



Delving into Roccaverano PDO cheese: A comprehensive examination of microbial diversity and flavour profiles compared to non-PDO cheeses

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ABSTRACT

Roccaverano Protected Designation of Origin (PDO) is a fresh soft cheese produced in Roccaverano area (Italy). This study aimed to evaluate Roccaverano PDO microbiota, together with aromatic profile and sensory analysis to be compared with 15 non-PDO cheeses of the same type. Microbiota was evaluated through shotgun metagenomics sequencing, while GC-MS analysis was conducted to study volatile organic compounds (VOCs) presence and concentration. Sensory analyses were conducted through ONAF (Italian National Organization of Cheese Tasters) evaluation parameters followed by flash profile sensory analysis of selected cheeses.

The results demonstrated *Lactococcus lactis* predominance in both non-PDO and PDO cheeses, while *Streptococcus thermophilus* was more abundant in non-PDO group. A higher abundance of *Kluyveromyces lactis* was observed in Roccaverano PDO, which exhibited greater fungal diversity compared to non-PDO cheeses. Metagenome-Assembled Genomes of 26 *L. lactis* and 19 *Leuconostoc mesenteroides* showed absence of significant differences in terms of average nucleotide identity and pangenomes partitions. The ONAF sensory evaluation demonstrated a higher average score of Roccaverano PDO group. Flash profile analysis demonstrated that lactic aroma/odour, acid, astringent, vegetal odour, exotic fruit and fermented aroma, hazelnut flavour and sweet were associated with high ONAF scores. The concentration of butanoic acid, 2-methyl-, ethyl ester and butanoic acid, 3-methyl- (sweat, acid, rancid related) were higher in PDO cheeses, while reads related to butanoate metabolism were less abundant compared to non-PDO samples. Several fungal species (included *K. lactis*) were associated with astringents, acid and chalky flavours. Roccaverano PDO demonstrates unique characteristics even maintaining a certain degree of variability between samples.

1. Introduction

Protected Designation of Origin (PDO) is a geographical indication of the European Union to preserve the origin designation of foods (Dias and Mendes, 2018). Italian PDOs include 174 foods (<https://www.tmdn.org/giview/gi/search>, accessed 06 May 2024), including Roccaverano PDO (formerly *Robiola di Roccaverano* PDO) (EC, 1996). Roccaverano PDO is a fresh soft cheese produced in Roccaverano area (Asti and Alessandria Provinces, Italy) with raw whole goat's milk or with the addition of raw whole cow's and/or ewe's milk in a variable ratio with at

least 50 % of goat's milk (Biolcati et al., 2020). *Camosciata delle Alpi* (national breed) and *Roccaverano* (local breed) are the only goat breeds from which the milk to produce Roccaverano PDO comes (<https://roccaveranodop.it/en/homepage/>, accessed 06 May 2024). Natural starter cultures (NSCs) are used in the production of this cheese without additional starter cultures, and consumed after 4–15 days of ripen (Biolcati et al., 2020). The use of NSCs and raw milk enable the obtainment of cheeses with a characteristic microbiota. Studies on the microbiota of the Roccaverano PDO were conducted to evaluate the composition of cheese microbiota (Biolcati et al., 2022, 2020).

Abbreviations: Average nucleotide identity, ANI; directly from the producer, DFP; fold changes, FC; Flash profile, FP; γ -aminobutyric acid, GABA; GC-MS, gas chromatography-mass spectrometry; Kyoto Encyclopedia of Genes and Genomes, KEGGs; Kruskal-Wallis test, K-W; large-scale distribution, LSDi; Metagenome-Assembled Genomes, MAGs; Mann-Whitney, M-W; Non-metric Multi-Dimensional Scaling, NMDS; Natural starter culture, NSC; ONAF, Italian National Organization of Cheese Tasters; Principal Component Analysis, PCA; Principal Coordinates Analysis, PCoA; Protected Designation of Origin, PDO; purchase to local market, PLM; parts per billion, ppb; Total sum scaling, TSS; Volatile Organic Compounds, VOCs.

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Lactococcus lactis was found to be the prevalent species in raw milk and NSCs, while *Leuconostoc mesenteroides* was the predominant species in cheeses of ripened for 5 and 15 days (Biolcati et al., 2020). The study of fungal isolates revealed a prevalence of *Kluyveromyces* spp. in NSC while in cheese *Geotrichum candidum* and *Yarrowia lipolytica* were prevalent (Biolcati et al., 2022, 2020). Roccaverano cheese core microbiota is characterized by *L. lactis*, *G. candidum*, and *Kluyveromyces marxianus* (Biolcati et al., 2022). Microorganisms influence the fermentation and ripen food processes, facilitating the development of molecules that influence the sensorial characteristics of the product (Sharma et al., 2020). Bacterial pathways, including those associated with lactic acid bacteria, can affect the production of volatile organic compounds (VOCs), which consequently influence the sensory characteristics of cheeses (Feng et al., 2024). The interconnection between cheeses microbiome and its sensory characteristics underscores the importance of evaluating microbiome composition to study potential correlations between cheese factors and microorganisms. Although the microbiome of Roccaverano PDO has been assessed through metagenomic approaches, cheeses characteristics are influenced not only by microbial species but also by strains and specific metabolic pathways (Yang et al., 2021). Specific strains characteristics can be evaluated through shotgun metagenomic analysis allowing the evaluation of microbiome composition and microbiota pathways (Walsh et al., 2023). Moreover, several bacterial strains can be detected in the same food matrix (Yang et al., 2021). The shotgun approach has been used for several foods, including cheeses (Fontana et al., 2023). However, shotgun metagenomic studies focused on Roccaverano PDO have been not conducted. Considering the importance of PDO designation for foods certification and its influence on the economic values of PDO foods (University of Parma, 2023), studying PDO products is of economic and scientific interest. Moreover, PDO designation can promote the development of rural areas, including social improvements linked to tourism and trade (Marescotti, 2003). Considering the importance of Roccaverano PDO for its production area, aim of this study is the comparison of Roccaverano PDO cheeses with non-PDO cheeses of the same type. The study includes Roccaverano PDO cheeses collected from the fifteen producers of Roccaverano Consortium. Moreover, the inclusion of commercial competitor non-PDO cheeses allowed the evaluation of artisanal and not artisanal cheeses to be compared with Roccaverano PDO. The cheeses were subjected to microbiome evaluation through shotgun metagenomic approach. Microbiota data were analysed with sensory and chemical analysis (volatile organic compounds), to determine possible correlations between different cheeses characteristics and to evaluate the presence of potential markers specific to Roccaverano PDO cheese.

2. Materials and methods

2.1. Cheeses sampling and samples storage

The cheese samples were collected between May 3rd and June 6th, 2023, selecting 15 Roccaverano PDO cheeses and 15 soft-cheeses of the same type (*robiola*) and seasoning (10 days) (Table 1). PDO samples were collected from 15 producers affiliated with the Roccaverano PDO consortium.

All PDO were produced with pure raw goat's milk, while non-PDO samples were produced with pure raw goat's milk or pasteurized (Table 1). The samples were obtained from producers, local market, and large-scale distribution.

The samples collection was performed including the rind and obtaining a slice that encompasses the various sections of the cheese wheel. The sampling procedure was conducted storing 30 g of cheese destined to VOCs analysis at -20°C . Ten grams of cheese from each sample were homogenized in Ringer's solution (96,724, Millipore, U.S.A) (1:10 ratio) for 1 min using a Stomacher® 400 Circulator (LAB blender 400; PBI, Italy). A total of 1.8 ml, destined to DNA extraction, was centrifugated ($7000 \times g$ for 10 min) and stored at -20°C after

Table 1

Information about 39 cheese samples object of study. Information about cheese samples is reported in the table below including samples codes, PDO or non-PDO, sampling data and area. Samples with same code number were collected from the same producer. The asterisk near samples codes indicates samples from the same producer and distributor. The table include information about the sensory test performed: flash profile (FP) or ONAF standard evaluation (ONAF). The type of distribution from where the cheeses were obtained is indicated as directly from the producer (DP), large-scale distribution (LSDi) and purchase to local market (PLM). The symbols “°” next to the non-PDO sample names indicate the use of pasteurized milk, PDO cheeses were produced with pure raw goat's milk.

