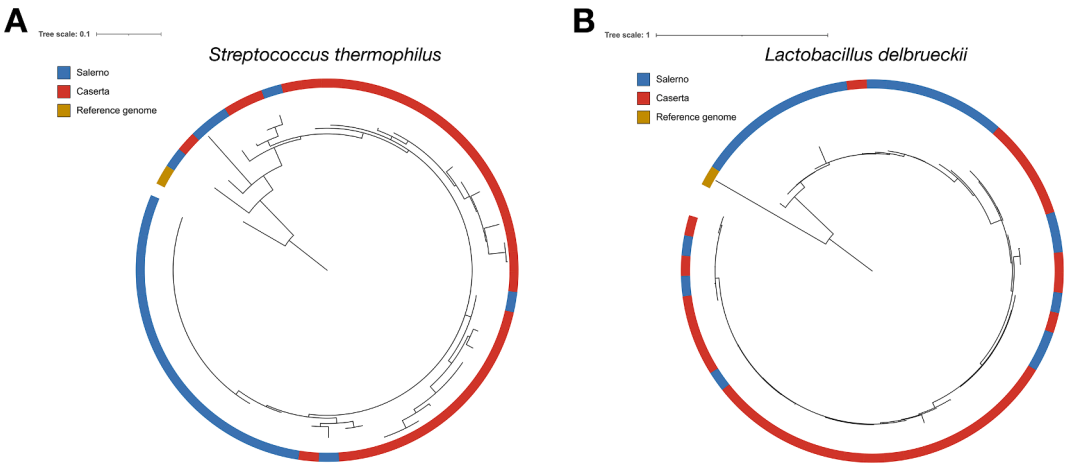

Fig. 4. Phylogenetic analysis of microbial strains composing the core microbiome of PDO BM. Phylogenetic trees showing the putative strains of *Streptococcus thermophilus* (A) and *Lactobacillus delbrueckii* (B) detected in the PDO Buffalo Mozzarella cheese metagenomes through StrainPhlAn version 4.0.2. Putative strains are color-coded according to the province of the cheesemaking facility, whereas reference genomes downloaded from the NCBI databases are colored in gold. Accession numbers: GCF_903886475.1 *Streptococcus thermophilus*; GCF_006740305.1 *Lactobacillus delbrueckii*.

3.2. Variations in flavor complexity between Salerno and Caserta PDO BM

The same statistical tests, distance calculations, and clustering methods, described above were computed also on emission rates of VOCs expressed as p.p.b. PDO BM showed a complex flavor profile. Indeed, we identified 29 VOCs in our samples, as shown in Figs. 5 and 6. The different microbiome led to significant differences in flavor profiles between the two PDO provinces (Permutational Multivariate ANOVA, $p < 0.05$). However, a more complex VOCs pattern was found in samples from Salerno. In particular, absolute abundances of ethylacetate, 2-nonanone, hexanal, diacetyl, acetic acid, decanoic acid, butanoic acid, acetone, pentanal, nonanal, dodecanoic acid, hexanol, and ethylhexanoate were significantly higher in Salerno samples (Table 2 and Supplementary Fig. S2). In addition, the proportion of VOCs in the two groups was different. Indeed, isoamylalcohol accounted on average for 13.3 % of the VOCs found in Mozzarella cheeses from Caserta, whereas it represented 4.8 % of the VOCs of samples from Salerno (Fig. 6). The VOCs detected through the volatilome analysis were also queried against the FlavorDB, to explore the possible role of these molecules in flavor profiles (Fig. 6). In particular, while both provinces exhibited common levels of VOCs responsible for fruity and sweet odors, Caserta PDO BM presented notable oily and burnt aromas reminiscent of whisky, complemented by malt undertones. Conversely, Salerno PDO BM showed acidic and mildly sour nuances, besides coconut and herbal accents, with additional elements of sweetness and woodiness (Fig. 6). The networks analysis illustrating the Spearman rank correlation tests was used to explore the possible co-occurrence patterns of taxa and VOCs abundance from the two areas of production (Supplementary Fig. S3). The correlation strengths varied, with thicker edges representing stronger correlations. Positive and negative correlations were differentiated by the usage of green and pink edges respectively. Within the dataset of Caserta, a diverse array of microbial species exhibited both positive and negative correlations (Supplementary Fig. s3). Between the positive correlations, we found that *Lactococcus lactis* abundance was positively correlated with ethylhexanoate and acetoin, while *Streptococcus equinus* co-occurred with acetoin and 2-nonanone and *Streptococcus salivarius* with heptanal. Moreover, some microbial contaminants like *Moraxella osloensis* positively correlated with ethylbutanoate (Supplementary Fig. s3A). Conversely, *Lactococcus piscium* co-excluded with acetone, 2-methylbutanal, 3-methylbutanal, isoamylalcohol, and ethylhexanoate in Caserta samples (Supplementary Fig. S3A). Within Salerno dataset, the array of positive correlations included *Lb. delbrueckii* with 2-methylbutanal, butanoic and hexanoic acids and *Lb. helveticus* with 2-nonanone, diacetyl and 1-octen-3-ol. On the contrary, *Limosilactobacillus fermentum* was negatively associated with 2-methylbutanal, while *St. thermophilus* only showed negative correlations with pentanal, acetone, 2-heptanone, 2-nonanone, 1-octen-3-ol, hexanoic, decanoic and dodecanoic acid (Supplementary Fig. S3B).

3.3. Integration of microbiome and volatilome profiles drives clustering of BM from different production area

To better understand the differences in microbial profiles and VOCs between PDO BM samples from Caserta and Salerno, we employed a Between-Class Discriminant Analysis (BCA) analysis, which allowed us the clustering of the data based on geographical origin, integrating microbial taxonomic profiles and VOCs data (Fig. 7). In addition, we employed Linear Discriminant Analysis Effect Size (LEfSe) analysis to identify which microbial taxa and VOCs drive the differences between the samples from the two provinces, thus highlighting biomarkers that are characteristic of each geographical area. The LEfSe analysis identified several taxa as biomarkers of PDO BM produced in Salerno, including *Liquorilactobacillus ghanensis* and *Pediococcus parvulus*. On the contrary, *Lactococcus lactis*, *Streptococcus suis*, *Streptococcus parauberis*, *Lactococcus raffinolactis*, and *Enterococcus italicus* were identified as markers of Caserta BM (Supplementary Fig. S4). Additionally, the same approach was applied to investigate the VOCs as biomarkers. Interestingly, all the VOCs identified by LEfSe as discriminant of the two production area were recognized as biomarkers of PDO BM from Salerno (Supplementary Fig. S5).

