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**Microbial diversity, morpho-textural characterization and volatilome profile of the Portuguese thistle-curdled cheese *Queijo da Beira Baixa* PDO**

Federica Cardinali<sup>1</sup>, Roberta Foligni<sup>1</sup>, Ilario Ferrocino<sup>2</sup>, Joanna Harasym<sup>3</sup>, Agnieszka Orkusz<sup>3</sup>, Irene Franciosa<sup>2</sup>, Vesna Milanović<sup>1</sup>, Cristiana Garofalo<sup>1</sup>, Cinzia Mannozi<sup>1</sup>, Massimo Mozzon<sup>1</sup>, Luca Cocolin<sup>2</sup>, Andrea Osimani<sup>1,\*</sup>, Lucia Aquilanti<sup>1</sup>

<sup>1</sup> Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche, via Brecce Bianche, Ancona, Italy

<sup>2</sup> Department of Agricultural, Forest, and Food Science, University of Turin, Largo Paolo Braccini 2, Grugliasco, Torino, Italy

<sup>3</sup> Department of Biotechnology and Food Analysis, Wrocław University of Economics and Business, Komandorska 118/120, 53-345 Wrocław, Poland

\* Corresponding authors:

- Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche, via Brecce Bianche, 60131, Ancona, Italy. E-mail address: [a.osimani@univpm.it](mailto:a.osimani@univpm.it) (AO)

## ABSTRACT

The aim of the present study was to characterize the bacterial and fungal communities naturally occurring in *Queijo da Beira Baixa* PDO cheese samples produced in Castelo Branco district (Beira Baixa Region, Portugal) through viable counts and metataxonomic analyses. Physico-chemical and morpho-textural analyses were also carried out, together with the analysis of volatile organic compounds (VOCs) via Headspace Solid-Phase Microextraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS) analysis. In the analyzed samples, pH values ranged between  $4.72 \pm 0.15$  and  $5.85 \pm 0.02$ , whereas total titratable acidity was comprised between  $12.30 \pm 0.42$  and  $23.35 \pm 1.77$  mL of 0.1 NaOH. Values of acetic acid ranged between  $0.09 \pm 0.02$  and  $0.34 \pm 0.03$  g  $100$  g<sup>-1</sup>, whereas lactic acid content was comprised between  $0.64 \pm 0.00$  and  $1.95 \pm 0.16$  g  $100$  g<sup>-1</sup>. Water activity ranged between  $0.928 \pm 0.04$  and  $0.961 \pm 0.05$ . Specific volume ranged from  $1.09 \pm 0.08$  to  $1.32 \pm 0.02$  g mL<sup>-1</sup>. The results of color analyses on the cheese samples showed lightness comprised between  $79.35 \pm 0.94$  and  $88.02 \pm 0.36$ , greenish tones varying from  $-2.71 \pm 0.12$  to  $-4.87 \pm 0.06$ , and yellowish tonality comprised between  $15.30 \pm 0.10$  and  $25.94 \pm 0.29$ , chroma varied from  $15.58 \pm 0.10$  to  $26.39 \pm 0.29$ . Texture profile analysis showed hardness ranging between  $38.3 \pm 9.6$  N and  $68.55 \pm 7.5$  N, and springiness ranging between  $0.198 \pm 0.034$  and  $0.356 \pm 0.117$ . Cohesiveness, chewiness, and resilience ranged between  $0.199 \pm 0.004$  and  $0.282 \pm 0.005$ ,  $2.3 \pm 0.1$  and  $4.9 \pm 3.0$ , and  $0.088 \pm 0.006$  and  $0.152 \pm 0.109$ , respectively. As for lactic acid bacteria, presumptive lactococci, thermophilic cocci, and lactobacilli, counts up to 9 Log cfu g<sup>-1</sup> were detected. Whereas coagulase-negative cocci showed counts up to 7 Log cfu g<sup>-1</sup>. Enterococci counts were up to 6 Log cfu g<sup>-1</sup>; whereas Enterobacteriaceae showed viable counts up to 4 Log cfu g<sup>-1</sup>. Finally, counts of eumycetes showed values up to 4 Log cfu g<sup>-1</sup>. The results of metataxonomic analysis of bacteria showed the dominance of *Lactococcus lactis* in all the samples. Moreover, other taxa were detected, including *Lactiplantibacillus plantarum*, *Loigolactobacillus coryniformis*, *Lactococcus piscium*, *Streptococcus thermophilus*, and *Lacticaseibacillus zae*. Mycobiota was characterized by the presence of *Candida sake*, *Ustilago*, *Cladosporium variabile*, *Starmerella*, *Debaryomyces hansenii*, and *Pichia kluyveri*. In the analyzed *Queijo da Beira Baixa* PDO cheese samples, carboxylic acids (2-methyl propanoic, butanoic, 2-methyl butanoic, 3-methyl butanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids) represented the most detected VOCs, followed by esters (isobutyl acetate, 2-butyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate), carbonyl compounds (2-butanone, 3-methyl butanal, 3-hydroxybutan-2-one, 2-heptanone, benzaldehyde, phenylacetaldehyde, 2-nonanone, dodecanal), and alcohols (ethanol, 2-propanol, 3-methyl-butanol).

**Keywords:** *Queijo de Castelo Branco*; lactic acid bacteria, *Lentilactobacillus lactis*; *Lactococcus piscium*; volatile organic compounds.

## 1. Introduction

In Southern European countries, the production of artisan regional cheeses represents a gastronomic heritage of great importance. The high value of cheese was already known during the Roman Empire, when rents, tolls, and charges were used to be paid with such food (Freitas & Malcata, 2000). Today, artisan cheeses produced in the countries of the Mediterranean Region are still linked to ancient traditions that must not be lost. The biogeographical region of the Mediterranean Sea includes seven European Member States, either partially (France, Portugal, Italy, Spain) or completely (Greece, Malta, Cyprus). The Mediterranean Region has specific features, including a climate characterized by hot, dry summer, and humid, cool winter, and a generally hilly landscape. Such well-defined environmental conditions allow peculiar and unique cheese types to be produced.