Code	PDO/ non-PDO	Sampling data	Sensory test	Area	Distribution
R1*	PDO	03 May 2023	ONAF	Roccaverano area	DP
R2*	non-PDO	03 May 2023	ONAF	Roccaverano area	DP
R3*	non-PDO	03 May 2023	ONAF	Roccaverano area	DP
R4	PDO	03 May 2023	ONAF	Roccaverano area	DP
R5	PDO	03 May 2023	ONAF	Roccaverano area	DP
R6	PDO	03 May 2023	ONAF	Roccaverano area	DP
R7	PDO	03 May 2023	ONAF	Roccaverano area	DP
R8	PDO	03 May 2023	ONAF	Roccaverano area	DP
R9	PDO	03 May 2023	ONAF	Roccaverano area	DP
R10	PDO	03 May 2023	ONAF	Roccaverano area	DP
R11	non-PDO	10 May 2023	ONAF	Cuneo Province	DP
R12°	non-PDO	10 May 2023	ONAF	Bergamo Province	LSDi
R13	non-PDO	10 May 2023	ONAF	Cuneo Province	LSDi
R14°	non-PDO	10 May 2023	ONAF	Cuneo Province	LSDi
R15	non-PDO	10 May 2023	ONAF	Cuneo Province	DP
R16	PDO	10 May 2023	ONAF	Roccaverano area	LSDi
R17	non-PDO	10 May 2023	ONAF	Roccaverano area	DP
R18	PDO	10 May 2023	ONAF	Roccaverano area	DP
R19	PDO	10 May 2023	ONAF	Roccaverano area	DP
R20	non-PDO	10 May 2023	ONAF	Cuneo Province	PLM
R21°	non-PDO	10 May 2023	ONAF	Cuneo Province	PLM
R22°	non-PDO	30 May 2023	ONAF	Cuneo Province	LSDi
R23	PDO	30 May 2023	ONAF	Roccaverano area	DP
R25°	non-PDO	30 May 2023	ONAF	Lombardia	LSDi
R26	non-PDO	30 May 2023	ONAF	Cuneo Province	DP
R27	non-PDO	30 May 2023	ONAF	Cuneo Province	LSDi
R28	PDO	30 May 2023	ONAF	Roccaverano area	DP
R29	PDO	30 May 2023	ONAF	Roccaverano area	DP
R30	PDO	30 May 2023	ONAF	Roccaverano area	DP
R31	non-PDO	30 May 2023	ONAF	Treviso Province	LSDi
F1	PDO	06 June 2023	FP	Roccaverano area	DP

(continued on next page)

Table 1 (continued)

Code	PDO/ non-PDO	Sampling data	Sensory test	Area	Distribution
F2	non-PDO	06 June 2023	FP	Roccamerano area	DP
F5	PDO	06 June 2023	FP	Roccamerano area	DP
F19	PDO	06 June 2023	FP	Roccamerano area	DP
F20	non-PDO	06 June 2023	FP	Cuneo Province	PLM
F25°	non-PDO	06 June 2023	FP	Lombardia	LSDi
F26	non-PDO	06 June 2023	FP	Cuneo Province	DP
F27	non-PDO	06 June 2023	FP	Cuneo Province	LSDi
F29	PDO	06 June 2023	FP	Roccamerano area	DP

removing the supernatant. The remaining portions of cheese wheels were used for sensory analyses, which were conducted within four hours of samples collection for metagenomics and VOCs analysis.

Eight cheese samples (4 PDO and 4 non-PDO) were selected on the base of their higher and lower ONAF sensory score and subjected to a flash profile (FP) sensory test (Table 1). An additional non-PDO cheese (F2) was included considering the production of Roccamerano PDO from the same dairy.

2.2. DNA extraction and shotgun sequencing

Total DNA from cheese samples was extracted using DNeasy PowerFood Microbial Kit (21000–100-MON, Qiagen, Germany) following kit instructions. The nucleic acids eluted in 100 µl of the kit elution buffer have been treated with 2 µl of RNaseA 10 mg/ml (G118, Applied Biological Materials Inc. (abm), Canada) for 45 min at 37 °C. The DNA quality was evaluated with NanoDrop (ND 1000, Thermo Fisher Scientific, U.S.A) and an electrophoretic run of an agarose gel 0.8 % (w/v) in Tris-acetate-EDTA 1X (100 V for 30 min) using GelRed as stain (41,001, Biotium, U.S.A). Good quality samples have been stored at –20 °C.

Shotgun metagenomics sequencing (paired-end 150 bp, 9 Gb per samples) was performed on Illumina Novaseq 6000 machine by Novogene company (Cambridge, United Kingdom). After Qubit 2.0 quantification, 1 µg of DNA was used for library preparation using NEBNext® library prep Kit, randomly fragmented and then polished, A-tailed, and ligated with the NEBNext adapter for Illumina sequencing, and PCR enriched. The purification PCR products was performed with AMPure XP system. The libraries were analysed by Agilent 2100 Bioanalyzer and quantified using real-time PCR. The reads were quality filtered with Solexa QA++ software (Cox et al., 2010), sequences below 60 bp and dereplicated sequences were removed by Prinseq (Schmieder and Edwards, 2011). Raw sequence reads are available on Sequence Read Archive (National Center for Biotechnology Information; NCBI) under the accession number PRJNA1160470 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1160470>; link accessed on October 10, 2024).

2.3. Bioinformatics analysis

The subsequent bioinformatics tools were employed with default settings unless differentially specified. Raw sequence reads were quality filtered with AdapterRemoval vr. 2.3.3 (min. quality 30) (Schubert et al., 2016), while host reads were removed with Bowtie2 vr. 2.5.3 (Langmead and Salzberg, 2012), detecting goat's reads using the genome assembly GCF_001704415.2 (NCBI; https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_001704415.2/; link accessed on April 2, 2024). Metaphlan3 vr. 3.0 (Truong et al., 2015) was used to obtain the number of reads corresponding to bacterial species in the samples.

Information about fungi was obtained comparing the Eukdetect database (https://figshare.com/articles/dataset/Eukdetect_database/12670856; link accessed on April 2, 2024) (Lind and Pollard, 2021) with the reads using Bowtie2. The assemblies were performed using SPAdes vr. 3.15.5 (meta option) (Bankevich et al., 2012), eliminating the contigs below 500 bp. Assembly quality control was performed with Quast vr. 5.2.0 (Gurevich et al., 2013), while genome reconstruction with Metabat2 (Kang et al., 2019) after contig mapping on the reads with Bowtie2. CheckM vr. 1.2.2 (Parks et al., 2015) was used to check MAGs completeness and contamination maintaining high and medium quality bins (>90 % completeness, <5 % contamination). PhyloPhlAn vr. 3.1 (Asnicar et al., 2020) was used to identify bins taxonomy. Tormes vr. 1.3.0 (Quijada et al., 2019) was used for bins pangenome analysis of MAGs species to obtain information about core/accessory genes and presence of possible antibiotic resistance related genes. Average nucleotide identity (ANI) was calculated using ANIclustermap vr. 1.3.0 (<https://github.com/moshi4/ANIclustermap>; link accessed on April 2, 2024).