3.4. Comparative functional analysis of PDO BM microbiomes

Functional annotation of predicted genes was performed through the calculation of KEGG pathway completeness. This analysis provided a comprehensive view of the functional potential of the microbial communities present in the PDO BM samples. By assessing the completeness of various metabolic pathways, we could infer the metabolic capabilities of the microbiomes in the different production areas, revealing distinct metabolic potential according to geographical origin of PDO BM.

The heatmap showing the metabolic potential of PDO BM from Caserta and Salerno revealed that Caserta samples had more complete pathways (i.e., showed the presence of more genes related to that pathway) related to sulfur, amino acids (Val, Ile, Leu, Trp), lipid, methane metabolisms, and vitamins biosynthesis compared with

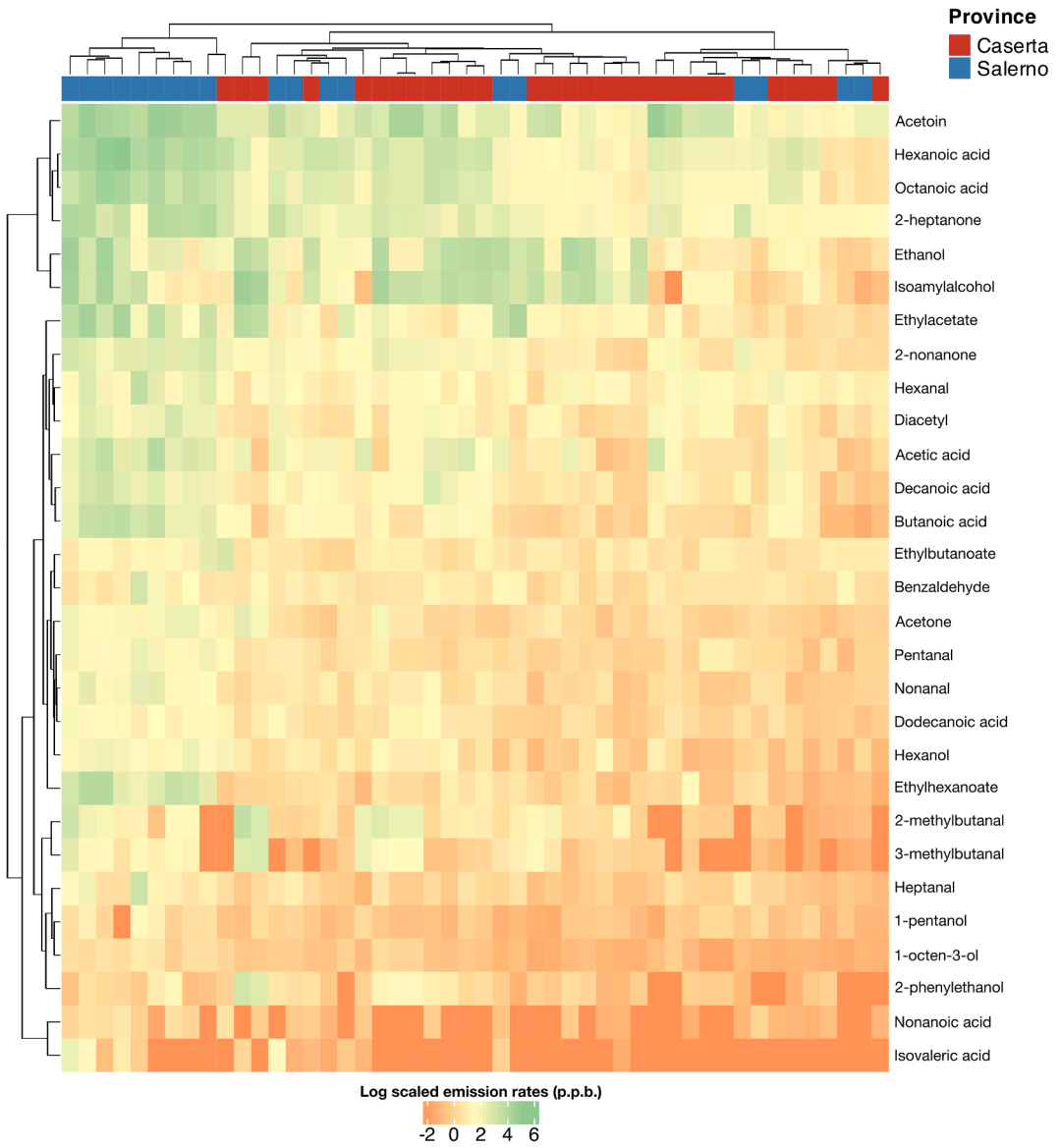

Fig. 5. Heatmap of VOC emission rates in PDO BM from Caserta and Salerno. Heatmap representing emission rates of VOC in PDO BM produced in Caserta and Salerno area. Column color bar is colored according to the area of production. The color scale indicates the Log scaled emission rates of VOCs expressed as p.p.b.

Salerno (Supplementary Fig. S6).

4. Discussion

Our work aimed to shed light on the intricate interplay among the microbiome of BM cheese, their distinctive VOC profiles, and the link with the area of production. Consistently with previous works (De Filippis et al., 2014; Ercolini et al., 2012), we found that BM cheeses from both the production areas were dominated by the three main species of thermophilic LAB, i.e., *St. thermophilus*, *Lb. delbrueckii* and *Lb. helveticus*, representing the core microbiome of the PDO BM. These thermophilic LAB species, which are distinctive of NWCs used in PDO BM production, rapidly acidify the curd, and have a critical role in the flavor profile of the final products (Silva et al., 2020). Although the three core taxa were present at similar abundance in BM produced in the two production area, a significantly higher microbial diversity was found in BM from Caserta, which showed a higher prevalence of several sub-dominant taxa, such as *Moraxellaceae* (e.g., *A. johnsonii*, *M. osloensis*) and *Streptococcaceae* (*Lc. lactis*, *Lc. piscium* and *S. parauberis*), previously reported as common members of the raw milk microbiota (O'Sullivan & Cotter, 2017), suggesting a strong influence of the raw milk microbiome on the final cheese microbiome in Caserta, that may be linked to differences in the pasteurization or thermization process. Notably, *Lc. lactis* emerged as the most abundant microbial species in Caserta, underscoring its role as a biomarker of the Caserta group. This finding was statistically validated through Wilcoxon-Mann-Whitney's test (Table 1 and Fig. 2) and LEfSE analysis (Supplementary Fig. 3). The prevalence of *Lc. lactis* in Caserta BM may be due to specific environmental and production factors unique to that area, which merit further investigation. Although some members of the *Moraxellaceae* are frequently correlated with flavor compound in traditional cheeses (Zhang et al., 2022), the impact of *A. johnsonii* and *M. osloensis* on sensory properties of cheeses is not clear, since the metabolic potential of these species has not been deeply characterized. However, our results highlighted that the microbiome profiles are strongly correlated with the VOC abundance and shape the VOC pattern, potentially leading to different flavor profiles. Notably, this pattern encompasses various compounds known to contribute to flavor in BM (Curioni & Bosset, 2002; Mauriello et al., 2003; Natrella et al., 2020b), including esters (i.e. ethyl acetate, ethylhexanoate), known to give fruity and floral notes, 2-nonanone, associated with buttery and nutty