2.4. GC-MS analysis

The volatile organic compounds (VOCs) in cheese samples were extracted using headspace (HS) solid-phase microextraction (SPME) and analysed with gas chromatography–mass spectrometry (GC–MS) in three replicates. The analysis was performed in 20 ml screw-cap glass vials on 1 g of cheese homogenized with 1 ml of sodium chloride (27,800.291, VWR, U.S.A.) solution (30 % w/v) in ultrapure water and 10 µl of 1,3,5-triisopropylbenzene (53.5 ppm in methanol) as an internal standard. HS-SPME followed by GC/MS was performed following the SPME extraction (vial with the sample was maintained for 10' at 40 °C for the equilibrium, than a Carboxen/Polydimethylsiloxane/Divinylbenzene (CAR/PDMS/DVB), 1 cm, 50/30 µm film thickness (Supelco, Bellefonte, PA, USA) fiber was exposed to the headspace for 30' at 40 °C using an SPME autosampler (PAL System, Combi PAL, Zwingen, Switzerland), followed by the removing of the fiber that was inserted into injector in splitless mode at 260 °C for 2 min. GC–qMS analysis was performed using a Shimadzu GC-2010 gas chromatograph equipped with a Shimadzu QP-2010 Plus quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a StabilWAX-MS capillary column with 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness (Restek, Italy). The temperature programme started at 40 °C and was maintained for 2 min, then increased at a rate of 4 °C/min to 200 °C, and finally increased at a rate of 10 °C/min to 250 °C (held for 5 min). The flow rate of the carrier gas (He) was 1 ml/min. The injection port temperature was 260 °C, the ion source temperature was 200 °C and the interface temperatures was 250 °C. Detection was performed using electron ionisation at 70 eV with a scan range of 33–350 m/z. Tentative identification was performed using mass spectral matching against the NIST 17 MS library, while analyte identification was confirmed using analytical standards, when available.

Semiquantitative measurements (ppb) were derived by assessing the m/z peak area of the quantifier ion for each identified compound, normalized against the total peak area, 189 m/z ion intensity of the included internal standard.

2.5. Sensory analysis

The sensory analysis of cheeses was performed by ONAF (Official National Tasters of Cheeses) expert using the sensory Official ONAF card for cheese evaluation. For each sample was evaluated the appearance, the pasta colour, the holes, the structure, the smell/aroma, the taste and structure in mouth. Evaluation was performed using scores between 4 and 10 for the first four parameters and scores between 8 and 20 for the last three. Sensory analysis was performed in a sensory room at 20 °C with white light. Natural water was used to rinse the mouth between the samples. Cheeses were coded with a numeric code and examined in a

randomized order. No information was provided to tasters on cheese classification. Cheeses with higher and lower total scores (Table 1) were selected to perform the flash profile analysis (Bredie et al., 2018).

2.6. Statistical analysis

Statistical analysis about taxonomy and KEGGs-VOCs were conducted using MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml>, 2024-03-08) (Lu et al., 2023) and Microeco v. 1.4.0 (Liu et al., 2021) in RStudio v. 2023.09.1 (R v. 4.3.2). Multivariate statistics was performed in RStudio using Vegan v. 2.6–4. Homogeneity tests were performed in R using Shapiro–Wilk's W and Modified Levene's tests (Brown–Forsythe test). Kruskal–Wallis test (K–W), Mann–Whitney (M–W) and two-sample *t*-test were performed to evaluate differences between two groups, for nonparametric (K–W; M–W) and parametric data (*t*-test).

3. Results

3.1. Bacterial microbiome composition

The 97.9 % of reads taxonomic units count were related to Firmicutes phylum (st. dev. 4.8) (Supplementary table 1). Twenty-eight bacterial family were detected in both PDO and non-PDO samples. Microbiome analysis revealed a high abundance of bacteria in the *Streptococcaceae* family (average 91.2 %, st. dev. 16.2), followed by *Leuconostocaceae* (average 5.2 %, st. dev. 7.2) and *Lactobacillaceae* (average 1.2 %, st. dev. 6.6). Linear Discriminant Analysis (LDA) (Supplementary fig. 1A) scores (LDA score 3.9) indicated that *Pseudomonadaceae* were significantly more abundant in non-PDO group (average of 15.2 %, dev. st. 4.6) compared to PDO group (average 0.01 %, dev. st. 0.03). Similarly, the genus *Pseudomonas* spp. (LDA score 3.9) was significantly more abundant in non-PDO group (0.33 % st. dev. 0.79 compared to PDO 0.01 % st. dev. 0.03) while *Acinetobacter* spp. (LDA score 3.4) was more abundant

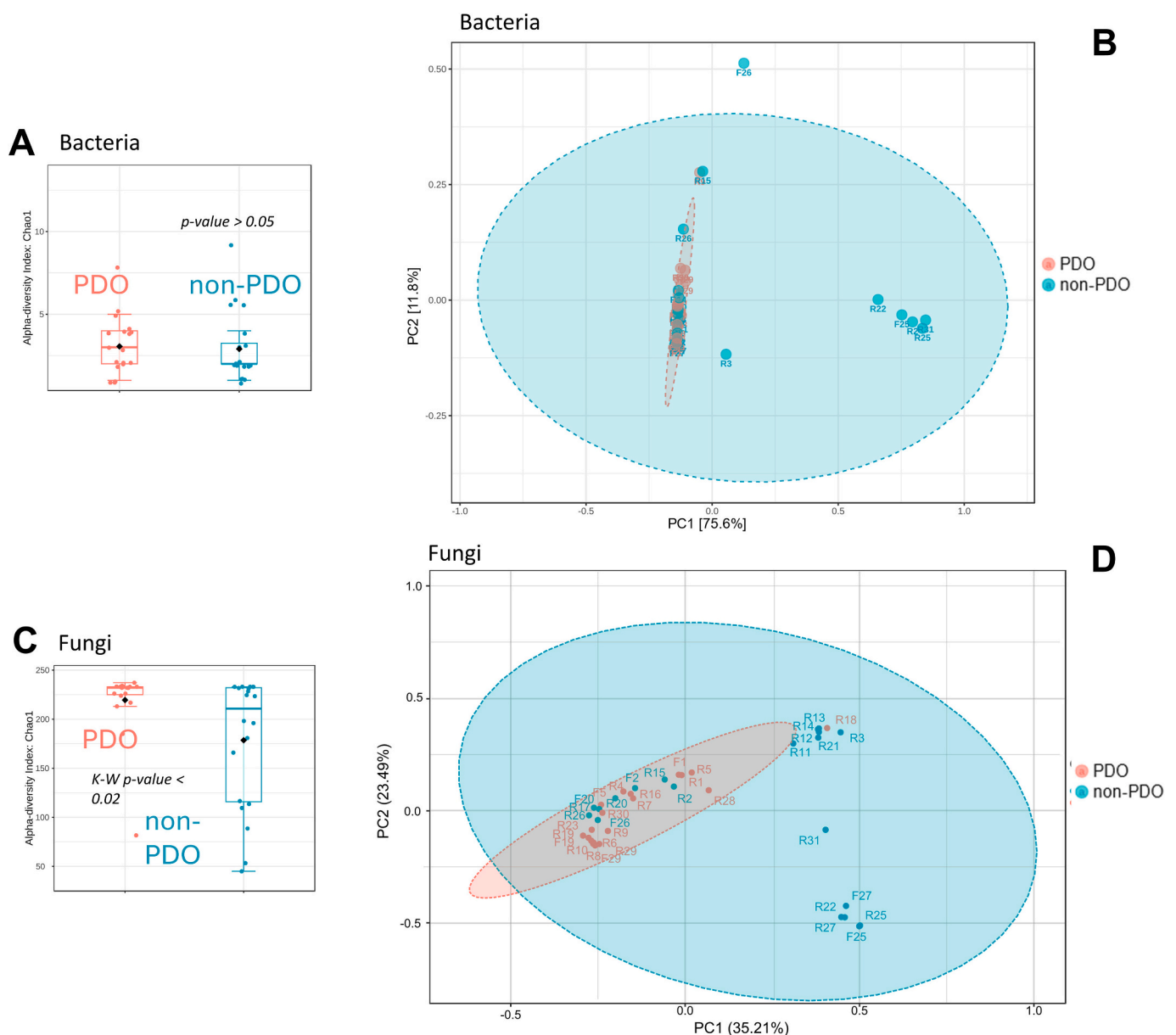


Fig. 1. Plots about Alfa-diversities (A, C) and PCoA about beta-diversities (B, D). The figure shows α -diversities of bacteria (A) and fungi species (C). Fungi α -diversities resulted statistically different between PDO and non-PDO groups. Bacteria species β -diversity (B) showed separation between groups (PC1 75.60 %) while fungi β -diversity (D) demonstrated a lower separation degree between groups (PC1 35.21 %).

in PDO samples (average 21.4 %, dev.st. 33.1 %) than in the non-PDO (average 4 %, dev.st. 10.9 %) (Supplementary fig. 1).

The cheeses microbiome included 124 bacterial species (Supplementary table 1). The bacterial core microbiome of both PDO and non-PDO cheeses was represented by four lactic acid bacteria: *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactococcus raffinolactis* and *Leuconostoc pseudomesenteroides*. Core microbiome of PDO cheeses included *Streptococcus parauberis*, while non-PDO core microbiome was characterized by *L. lactis*, *Streptococcus thermophilus*, *L. mesenteroides* and *L. piscium*. No significant differences were observed between groups α -diversities (p -value > 0.05) (Fig. 1A). Twenty-seven species (Supplementary table 2) were found only in PDO cheeses, including the following lactic acid bacteria: *Lactobacillus otakiensis*, *Enterococcus durans*, *Vagococcus teuberi*, *Lactobacillus curvatus*, *Pediococcus pentosaceus*, *Lactobacillus kefir-anofaciens*, *Lactococcus garvieae*, *Lactococcus petauri* and *Enterococcus italicus*. Forty-five species were detected exclusively in non-PDO cheeses (Supplementary table 2), including *Psychrobacter* sp., *Lactobacillus harbiniensis*, *Lactobacillus diolivorans*, *Lactobacillus kefir*, *Enterococcus*

devriesei, *Marinilactibacillus psychrotolerans*, *Enterococcus mundtii*, *Lactobacillus sakei* and *Lactobacillus delbrueckii*. *L. lactis* represents the most abundant bacterial species in both groups (Fig. 2A, B, C). *S. thermophilus* (\log_2 fold changes (FC) 4.46) and *L. piscium* (\log_2 FC 1.83) were found to be statistically more abundant in non-PDO cheeses (p -value [FDR] < 0.05) (Fig. 2C) (Supplementary table 3). *S. parauberis* was more abundant in PDO cheeses (\log_2 FC 1.97, p -value [FDR] < 0.05) (Fig. 2C). Bacterial β -diversity demonstrates similarities between PDO samples; however, some non-PDO samples are not separated from PDO group (Fig. 1B).

3.2. Fungi microbiome composition

Fungi taxonomy evaluation showed that 77.5 % and 68 % of the fungi reads were related to Saccharomycetales order in PDO and non-PDO groups respectively, with *Saccharomycodaceae* as more numerous family (40.6 % in PDO and 19.1 % in non-PDO). The cheeses fungi microbiome was characterized by 233 species (Fig. 3A, B;

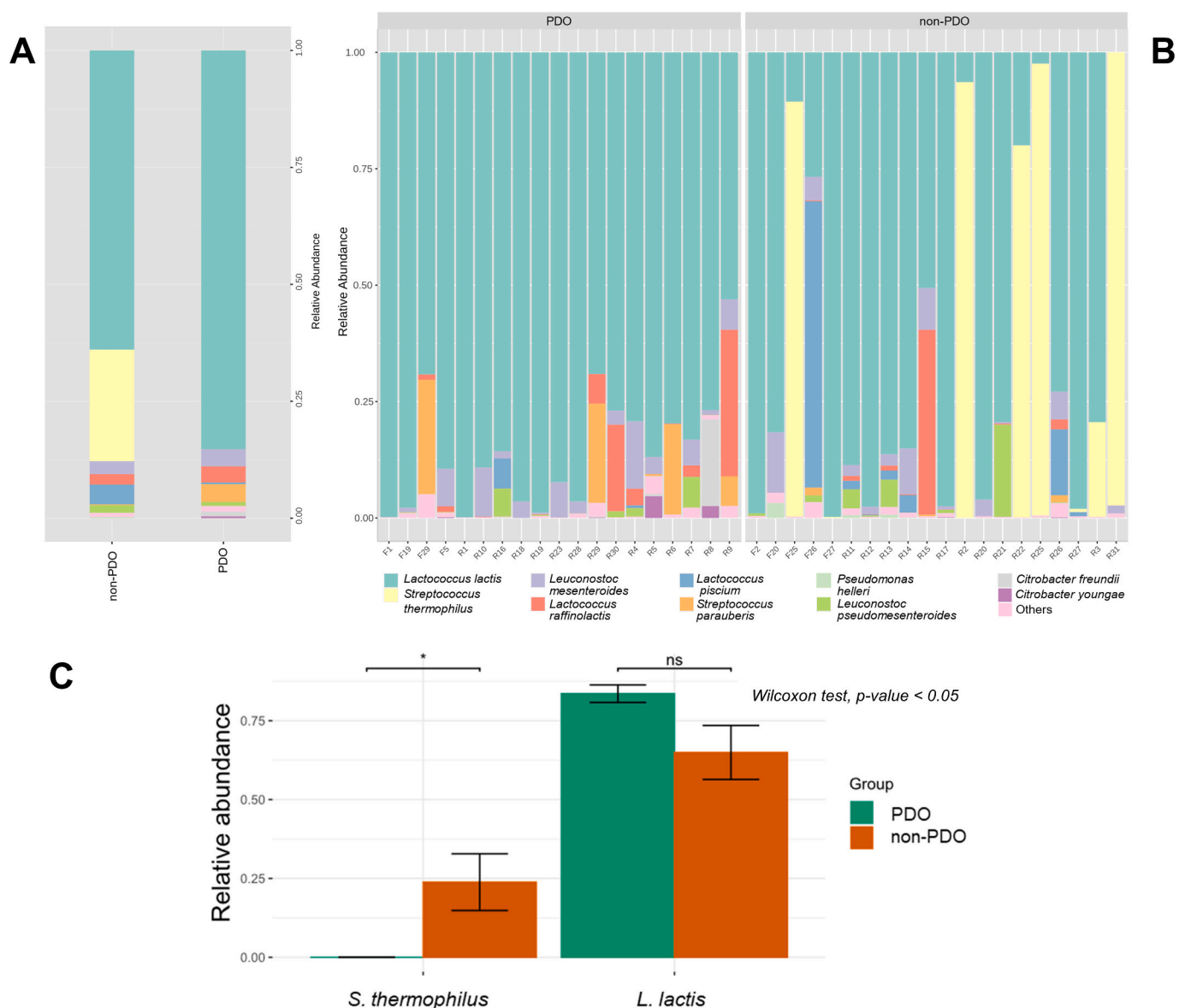


Fig. 2. Microbiome composition related to bacterial taxa The figure shows plots about bacterial species relative abundances of total sum scaling (TSS) (A, B). In plots A and B (common colours legend), are shown the ten most abundant species. The total abundances related to PDO and non-PDO groups are reported in the plot A while plot B shows abundances for each sample. The plot C shows Wilcoxon test results (filter_thres = 0.01, Microeco package) of bacterial taxa. *S. thermophilus* resulted statistically different between PDO and non-PDO groups (p -value < 0.05).