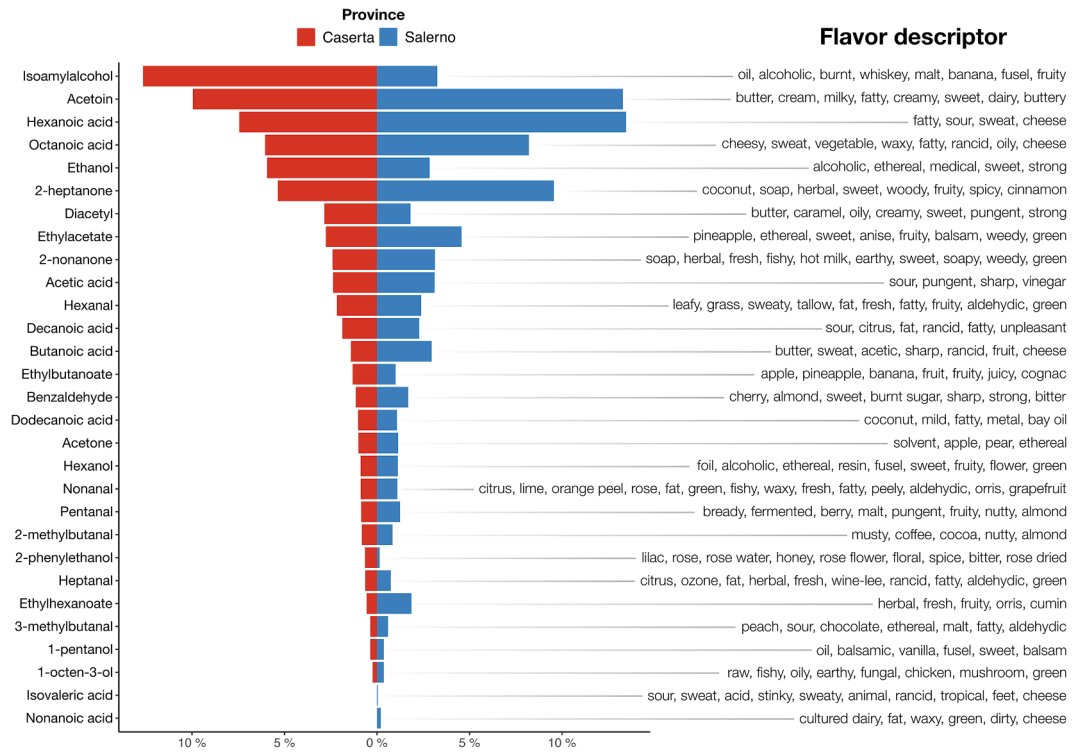

Fig. 6. Bar chart of VOC abundances and their flavor descriptors in PDO BM from Caserta and Salerno. Bar chart showing the average relative abundance of each Volatile Organic Compound (VOC) in PDO Buffalo Mozzarella cheese samples from Caserta (red) and Salerno (blue). For each VOC the flavor descriptor is also reported, in accordance with information collected from the FlavorDB (https://cosylab.iiitd.edu.in/flavordb/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nuances, aldehydes (e.g., hexanal, pentanal, and nonanal), contributing to grassy and fatty aromas, acids (acetic acid, decanoic acid, butanoic acid), playing a role in the characteristic cheesy traits (Cincotta et al., 2021; Natrella et al., 2020a; Sacchi et al., 2020; Ziino et al., 2005). In particular, a comparative study found that BM tended to contain higher levels of butanoic acid compared to other cheese types (Chambers et al., 2005). Interestingly, although BM from Caserta showed higher microbial taxonomic and functional diversity and richness, a more complex VOC pattern was identified in Salerno and almost all the VOCs identified were more abundant in these samples. This apparent paradox highlights the role of the underlying ecological mechanisms influencing the flavor profiles of BM cheese. However, the pathways involved in the synthesis of non-volatile L-amino acids contributing to the bitter taste in cheeses (i.e., histidine, valine, isoleucine, leucine, and tryptophan, (Kilcawley, 2017) were more complete in PDO BM from Caserta. These L-amino acids might be precursors of some VOCs, as in the case of isoamylalcohol, present in greater quantities in the Caserta samples, which can derive from the catabolism of branched chain amino acids. Indeed, L-leucine serves as a precursor for 3-methylbutanal due to the activity of microbial enzymes involved in the Ehrlich pathway (Duensing et al., 2024; Illikoud et al., 2018). The aroma of BM has been extensively explored in prior studies, in which 1-octen-3-ol, nonanal, other aldehydes and ketones like acetoin are reported between the most aroma-active VOCs of water buffalo mozzarella cheese (Curioni & Bosset, 2002). Indeed, BM cheese have very specific sensory attributes. The key sensory attributes to characterize BM cheese were identified as cohesion, acidity, saltiness, a yoghurt-like aroma, and a crumbly texture (Pagliarini et al., 1997). Additionally, it has been suggested that mozzarella cheese should possess a delicate balance of saltiness, buttery richness, and a subtle milky aroma (Chen et al., 2008). SLAB taxa, such as *Lb. delbrueckii*, *Lb. helveticus* and *St. thermophilus,* usually present in traditional NWC, have a pivotal role in developing the aroma bouquet of BM. For instance, laboratory-scale BM production with different starter cultures (thermophilic SLAB or a more complex starter including other species) or acidification with the addition of citric acid, highlighted that BM produced using the thermophilic starter yielded the highest flavour scores (Coppola et al., 1990). However, it must be also considered that the sensory profiles of PDO BM is also affected by the type of forage used for the feeding. Indeed, the inclusion in the buffalo diet of fresh sorghum increased the unsaturated fatty acids and decreased the saturated fatty acids amount, however, rather than sensory descriptors of aroma, texture attributes were the most influenced (Uzun et al., 2018). In fact, the authors reported a significant difference for descriptors such as tenderness, elasticity, juiciness, chewiness and cohesiveness. Furthermore, it has been reported that the replacement of preserved forage, such as maize silage, with fresh forage in the buffalo diet implies a different amount of VOCs, due to the effect of the animal diet on the composition of fatty acids (Sacchi et al., 2020). Interestingly, several minor taxa in BM from Caserta were found, possibly coming from the production environment or from the materials used (e.g., brine, milk). Although these taxa were highlighted as markers of BM from Caserta and contributed to the overall taxonomic and functional diversity of the metagenome, they probably have a small contribution in shaping the BM flavor bouquet. *Pseudomonas*, *Moraxella*, and *Acinetobacter* genera, in particular, are frequently identified in raw milk samples and have been associated with seasonality and geography (Yap et al., 2024). While not providing desirable metabolites, some of these taxa might be linked to off-flavours. Indeed, we found a negative correlation between *Acinetobacter johnsonii* and 2-heptanone, one of the key odourant of fresh cheeses (Cincotta et al., 2021), as previously reported (Feng et al., 2023). Therefore, low abundant but still metabolically active taxa might have competitive interactions with SLAB/NSLAB, with the latter potentially shifting from catabolic to competitive processes. Our results suggest low-abundance microbial contaminants in BM from Caserta might have inhibited flavor-producing species, leading to a less complex flavor pattern in these products. Nevertheless, we suggest that PDO BM