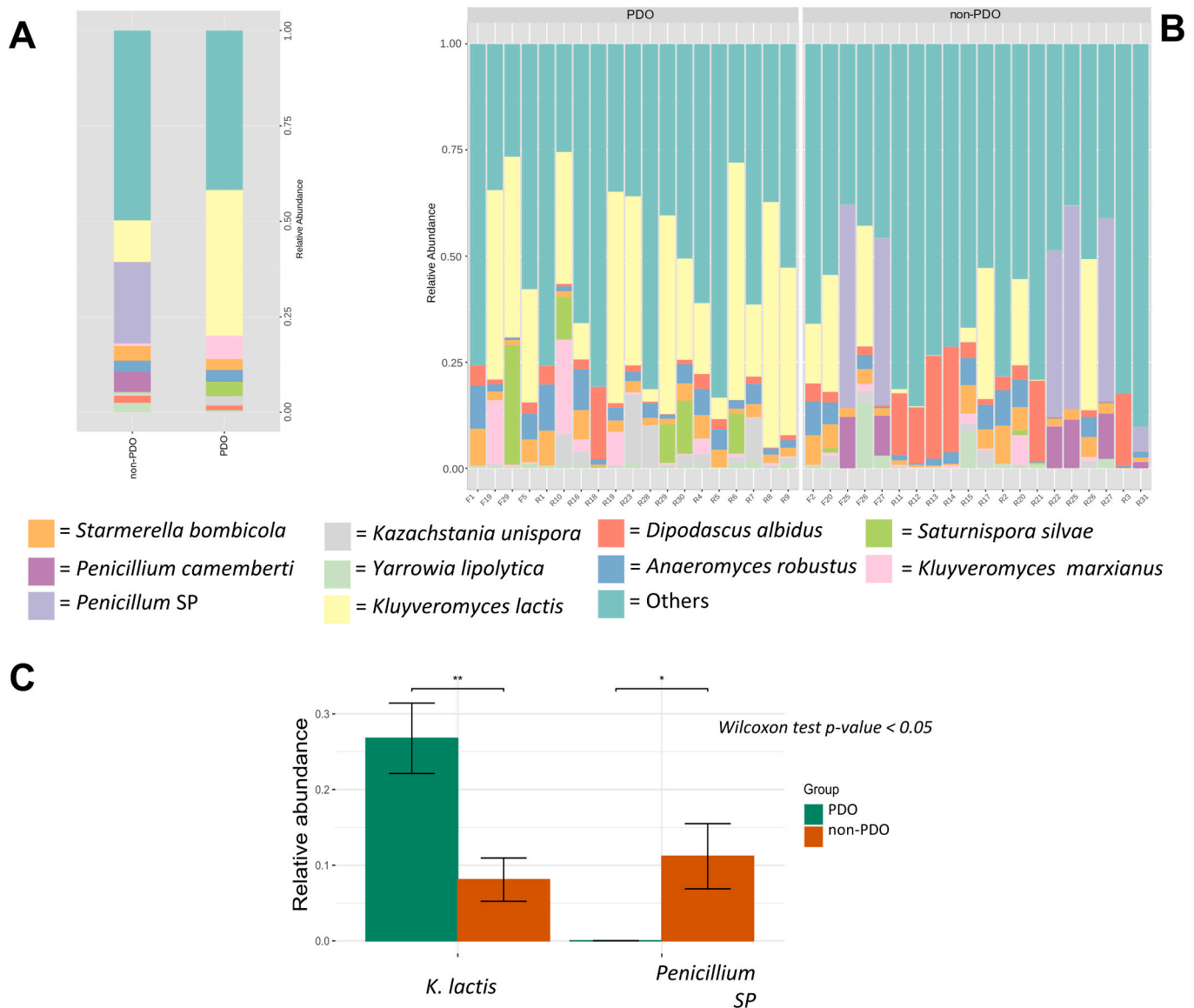


Fig. 3. Microbiome composition related to fungi taxa. The figure shows plots about fungal species relative abundances of total sum scaling (TSS) (A, B). In plots A and B (common colours legend), are shown the ten most abundant species. The total abundances related to PDO and non-PDO groups are reported in the plot A while plot B shows abundances for each sample. The plot C shows Wilcoxon test results (filter_thres = 0.01, Microeco package) of fungal taxa. *K. lactis* and *Penicillium sp.* resulted statistically different between PDO and non-PDO groups (p -value < 0.05). A single asterisk (*) and double asterisks (**) indicate a p -value of < 0.05 and < 0.01, respectively.

Supplementary table 4). No species were found exclusively in the PDO or non-PDO group. Average percentages demonstrates that *Kluyveromyces lactis* represents the 17.39 % (st. dev. 19.01) of the total fungi reads. The composition of the fungal microbiome varied between samples, with *K. lactis*, *Anaeromyces robustus*, and *Starmarella bombicola* exhibiting high prevalence in the core microbiome (> 0.8).

Fungal α -diversity resulted significantly higher in PDO cheeses ($K-W$, p -value < 0.02), with a higher variability in non-PDO group (Fig. 1C).

LDA scores showed a high correlation of Roccaverano PDO samples with *Saccharomycodaceae* and *K. lactis* with LDA scores of 5 and 4.9 respectively, however *K. lactis* relative abundance resulted variable between PDO samples (average 27.04 %, st. dev. 20.43) (Supplementary fig. 1B). The abundance of *K. lactis* was higher in PDO group while *Penicillium sp.*, was more abundant in non-PDO samples (Wilcoxon test, p -value < 0.05) (Fig. 3C). Eighty-eight species were differentially abundant in PDO and non-PDO groups (p -value [FDR] < 0.05) (Supplementary table 3). Fourteen species with a \log_2FC above 2 were

associated to non-PDO group *Thielaviopsis ethacetica*, *Eremothecium coryli*, *Hanseniaspora nectarophila*, *Candida intermedia*, *Chaetothyriales sp.*, *Tremella mesenterica*, *Neurospora sublineolata*, *Rhizopogon vinicolor*, *Preussia sp.*, *Allomyces macrogynus*, *Yarrowia lipolytica*, *Hesseltinella vesiculosa*, *Debaryomyces hansenii* and *Candida hawaiiiana*. Positive associations with PDO group ($\log_2FC > 2$) were observed for *Kluyveromyces dobzhanskii*, *K. lactis*, *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Kazachstania unispora*, *Trichosporon faecale* and *Saturnispora silvae*. Fungal β -diversity showed separation between samples with a low principal component 1 (PCoA; 35.21 %) demonstrating a not clear separation between PDO and non-PDO samples (Fig. 1D).

3.3. Comparison of PDO and non-PDO MAGs and KEEGs

Metagenomes assembly allowed the obtainment of 26 *L. lactis* (9 in PDO samples and 17 in non-PDO samples) and 19 *L. mesenteroides* (9 in PDO samples and 10 in non-PDO samples) MAGs. In *L. lactis* two core genes (present in 99–100 % of the samples) were detected, while most of

the genes were grouped in shell ($n = 3288$) and cloud ($n = 4154$) partitions. *L. mesenteroides* was characterized by 461 core genes, while shell and cloud genes resulted 1395 and 1484 respectively. The average nucleotide identity (ANI) and pangenome analysis demonstrated a low separation between MAGs from PDO and non-PDO samples (Fig. 4). Four MAGs (R2.2, F2.4 and R2.5, F2.2) demonstrates a persistence of *L. lactis* in cheese sampled in different days (comparison with FP samples) (Table 1 and Fig. 4A, B).

Part of the genes was found only in MAGs from a specific cheese group. *L. lactis* MAGs were characterized by 78 genes found only in PDO samples and 260 in non-PDO samples. At least one copy of cation-proton antiporters CtpE gene (calcium supplier for cell surface integrity) (Gupta et al., 2017) was found in all *L. lactis* MAGs, however R10.10 (PDO) was characterized by *ctpE3* and 4 (Poolman, 2023). MAG R29.14 (PDO) contained *fetA* and *fetB* (iron homeostasis). This same MAG also showed *nisZ* linked to nisin synthesis (Cheigh et al., 2005), which not found in other *L. lactis* MAGs. Similarly, *lcnB*, related to lactococcin bacteriocin production (Venema et al., 1993) was found only in R5.6 (PDO). *L. mesenteroides* was characterized by 130 and 77 genes respectively present only in non-PDO and PDO MAGs. F5.9 and R30.9 contains *clcA* (H^+/Cl^- antiporter ClcA), while apparently no other genes present in only one group presented function technologically relevant for cheese (Poolman, 2023; Wang et al., 2018).