Table 1
Microbial species abundances in PDO BM by production province. This table summarizes the microbial species detected in the Protected Designation of Origin Buffalo Mozzarella samples, categorized by the province of production. The microbial profiles were generated using the MetaPhlAn 3.0 tool and are expressed as relative abundances. The statistics included are the mean and standard deviation (SD) for each species, along with the corresponding p-values derived from Wilcoxon tests. Significant differences ($p < 0.05$) between provinces are indicated with asterisks.

Microbial species	Province		p-value[2]	q-value[3]
	Caserta, N=35[1]	Salerno, N=22[1]		
Lactococcus lactis	0.51677 (0.84231)	0.00365 (0.01209)	<0.001***	**<0.001**
Moraxella osloensis	0.05945 (0.07822)	0.00456 (0.01201)	<0.001***	**<0.001**
Streptococcus suis	0.08603 (0.18253)	0.00491 (0.02059)	<0.001***	**<0.001**
Acinetobacter johnsonii	0.03295 (0.05550)	0.00943 (0.03573)	0.002**	**0.008**
Macrococcus caseolyticus	0.02132 (0.06076)	0.00000 (0.00000)	0.002**	**0.009**
GGB15717 SGB23955	0.01599 (0.04507)	0.00051 (0.00169)	0.003**	**0.010**
Flavobacterium frigidarium	0.00113 (0.00475)	0.23837 (1.09445)	0.009**	**0.024**
Acinetobacter junii	0.01093 (0.02519)	0.00000 (0.00000)	0.011*	**0.026**
Acinetobacter guillouiae	0.01836 (0.03319)	0.00942 (0.03598)	0.015*	**0.033**
Pediococcus parvulus	0.00106 (0.00250)	0.00000 (0.00000)	0.018*	**0.033**
Liquorilactobacillus ghanensis	0.00000 (0.00000)	0.00092 (0.00267)	0.028*	**0.040**
Stenotrophomonas maltophilia	0.00000 (0.00000)	0.00159 (0.00440)	0.028*	**0.040**
Exiguobacterium acetylicum	0.05076 (0.22693)	0.00000 (0.00000)	0.028*	**0.040**
Escherichia coli	0.03253 (0.15188)	0.00337 (0.01318)	0.029*	**0.040**
Streptococcus parauberis	0.04588 (0.15201)	0.00276 (0.01039)	0.032*	**0.040**
Lactococcus raffinolactis	0.03803 (0.09264)	0.00450 (0.01374)	0.038*	**0.045**
Epilithonimonas bovis	0.02447 (0.05434)	0.00103 (0.00405)	0.041*	**0.045**
Enterococcus italicus	0.00524 (0.01727)	0.00000 (0.00000)	0.044*	**0.045**
Kaistella haifensis	0.02611 (0.05251)	0.01578 (0.06598)	0.045*	**0.045**

[1] Mean (SD).
[2] *p < 0.05; **p < 0.01; ***p < 0.001.
[3] False discovery rate correction for multiple testing.

Table 2
VOC emission rates in PDO BM by production province. This table summarizes the volatile organic compounds (VOCs) detected in the Protected Designation of Origin Buffalo Mozzarella samples, categorized by the province of production. The data are expressed as VOCs emission rates (p.p.b.). The statistics included are the mean and standard deviation (SD) for each VOC, along with the corresponding p-values derived from Wilcoxon tests. Significant differences ($p < 0.05$) between provinces are indicated with asterisks.

Volatile organic compounds	Province		p-value[2]	q-value[3]
	Caserta, N=29[1]	Salerno, N=19[1]		
Ethylhexanoate	1.00106 (0.81950)	12.05704 (16.16867)	<0.001***	**0.003**
Heptanal	0.75347 (0.42292)	3.27354 (5.66590)	<0.001***	**0.003**
Nonanal	1.29061 (0.78664)	4.05011 (3.54378)	0.001**	**0.005**
Benzaldehyde	1.67903 (0.61702)	3.59906 (4.06836)	0.001**	**0.005**
Nonanone	3.73528 (2.89215)	9.00094 (6.19303)	0.002**	**0.007**
Isovaleric acid	0.05441 (0.17834)	1.15283 (2.30079)	0.002**	**0.007**
Pentanal	1.34427 (0.72865)	3.68459 (2.83257)	0.003**	**0.008**
Octen-3-ol	0.37903 (0.27289)	0.85452 (0.60276)	0.004**	**0.009**
Heptanone	8.26333 (5.18976)	24.53672 (19.67933)	0.005**	**0.009**
Hexanol	1.50981 (1.42668)	3.39759 (2.77298)	0.010**	**0.016**
Butanoic acid	2.23123 (1.74398)	11.21551 (12.68791)	0.010*	**0.016**
Octanoic acid	9.25703 (6.84943)	26.82247 (26.97224)	0.018*	**0.026**
Hexanal	3.44468 (1.52552)	7.25485 (7.25359)	0.030*	**0.035**
Dodecanoic acid	1.42424 (0.90049)	2.64005 (1.81204)	0.030*	**0.035**
Nonanoic acid	0.21444 (0.33740)	0.57773 (0.59900)	0.031*	**0.035**
Hexanoic acid	12.57798 (9.29650)	44.29228 (47.38278)	0.035*	**0.035**
Ethylacetate	5.85175 (10.36478)	23.00714 (27.35253)	0.035*	**0.035**

[1] Mean (SD).
[2] *p < 0.05; **p < 0.01; ***p < 0.001.
[3] False discovery rate correction for multiple testing.

microbiome and volatilome may be considered as integral facet of the *terroir* that links these typical products with the specific area of production, demonstrating a clear link between the cheeses microbiome, volatilome, and the geographical origin of production. Several factors, including animal feeding, micro-climatic features of the territory (i.e. temperature and humidity) and slight variations in cheesemaking technology between facilities located in different production area might select specific taxa or strains with different metabolic potentials, resulting in cheeses with unique sensorial profiles (Turbes et al., 2016). Indeed, we observed a separation between the *Lb. delbrueckii* and *St. thermophilus* strains detected in the two production areas. These results are consistent with a previous work (Cardin et al., 2024), which highlighted that strains of the most relevant SLAB species were different between Caciotta short-medium ripened cheeses in different facilities. Similarly, Bozoudi et al. (2016), reported a clustering of microbial communities and SLAB/NSLAB strains according to the production area in Feta cheese. Moreover, Ercolini and collaborators (2005) identified three distinct clusters of *St. thermophilus* strains based on the *LacS* gene sequences, highlighting the genetic variability that can exist even within strains sourced from similar dairy products. Interestingly, while all *St. thermophilus* strains from water buffalo mozzarella cheese grouped into one cluster, strains from other dairy products such as yogurt and various cheeses were dispersed across different clusters (Ercolini et al., 2005). This genetic diversity suggests that different *S. thermophilus* strains adapt to different fermentation environments, possibly selected by the unique pressures in each type of dairy product. Moreover, De Filippis et al. (2014) revealed distinct *S. thermophilus* biotypes associated with different geographical regions. Specifically, they identified numerous sequence types of the *LacS* gene, indicating a notable intraspecies variability. This variability underscores the significant influence of geographical origin on the microbial composition of PDO BM, further supporting the concept of the existence of a microbial *terroir* in fermented food products. This genomic differences at strain level may also explain the differences in terms of sensory properties of PDO BM, despite the shared core microbiome. Our work focused on microbiome and volatilome profiling of BM samples collected within the same season, but the long-term stability of the starter culture and the related effect on the cheese profile is a critical concern (Parente et al., 2017). Indeed, further longitudinal studies are needed to assess the microbiome and volatilome consistency over time, also controlling conditions such as animal

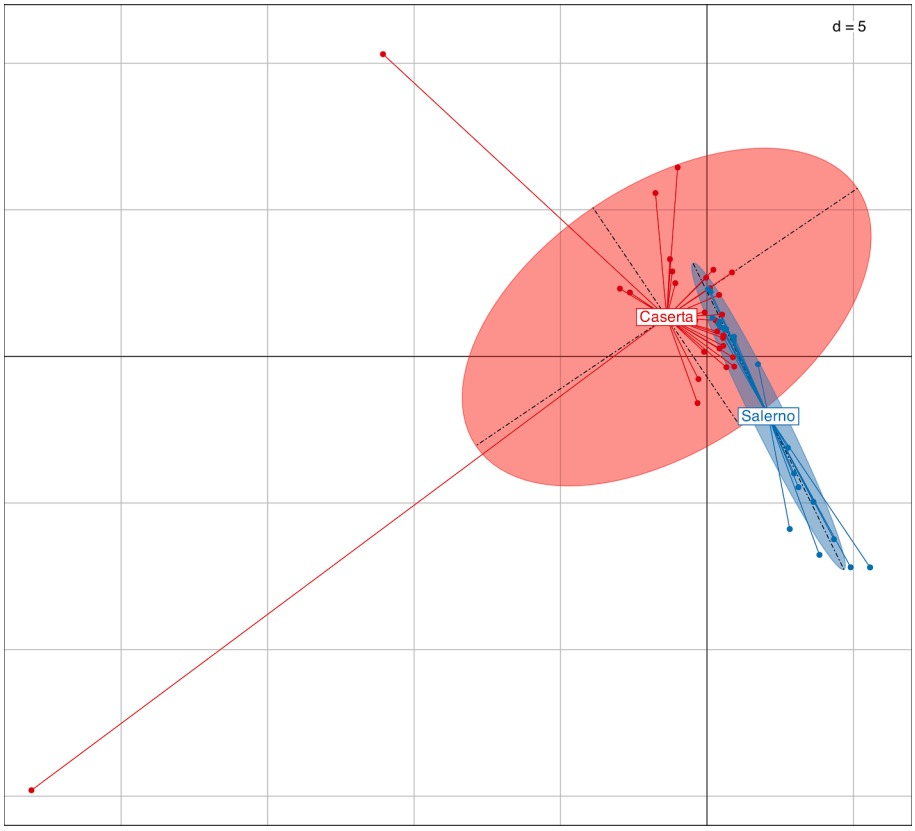

Fig. 7. Clusters from BCA of microbial and VOC profiles in PDO BM. The figure illustrates the two clusters coming out the Between-Class Discriminant Analysis (BCA), which integrates microbial taxonomic profiles with volatile organic compounds (VOCs) data of Protected Designation of Origin Buffalo Mozzarella samples produced in Caserta and Salerno provinces.

feeding, temperature, milk source, and starter culture. This will help to better understand the individual and combined role of each factor on the microbiome and volatile profiles of PDO BM. This will support the identification of consistent markers to be used in discriminating between PDO and non-PDO cheeses.