3.4. KEEGs and associated VOCs

Kyoto Encyclopedia of Genes and Genomes (KEEGs) demonstrates carbohydrate (average 21,586, st. dev. 4516) and amino acid (average 19,491, st. dev. 5683) metabolisms as the most abundant categories in PDO and non-PDO samples without significant differences. PCA analysis didn't show significant differences between PDO and non-PDO samples (Supplementary fig. 2). Seventy-five genes were related to Kyoto Encyclopedia of Genes and Genomes (KEEGs) with antibiotic resistances β -lactamase related functions without correlation with samples groups (PDO and non-PDO). Specific pathways demonstrated statistical differences in their abundances between PDO and non-PDO (M-W; p -value < 0.05). Lower abundances in PDO samples were observed for alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine biosynthesis, phenylalanine tyrosine and tryptophan biosynthesis, cysteine and methionine metabolism, arginine and proline metabolism, and butanoate metabolism (M-W; p -value < 0.05) (Fig. 5A). Sixty-two KEEGs pathways were found to be differentially abundant (\log_2 FC > 1.5 or < -1.5; p -value [FDR] < 0.05) between PDO and non-PDO samples. Among these pathways, some related to carbohydrate metabolisms (dolichyl-phosphate-mannose-protein mannosyltransferase, transaldolase, D-sedoheptulose 7-phosphate isomerase) and organic acid (isocitrate lyase) metabolisms were more abundant in PDO samples. Four PTS system related to sucrose and mannose transport demonstrated a negative \log_2 FC with PDO samples. Others twenty-four KEEGs related showed negative \log_2 FC (< -1.5) with PDO cheeses

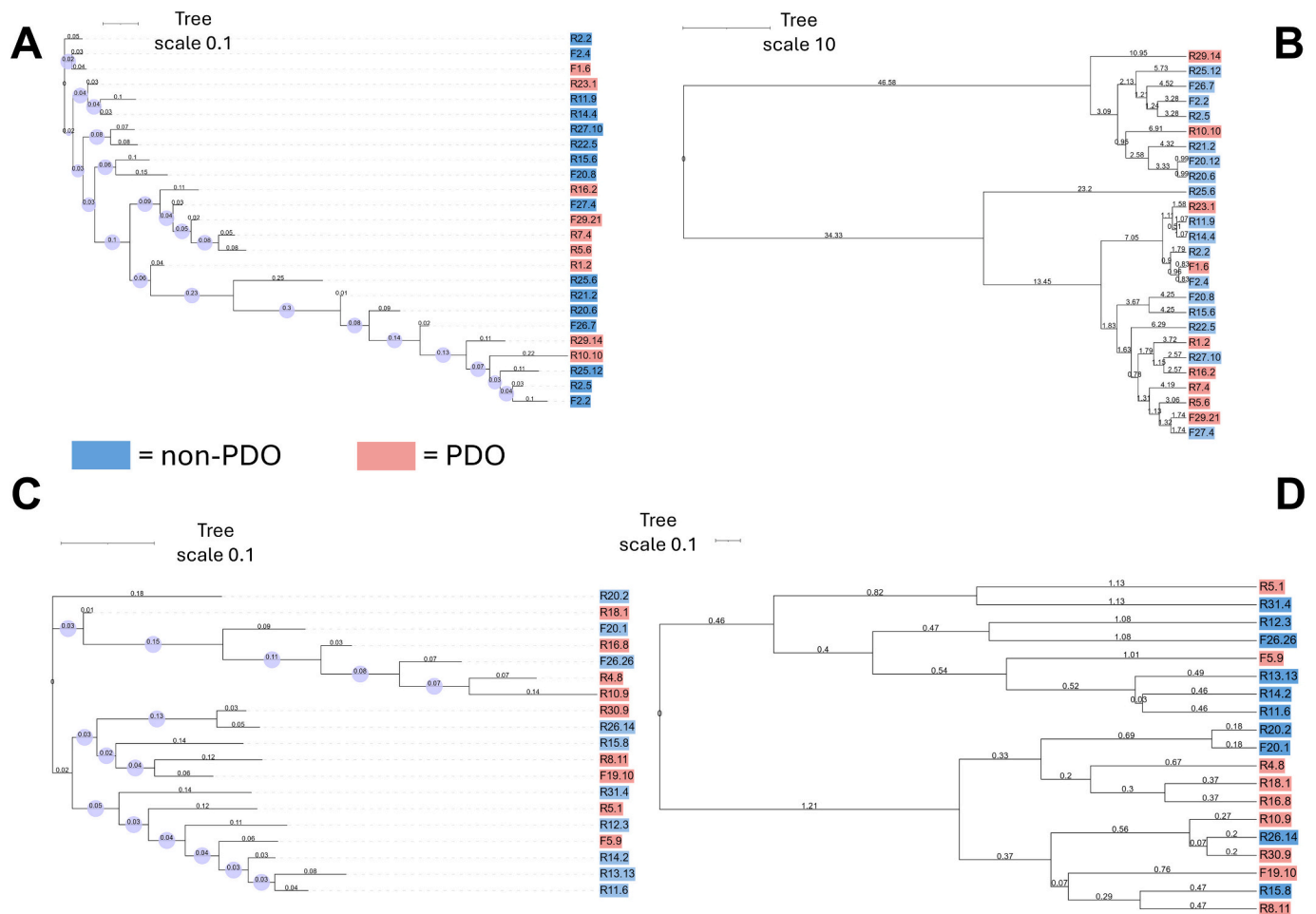


Fig. 4. Dendrograms about MAGs comparison. Pangenome dendrograms (Roary core genomes) are shown in panels A, C and dendrograms about ANI analysis (B, D) of *L. lactis* (A, B) and *L. mesenteroides* (B, D). The figures show PDO and non-PDO MAGs comparison, panels A and C shown bootstrap values between 0.98 and 1 (A) and 0.5–1 (C).

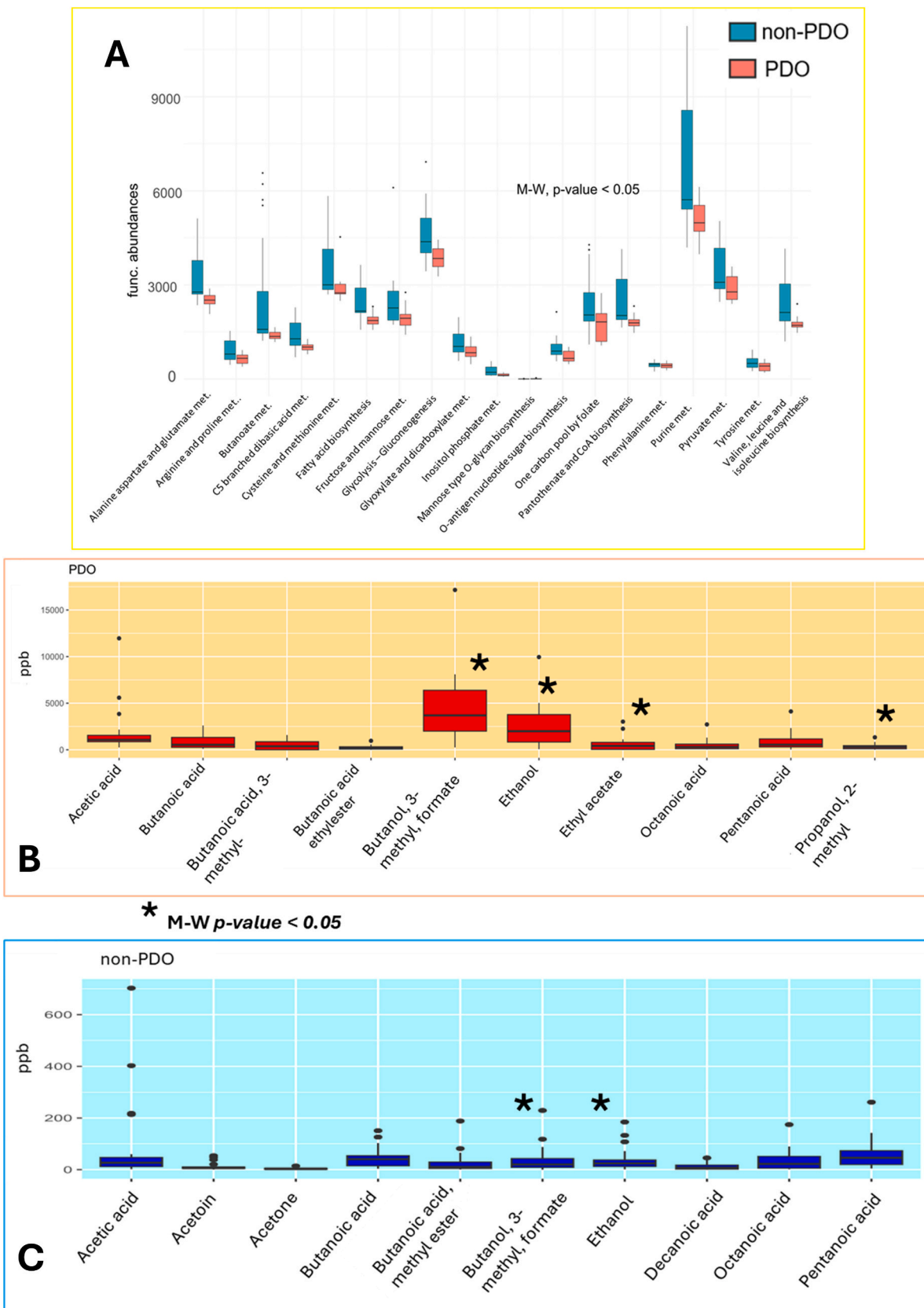


Fig. 5. Pathways statistically different between PDO and non-PDO samples (A) and ten most abundant VOCs detected in PDO (B) and non-PDO (C) cheeses. The plot A shows functional abundances statistically different of pathways (M-W, P -value < 0.05) in PDO and non-PDO cheeses. The plots B and C shown ten most abundant VOCs (ppb) in PDO and non-PDO cheeses respectively. M-W statistical differences are indicated by asterisks (*).