5. Conclusions

The complexity of the aromatic profile in Salerno BM, despite lower microbial and genetic diversity, raises questions about the role of specific microbial strains and their metabolic activities, that deserves further investigations. In addition, even though we confirmed the existence of a core microbiome, we highlighted the presence of strains potentially linked with the *terroir* of BM cheese and releasing distinctive VOCs. Besides being important for understanding the role of production environment in shaping the cheese microbiome and volatilome, these results also open new clues on the possibility of using microbial and VOC patterns as a fingerprint of the cheese production area, developing novel models to trace its origin, also integrating these data within advanced machine learning techniques. This may lead to an innovative biotechnological tool to safeguard the traceability and quality of PDO cheeses, contributing to counteract food frauds.

CRediT authorship contribution statement

Raffaele Magliulo: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Vincenzo Valentino:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Andrea Balivo:** Writing – review & editing, Formal analysis. **Alessia Esposito:** Formal analysis. **Alessandro Genovese:** Writing – review & editing, Supervision, Resources. **Danilo Ercolini:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Francesca De Filippis:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Accession numbers for sequence download have been provided in the paper

Acknowledgments

This work was partially supported by the Italian Ministry of Foreign Affairs and International Cooperation, with a grant to the project FOODMICROHERITAGE (VN21GR09). The work was also supported by the National Recovery and Resilience Plan (NRRP) funded by the European Union – NextGenerationEU, Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of MUR (CN_00000033, CUP E63C22000990007, Project "National Biodiversity Future Center – NBFC") and Mission 4 "Education and Research" Component 2: from - research to business, Investment 3.1: Fund for the realization of an integrated system of research and innovation infrastructures (IR0000033, CUP I83C22001040006, Project METROFOOD-IT, D.M. Prot. n.120 del 21/06/2022).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114798.

References

Afshari, R., Pillidge, C. J., Dias, D. A., Osborn, A. M., & Gill, H. (2020). Cheesomics: The future pathway to understanding cheese flavour and quality. *Critical Reviews in Food Science and Nutrition, 60*(1), 33–47. https://doi.org/10.1080/10408398.2018.1512471

Aramaki, T., Blanc-Mathieu, R., Endo, H., Ohkubo, K., Kanehisa, M., Goto, S., & Ogata, H. (2020). KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics (Oxford, England), 36*(7), 2251–2252. https://doi.org/10.1093/bioinformatics/btz859

Balivo, A., Cipolletta, S., Tudisco, R., Iommelli, P., Sacchi, R., & Genovese, A. (2023). Electronic nose analysis to detect milk obtained from pasture-raised goats. *Applied Sciences (Basel, Switzerland), 13*(2), 861. https://doi.org/10.3390/app13020861

Barham, E. (2003). Translating terroir: The global challenge of French AOC labeling. *Journal of Rural Studies, 19*(1), 127–138. https://doi.org/10.1016/s0743-0167(02)00052-9

Beghini, F., McIver, L. J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., Mailyan, A., Manghi, P., Scholz, M., Thomas, A. M., Valles-Colomer, M., Weingart, G., Zhang, Y., Zolfo, M., Huttenhower, C., Franzosa, E. A., & Segata, N. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *ELife, 10*. https://doi.org/10.7554/elife.65088

Blanco-Míguez, A., Beghini, F., Cumbo, F., McIver, L. J., Thompson, K. N., Zolfo, M., Manghi, P., Dubois, L., Huang, K. D., Thomas, A. M., Nickols, W. A., Piccinno, G., Piperni, E., Punčochář, M., Valles-Colomer, M., Tett, A., Giordano, F., Davies, R., Wolf, J., & Segata, N. (2023). Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. *Nature Biotechnology, 41*(11), 1633–1644. https://doi.org/10.1038/s41587-023-01688-w

Bougeard, S., & Dray, S. (2018). Supervised multiblock analysis in *R* with the ade4 package. *Journal of Statistical Software, 86*(1). https://doi.org/10.18637/jss.v086.i01

Bozoudi, D., Torriani, S., Zdragas, A., & Litopoulou-Tzanetaki, E. (2016). Assessment of microbial diversity of the dominant microbiota in fresh and mature PDO Feta cheese made at three mountainous areas of Greece. *Lebensmittel-Wissenschaft Und Technologie [Food Science and Technology], 72*, 525–533. https://doi.org/10.1016/j.lwt.2016.04.039

Brunschwig, G., Michelin, Y., Roussel, V., Delbruel, B., & Valadier, G. (1999). Development of a tool to delimit and characterize the "terroirs" of dairy farming in the cheese production area of the "Massif Central"(France). *Systems of Sheep and Goat Production: Organization of Husbandry and Role of Extension Services*, 109. Retrieved from CiteSeerX: https://citeseerx.ist.psu.edu/document?repid=rep1&type=pdf&doi=99100d9f2d31492bf077a64c6c1ce62d13f05f5b.

Cardin, M., Cardazzo, B., Coton, M., Carraro, L., Lucchini, R., Novelli, E., Coton, E., & Mounier, J. (2024). Ecological diversity and associated volatilome of typical mountain Caciotta cheese from Italy. *International Journal of Food Microbiology, 411*, Article 110523. https://doi.org/10.1016/j.ijfoodmicro.2023.110523

Chambers, D. H., Chambers, E., IV, & Johnson, D. (2005). Flavor description and classification of selected natural cheeses. In J. S. A. Edwards, B. Kowrygo, & K. Rejman (Eds.), *Culinary Arts and Sciences V: Global and National Perspectives* (pp. 641–654). Worshipful Company of Cooks Research Centre.

Chen, C., Wolle, D., & Sommer, D. (2008). Mozzarella. In *The Sensory Evaluation of Dairy Products* (pp. 459–487). US: Springer. https://doi.org/10.1007/978-0-387-77408-4_15.