(Supplementary table 5). Among these pathways, spermidine/putrescine transport system permease and substrate-binding protein, as well as a urease subunit alfa.

In PDO and non-PDO cheeses 343 VOCs were detected. Among ten most abundant VOCs in both groups (Fig. 5B, C; Supplementary table 6) 1-butanol, 3-methyl-, formate and ethanol concentrations resulted higher in PDO samples (M-W, p -value < 0.05 and \log_2 FC 3620 and 1380 respectively). PDO cheeses demonstrated a higher concentration of ethyl acetate and 1-propanol, 2-methyl- (M-W, p -value < 0.05 and \log_2 FC 436 and 281 respectively). Other molecules showed a higher concentration (\log_2 FC > 100) in PDO cheeses. These molecules were pentanoic acid, 3-methyl- (\log_2 FC 177), butanoic acid, 3-methyl- (\log_2 FC 304), butanol, 3-methyl-, acetate (\log_2 FC 232), phenylethyl alcohol (\log_2 FC 129). VOCs with negative \log_2 FC were less numerous in PDO, 4-pentadiene showed the greater \log_2 FC (\log_2 FC -31).

3.5. Sensory analysis and correlations with microbiota and VOCs

PDO group demonstrated a higher total score in ONAF sensory test with an average score of 79.7 (st. dev. 6.5) for PDO cheeses and 72.7 (st. dev. 9.4) for non-PDO cheeses (t -test, p -value < 0.05) (Supplementary table 7).

Multivariate analysis that included sensory ONAF evaluation, VOCs concentration and microbiome composition demonstrated the influence

of some variable in the characterization of PDO and non-PDO (Non-metric Multi-Dimensional Scaling (NMDS), arrows with p -value < 0.002) (Supplementary fig. 3). Aspect, colour, structure, and odour structure indicated a positive association with PDO cheeses. *K. lactis* confirmed the positive correlation with PDO, while *L. lactis* contributed to the grouping of PDO cheeses. *S. thermophilus* demonstrated a higher abundance in non-PDO samples.

The analysis of samples subjected to FP sensory evaluation revealed association between sensory characteristics and VOCs-microbiome data (Fig. 6, Supplementary fig. 4). Vegetal odour, exotic fruit flavour, lactic aroma, astringent, astringent odour, acid and chalky were related to PDO group while rancid aroma, fungus/mould odour and adhesive to non-PDO cheeses that showed low ONAF evaluation scores (Fig. 6, Supplementary fig. 4). Two cheeses with high evaluation scores were associated with lactic aroma (Fig. 6). F1 and F2 (respectively PDO and non-PDO, high ONAF evaluation scores) from the same producer resulted positioned in the same PCA plot area (Fig. 6). The sensory characteristics of vegetal odour and exotic fruit flavour were positioned in the same plot area, along with astringent, lactic aroma and astringent odour, acidity and chalkines. Among the mentioned relevant VOCs, as butanoic acid, 3-methyl-, ethyl ester and 1-butanol, 3-methyl-, formate were present in the same area characterized by vegetal odour, exotic fruit, astringent and lactic aroma.

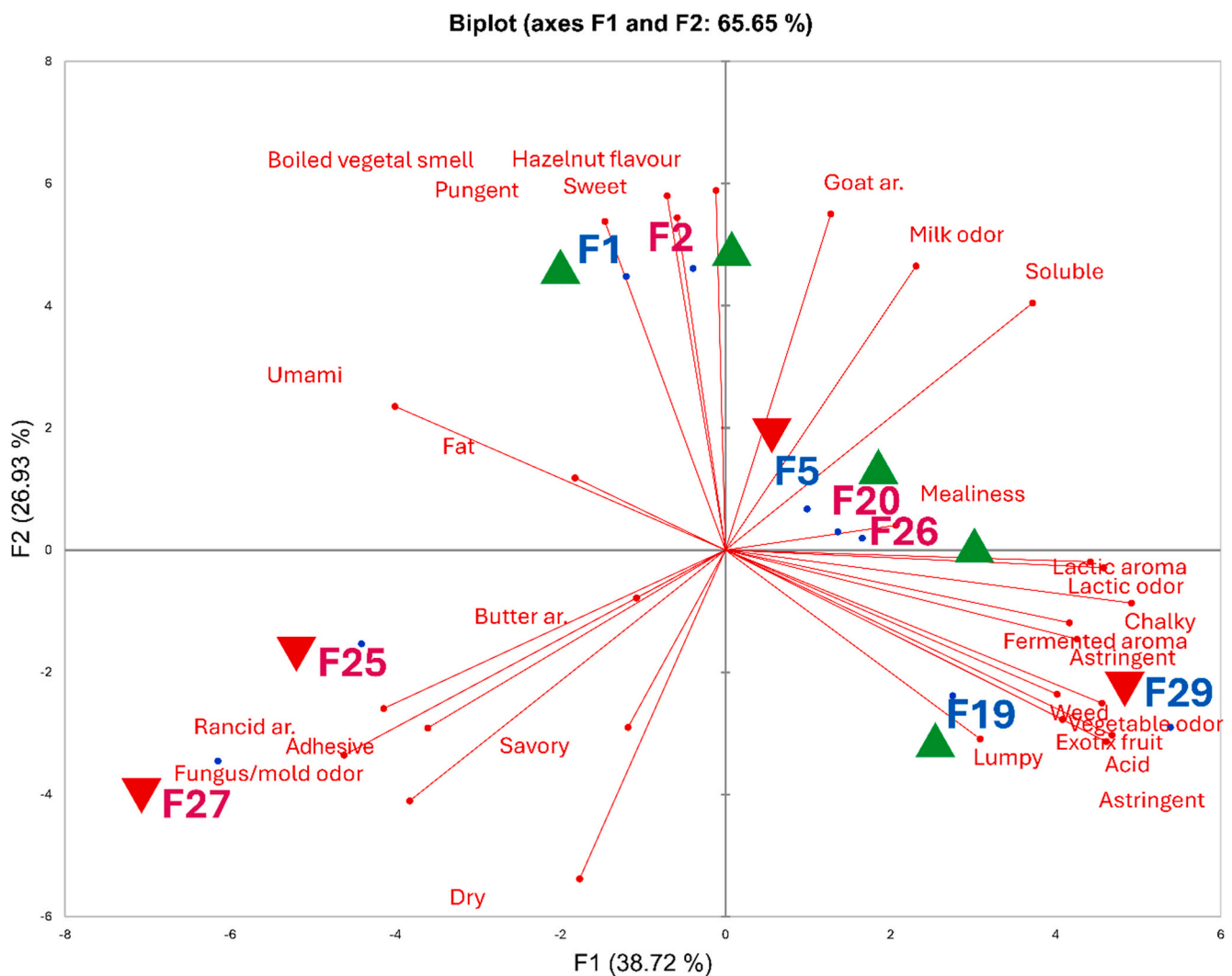


Fig. 6. PCA about FP sensory analysis. The cheeses were selected for their higher (green triangles) or lower (red triangles) ONAF evaluation scores. PDO are indicated by blue names, while non-PDO by violet names.