Cincotta, F., Condurso, C., Tripodi, G., Merlino, M., Prestia, O., Stanton, C., & Verzera, A. (2021). Comparison of lactose free and traditional mozzarella cheese during shelf-life by aroma compounds and sensory analysis. *Lebensmittel-Wissenschaft Und Technologie [Food Science and Technology], 140*(110845), Article 110845. https://doi.org/10.1016/j.lwt.2020.110845

Coppola, S., Villani, F., Coppola, R., Coppola, R., Parente, E., & Parente, E. (1990). Comparison of different starter systems for water-buffalo Mozzarella cheese manufacture. *Dairy Science & Technology, 70*(5–6), 411–423. https://doi.org/10.1051/lait:19905-631

Cunha, J. T., Ribeiro, T. I. B., Rocha, J. B., Nunes, J., Teixeira, J. A., & Domingues, L. (2016). RAPD and SCAR markers as potential tools for detection of milk origin in dairy products: Adulterant sheep breeds in Serra da Estrela cheese production. *Food Chemistry, 211*, 631–636. https://doi.org/10.1016/j.foodchem.2016.05.109

Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as determined by gas chromatography-olfactometry. *International Dairy Journal, 12*(12), 959–984. https://doi.org/10.1016/s0958-6946(02)00124-3

De Filippis, F., La Storia, A., Stellato, G., Gatti, M., & Ercolini, D. (2014). A selected core microbiome drives the early stages of three popular Italian cheese manufactures. *PloS One, 9*(2). https://doi.org/10.1371/journal.pone.0089680. Article e89680.

Dray, S., & Dufour, A.-B. (2007). Theade4Package: Implementing the duality diagram for ecologists. *Journal of Statistical Software, 22*(4). https://doi.org/10.18637/jss.v022.i04

Duensing, P. W., Hinrichs, J., & Schieberle, P. (2024). Formation of key aroma compounds during 30 weeks of ripening in Gouda-type cheese produced from pasteurized and raw milk. *Journal of Agricultural and Food Chemistry, 72*(19), 11072–11079. https://doi.org/10.1021/acs.jafc.4c01814

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Computational Biology, 7*(10), Article e1002195. Doi: 10.1371/journal.pcbi.1002195.

Ercolini, D., De Filippis, F., La Storia, A., & Iacono, M. (2012). "remake" by high-throughput sequencing of the Microbiota involved in the production of water buffalo mozzarella cheese. *Applied and Environmental Microbiology, 78*(22), 8142–8145. https://doi.org/10.1128/aem.02218-12

Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G., & Coppola, S. (2004). PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. *Journal of Applied Microbiology, 96*(2), 263–270. https://doi.org/10.1046/j.1365-2672.2003.02146.x

Ercolini, D., Fusco, V., Blaiotta, G., & Coppola, S. (2005). Sequence heterogeneity in the lacSZ operon of Streptococcus thermophilus and its use in PCR systems for strain differentiation. *Research in microbiology, 156*(2), 161–172. https://doi.org/10.1016/j.resmic.2004.09.005

Feng, J., Zheng, Y., Zhang, X., Zhou, R., & Ma, M. (2023). Effect of supercritical carbon dioxide on bacterial community, volatile profiles and quality changes during storage of Mongolian cheese. *Food Control, 143*(109225), Article 109225. https://doi.org/10.1016/j.foodcont.2022.109225

Galli, B. D. (2024). Sustainability implications and relevance of using omics sciences to investigate cheeses with protected designation of origin. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.13403

Garg, N., Sethupathy, A., Tuwani, R., Nk, R., Dokania, S., Iyer, A., Gupta, A., Agrawal, S., Singh, N., Shukla, S., Kathuria, K., Badhwar, R., Kanji, R., Jain, A., Kaur, A., Nagpal, R., & Bagler, G. (2018). FlavorDB: A database of flavor molecules. *Nucleic Acids Research, 46*(D1), D1210–D1216. https://doi.org/10.1093/nar/gkx957

Graham, E. D., Heidelberg, J. F., & Tully, B. J. (2018). Potential for primary productivity in a globally-distributed bacterial phototroph. *The ISME Journal, 12*(7), 1861–1866. https://doi.org/10.1038/s41396-018-0091-3

Gu, Z. (2022). Complex heatmap visualization. *IMeta, 1*(3). https://doi.org/10.1002/imt2.43

Illikoud, N., Klopp, C., Roulet, A., Bouchez, O., Marsaud, N., Jaffrès, E., & Zagorec, M. (2018). One complete and three draft genome sequences of four Brochothrix thermosphacta strains, CD 337, TAP 175, BSAS1 3 and EBP 3070. *Standards in Genomic Sciences, 13*(1). https://doi.org/10.1186/s40793-018-0333-z

Kilcawley, K. N. (2017). Cheese flavour. In P. F. Fox, T.éP. Guinee, T. M. Cogan, & P. L. H. McSweeney (Eds.), *Fundamentals of Cheese Science* (pp. 443–474). US: Springer. https://doi.org/10.1007/978-1-4899-7681-9_13.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods, 9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Lee, D.-Y., Park, S. H., Park, S.-E., Kim, E.-J., Kim, H.-W., Seo, S.-H., Cho, K.-M., Kwon, S. J., Whon, T. W., Min, S. G., Choi, Y.-J., Roh, S. W., Seo, H.-Y., & Son, H.-S. (2023). Comprehensive elucidation of the terroir of Korean kimchi through the study of recipes, metabolites, microbiota, and sensory characteristics. *Food Research International, 166*, Article 112614. https://doi.org/10.1016/j.foodres.2023.112614

Lee, J.-H., Diono, R., Kim, G.-Y., & Min, D. B. (2003). Optimization of solid phase microextraction analysis for the headspace volatile compounds of Parmesan cheese. *Journal of Agricultural and Food Chemistry, 51*(5), 1136–1140. https://doi.org/10.1021/jf025910+

Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research, 49*(W1), W293–W296. https://doi.org/10.1093/nar/gkab301

Levante, A., Bertani, G., Marrella, M., Mucchetti, G., Bernini, V., Lazzi, C., & Neviani, E. (2023). The microbiota of Mozzarella di Bufala Campana PDO cheese: A study across the manufacturing process. *Frontiers in Microbiology, 14*. https://doi.org/10.3389/fmicb.2023.1196879

Li, D., Liu, C.-M., Luo, R., Sadakane, K., & Lam, T.-W. (2015). MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct *de Bruijn* graph. *Bioinformatics, 31*(10), 1674–1676. https://doi.org/10.1093/bioinformatics/btv033

Mauriello, G., Moio, L., Genovese, A., & Ercolini, D. (2003). Relationships between flavoring capabilities, bacterial composition, and geographical origin of natural whey cultures used for traditional water-buffalo mozzarella cheese manufacture. *Journal of Dairy Science, 86*(2), 486–497. https://doi.org/10.3168/jds.s0022-0302(03)73627-3

Minervino, A. H. H., Zava, M., Vecchio, D., & Borghese, A. (2020). Bubalus bubalis: A Short Story. *Frontiers in Veterinary Science, 7*, Article 570413. https://doi.org/10.3389/fvets.2020.570413

Nanetti, E., Palladino, G., Scicchitano, D., Trapella, G., Cinti, N., Fabbrini, M., Cozzi, A., Accetta, G., Tassini, C., Iannaccone, L., Candela, M., & Rampelli, S. (2023). Composition and biodiversity of soil and root-associated microbiome in Vitis vinifera cultivar Lambrusco distinguish the microbial terroir of the Lambrusco DOC protected designation of origin area on a local scale. *Frontiers in Microbiology, 14*. https://doi.org/10.3389/fmicb.2023.1108036

Natrella, G., Faccia, M., Lorenzo, J. M., De Palo, P., & Gambacorta, G. (2020a). Evolution of volatile compounds from milk to curd during manufacturing of Mozzarella. *Mljekarstvo, 70*(1), 50–58. https://doi.org/10.15567/mljekarstvo.2020.0105

Natrella, G., Faccia, M., Lorenzo, J. M., De Palo, P., & Gambacorta, G. (2020b). Short communication: Sensory characteristics and volatile organic compound profile of high-moisture mozzarella made by traditional and direct acidification technology. *Journal of Dairy Science, 103*(3), 2089–2097. https://doi.org/10.3168/jds.2019-17059

O'Sullivan, O., & Cotter, P. D. (2017). Microbiota of raw milk and raw milk cheeses. In *Cheese* (pp. 301–316). Elsevier. https://doi.org/10.1016/b978-0-12-417012-4.00012-0.

Pagliarini, E., Monteleone, E., & Wakeling, I. (1997). Sensory profile description of Mozzarella cheese and its relationship with consumer preference. *Journal of Sensory Studies, 12*(4), 285–301. https://doi.org/10.1111/j.1745-459x.1997.tb00068.x

Parente, E., Cogan, T. M., & Powell, I. B. (2017). Starter cultures: General aspects. In *Cheese* (pp. 201–226). Academic Press. https://doi.org/10.1016/B978-0-12-417012-4.00008-9.

Pillonel, L., Ampuero, S., Tabacchi, R., & Bosset, J. (2003). Analytical methods for the determination of the geographic origin of Emmental cheese: Volatile compounds by GC/MS-FID and electronic nose. *European Food Research and Technology, 216*(2), 179–183. https://doi.org/10.1007/s00217-002-0629-4

Sacchi, R., Marrazzo, A., Masucci, F., Di Francia, A., Serrapica, F., & Genovese, A. (2020). Effects of inclusion of fresh forage in the diet for lactating buffaloes on volatile organic compounds of milk and mozzarella cheese. *Molecules (Basel, Switzerland), 25*(6), 1332. https://doi.org/10.3390/molecules25061332

Salzano, A., Manganiello, G., Neglia, G., Vinale, F., De Nicola, D., D'Occhio, M., & Campanile, G. (2020). A preliminary study on metabolome profiles of buffalo milk and corresponding mozzarella cheese: Safeguarding the authenticity and traceability of protected status buffalo dairy products. *Molecules (Basel, Switzerland), 25*(2), 304. https://doi.org/10.3390/molecules25020304

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics (Oxford, England), 27*(6), 863–864. https://doi.org/10.1093/bioinformatics/btr026

Siberchicot, A., Julien-Laferrière, A., Dufour, A.-B., Thioulouse, J., & Dray, S. (2017). Adegraphics: An S4 lattice-based package for the representation of multivariate data. *The R Journal, 9*(2), 198. https://doi.org/10.32614/rj-2017-042

Silva, L. F., Sunakozawa, T. N., Amaral, D. M. F., Casella, T., Nogueira, M. C. L., De Dea Lindner, J., Bottari, B., Gatti, M., & Penna, A. L. B. (2020). Safety and technological application of autochthonous Streptococcus thermophilus cultures in the buffalo Mozzarella cheese. *Food Microbiology, 87*, Article 103383. https://doi.org/10.1016/j.fm.2019.103383

Thioulouse, J., Dray, S., Dufour, A.-B., Siberchicot, A., Jombart, T., & Pavoine, S. (2018). Multivariate analysis of ecological data with ade4. *Springer, New York.*. https://doi.org/10.1007/978-1-4939-8850-1

Turbes, G., Linscott, T. D., Tomasino, E., Waite-Cusic, J., Lim, J., & Meunier-Goddik, L. (2016). Evidence of terroir in milk sourcing and its influence on Cheddar cheese. *Journal of Dairy Science, 99*(7), 5093–5103. https://doi.org/10.3168/jds.2015-10287

Uzun, P., Masucci, F., Serrapica, F., Napolitano, F., Braghieri, A., Romano, R., Manzo, N., Esposito, G., & Di Francia, A. (2018). The inclusion of fresh forage in the lactating buffalo diet affects fatty acid and sensory profile of mozzarella cheese. *Journal of Dairy Science, 101*(8), 6752–6761. https://doi.org/10.3168/jds.2018-14710

Yap, M., O'Sullivan, O., O'Toole, P. W., Sheehan, J. J., Fenelon, M. A., & Cotter, P. D. (2024). Seasonal and geographical impact on the Irish raw milk microbiota correlates with chemical composition and climatic variables. *MSystems, 9*(4). https://doi.org/10.1128/msystems.01290-23

Zhang, X., Zheng, Y., Feng, J., Zhou, R., & Ma, M. (2022). Integrated metabolomics and high-throughput sequencing to explore the dynamic correlations between flavor related metabolites and bacterial succession in the process of Mongolian cheese production. *Food Research International, 160*, Article 111672. https://doi.org/10.1016/j.foodres.2022.111672

Zhu, W., Lomsadze, A., & Borodovsky, M. (2010). Ab initio gene identification in metagenomic sequences. *Nucleic Acids Research, 38*(12), e132–e. https://doi.org/10.1093/nar/gkq275

Ziino, M., Condurso, C., Romeo, V., Giuffrida, D., & Verzera, A. (2005). Characterization of "Provola dei Nebrodi", a typical Sicilian cheese, by volatiles analysis using SPME-GC/MS. *International Dairy Journal, 15*(6–9), 585–593. https://doi.org/10.1016/j.idairyj.2004.07.024