4. Discussion

Bacterial microbiome evaluation demonstrated diversities between PDO and non-PDO cheeses. Roccaverano PDO demonstrated a lower abundance of *Pseudomonadaceae* and *Pseudomonas* spp., that are considered spoilage agents in fresh soft cheeses (Tirloni et al., 2021). However, PDO cheeses demonstrated a higher abundance of *Acinetobacter* spp. that is considered a common dairy contaminant (Johnson et al., 2022). *L. lactis* has been confirmed as the most abundant species in Roccaverano PDO (Biolcati et al., 2022). The absence of significant differences in term of α -diversity between PDO and non-PDO samples suggests a similar variety of bacterial composition. However, differences in terms of bacterial species were observed. The number of unique species was higher in non-PDO cheeses suggesting heterogeneity in this group. PDO cheeses were characterized by lactic acid bacteria already linked to cheese characteristics. Among these species, *L. otakiensis* and *E. durans*, isolated from cheese, demonstrate beneficial properties. *L. otakiensis* produces the inhibitory neurotransmitter γ -aminobutyric acid (GABA) (Ribeiro et al., 2018). Meanwhile, *E. durans* exhibit probiotic properties (Terzić-Vidojević et al., 2021). *S. thermophilus* was associated with non-PDO cheeses, probably due to the allowed addition of starter cultures (Grizon et al., 2023), which are not permitted in Roccaverano PDO. However, for non-PDO cheeses, it was not possible to confirm with certainty the absence of starter cultures, which may have contributed to the presence of *S. thermophilus*. The Roccaverano PDO cheeses, which are produced using NSC, demonstrated a higher abundance of *S. parauberis* when compared to non-PDO cheeses. This finding is consistent with the literature on *S. parauberis*, which has been linked to mastitis playing a role in cheese ripening (Dimov, 2023) and showing to be more abundant in artisanal goat's cheeses made with in-house starters compared to those produced with industrial or commercial starters (Nelli et al., 2023).

Bacterial β -diversity showed similarities between Roccaverano PDO and some non-PDO cheeses, suggesting that certain non-PDO cheeses share characteristics with Roccaverano PDO, despite lacking the PDO designation (Fig. 1B).

The evaluation of fungal taxa demonstrated a lower separation between PDO and non-PDO cheeses compared to bacterial taxa composition. The α -diversities suggested that fungal taxa composition was more heterogeneous in Roccaverano PDO compared to non-PDO cheeses (Fig. 1C). Roccaverano PDO showed a higher abundance of certain *Kluyveromyces* species, including *K. lactis*, which has been previously detected in Roccaverano PDO cheeses (Biolcati et al., 2020). The potential probiotic role of *Kluyveromyces* spp. has also been suggested for Roccaverano PDO, although this remains to be confirmed (Biolcati et al., 2020). Additionally, other fungal genera were more abundant in PDO cheeses. *S. silvae*, which was statistically more abundant in PDO samples, has also been identified in this cheese (Biolcati et al., 2020) (Fig. 3).

Metagenomic assembly allowed the obtainment of MAGs correspondent to the most abundant species. The high number of *L. lactis* MAGs accessory genes demonstrated high heterogeneity within this species, while *L. mesenteroides* demonstrated a exhibited a greater number of core genes, demonstrating a higher genomic similarity. No relevant differences between MAGs were observed in terms of isolation sources. The presence of cation-proton antiporters (CptE) and *clcA* antiporter, respectively in *L. lactis* and *L. mesenteroides* MAGs, suggested the presence of genetic traits linked to cheese-making conditions (Wang et al., 2018) that remains to be investigated. In the case of cation-proton antiporters the function can be related to acidity resistance (Poolman, 2023) or to calcium uptake (Gupta et al., 2017). Moreover, *L. lactis* from PDO demonstrated the presence of bacteriocins related genes (*lcnB* and *nisZ*) (Cheigh et al., 2005; Venema et al., 1993), increasing the importance of *L. lactis* as potential PDO safe food bio-preservatives (Bukvicki et al., 2020). *L. lactis* demonstrated similarities between MAGs found in cheese samples from the same cheese factory produced on different days (samples F/R 1–3) (Fig. 4). This suggests a persistence of *L. lactis* strains

linked to specific producers.

Metagenomics cheeses evaluation allowed the obtainment of information about microbiome pathways. The negative correlation of spermidine/putrescin pathways suggests lower metabolic activities related to these biogenic amines in PDO samples (Schirone et al., 2022). Moreover, genes related to butanoate pathway were less abundant in PDO samples, which showed a higher concentration of butanoic acid, 3-methyl- (isovaleric acid) and 1-butanol, 3-methyl-, acetate. This suggests the persistence of these VOCs due low metabolic consumption. This aspect suggests influence on cheese sensory characteristics. The VOC butanoic acid, 3-methyl- which demonstrated influences on cheese characteristics, was associated to sweat, acid, rancid odour (Wang et al., 2021). The presence of *K. lactis*, capable of metabolizing lactose (Varela et al., 2019), is significant. NMDS analysis has associated this yeast with specific sensory characteristics, including astringent, acidic, and chalky flavours (Supplementary fig. 4).

Other VOCs were linked to sensory characteristics evaluated by FP sensory analysis. Phenylethyl alcohol was associated to honey, rose odour in cheese (Wang et al., 2021). The concentration of this molecule was higher in PDO group, which was associated to vegetable odour in multivariate statistical analysis. Similarly, ethyl acetate, which exhibited pineapple, fruity, balsamic odours (Aprea, 2020), showed higher concentration in PDO samples that were also associated to vegetable odour and exotic fruit aroma. Among the common VOCs between PDO and non-PDO cheeses, octanoic acid resulted one of the ten molecules with the highest concentration. This VOC is related to goaty aroma (Aprea, 2020), while another highly concentrated VOC, butanoic acid, is associated to cheesy odour (Wang et al., 2021). This indicates that some VOCs with higher concentrations are linked to the typical sensory characteristics of cheese.

Sensory evaluation tests demonstrated significantly higher total scores for Roccaverano PDO compared to non-PDO competitors. The higher score for the PDO group indicates the assurance of PDO designation and compliance with the associated quality standards. The FP analysis revealed that some sensory characteristics, such as vegetal odour, exotic fruit flavour, astringent, and lactic aromas, as well as acid and chalky notes, seem to be associated. This is not surprising considering that characteristics such as acid and astringent odour can be interconnected.

5. Conclusions

The comparison of Roccaverano PDO with non-PDO competitors demonstrates that this cheese presents specific sensory, VOCs, and microbiome-related characteristics. Sensory evaluation demonstrates that PDO groups was characterized by higher evaluation scores, indicating the presence of VOCs linked to specific sensory attributes. However, it is important to note that some competitor cheeses have presented characteristics comparable to those of PDO cheese. Specific fungal and bacterial taxa demonstrated correlation with PDO or non-PDO cheeses, suggesting potential microbial markers for Roccaverano PDO. The multivariate analysis indicates that some VOCs and microorganisms were related to sensory characteristics, confirming the importance of microbiome for cheese characteristics. Variability in microbiome composition was observed also within PDO groups, demonstrating that specific cheese producers, raw materials and dairy plant can influence cheese microbiome, even within the same PDO regulation in the same area.

CRedit authorship contribution statement

Davide Buzzanca: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Manuela Giordano:** Writing – review & editing, Validation, Methodology, Formal analysis. **Elisabetta Chiarini:** Writing – original draft, Visualization, Investigation, Formal analysis. **Ilario Ferrocino:**

Writing – review & editing, Validation, Software, Methodology. **Luca Coccolin**: Writing – review & editing, Supervision, Resources, Project administration. **Giuseppe Zeppa**: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation. **Valentina Alessandria**: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Davide Buzzanca reports financial support was provided by Ministry of Education and Merit. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2024.111014>.

Data availability

Raw sequence reads are available on Sequence Read Archive (National Center for Biotechnology Information; NCBI) under the accession number PRJNA1160470 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1160470>).

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