Cheese is the result of milk coagulation followed by fermentation. Such food can be produced with either thermal-treated or raw milk. Moreover, a clotting agent is used to destabilize the casein micelles until a gel texture is formed. Among clotting agents, the most widely used in cheese manufacturing is calf rennet, that is generally obtained from the fourth stomach of suckling calves (Liu et al., 2021). Moreover, vegetable clotting agents can also be used, and, among these, those extracted from the plants *Lactuca sativa*, *Euphorbia*, *Streblus asper*, *Solanum*, *Ficus*, and thistles (Liu et al., 2021). These latter clotting agents refer to plants belonging to the tribe Cardueae Cass. = Cynareae Less. and encompass the genera *Carduus*, *Cirsium*, *Onopordum*, *Cynara*, *Scolymus*, *Silybum*, *Onopordum*, and *Carlina* (Aquilanti et al., 2011; Cardinali et al., 2017).

After coagulation, the cheese curd is formed and cheese whey release. Moreover, milk fat droplets are caught in the cheese matrix, whereas water, lactose, some minerals, and serum proteins (e.g., albumin) are lost in the whey (Britten & Giroux, 2022).

Although cheese ingredients are quite simple, cheese microbiology is critical for the definition of the overall cheese sensory traits that are the result of coexisting microbial species that interact with the food matrix and the environment, thus defining cheese quality and authenticity (Gobbetti et al., 2018).

Cheese fermentation is driven by the lactic acid bacteria naturally occurring in the raw milk or by microbial starter cultures that are added to the milk after its heat treatment. The metabolic activities of lactic acid bacteria, whether of natural origin or intentionally added, produce lactic acid as main organic acid, thus contributing to gel syneresis, whey expulsion, and curd formation (Wilkinson & LaPointe, 2020). Lactic acid bacteria utilize nutrients contained in the curd, as carbohydrates, organic acids, lipids, and proteins. Of note, proteolysis exerted by lactic acid bacteria in cheese leads to the production of free amino acids that strongly contribute to flavor development.

In cheese, yeasts occurring during ripening also contribute to the definition of sensory traits of the end-product. Indeed, cheese represents a suitable environment for yeasts due to the high salt concentration, temperature conditions, water activity values, and pH (Merchán et al., 2022). In cheese, yeasts metabolize lactose and galactose, and use succinic, lactic, and citric acids; moreover, yeasts exert potent proteolytic and lipolytic activities thus contributing to the development of cheese aroma (Merchán et al., 2022).

In order to valorize and protect the uniqueness of food products, the European Union established a series of geographical indication recognitions that include, the protected designation of origin (PDO), the protected geographical indication (PGI), and the geographical indication for spirit drinks and aromatized wines (GI). The PDO recognition is granted to food products that have the strongest link to the place in which they are manufactured.

Southern European countries are renowned for the manufacture of most of the ovine raw milk cheeses worldwide (Freitas & Malcata, 2000). Among those countries, Portugal accounts for 11 cheeses with PDO recognition including, *Queijo do Pico*, *Queijo de Cabra Transmontano/Queijo de Cabra Transmontano Velho*, *Queijo Rabaçal*, *Queijo Terrincho*, *Queijo de Azeitão*, *Queijo Serra da Estrela*, *Queijo de Nisa*, *Queijo S. Jorge*, *Queijo Serpa*, *Queijo de Évora*, and *Queijo da Beira Baixa* (eAmbrosia, 2022).

*Queijo da Beira Baixa* cheese has obtained the PDO recognition in the year 1996 under the names *Queijos da Beira Baixa*, *Queijo de Castelo Branco*, *Queijo Amarelo da Beira Baixa* and *Queijo Picante da Beira Baixa*, registered under Commission Regulation (EC) No 1107/96. Subsequently, in the year 2021, the European Commission has approved Portugal's application for amendments to the specification for the PDO into *Queijo da Beira Baixa*, registered under Commission Implementing Regulation (EU) 2021/245. Hence, *Queijo da Beira Baixa* PDO is the name given to cheeses of the Amarelo, Castelo Branco and Picante types. Among the *Queijo da Beira Baixa* types of cheese, the one produced in the Castelo Branco district (Beira Baixa Region, Portugal) has a semi-hard or semi-soft paste, yellowish color, and a strong flavor. The *Queijo da Beira Baixa* PDO cheese manufactured in Castelo Branco is produced with 100% raw ewe's milk from the Merino sheep breed (or other sheep breeds that are well adapted to the region), coagulated with infusion of cardoon (*Cynara cardunculus* L.) at 28 – 36 °C for 40 – 90 min. After coagulation, the curd is cut until the size of a grain of rice is reached. The maturation period is carried out at a temperature comprised between 8 to 14°C and 74-90% relative humidity for at least 40 days. The end product can be manufactured in two ranges of size: i) height 5-8 cm, weight 0.8-1.3 kg, and diameter 12-16 cm; ii) height 4-6 cm, weight 0.35-0.55 kg, and diameter 8-10 cm.

To the authors' knowledge, the information on the physico-chemical characteristics of *Queijo da Beira Baixa* PDO cheese is limited to the studies by Mata (1989) and Marques (1991), and only one published paper deals with the characterization of the volatiles of *Queijo da Beira Baixa* from Castelo Branco (Ferreira, Pinho, & Sampaio, 2009). To date, no published studies on the microbial diversity of *Queijo da Beira Baixa* PDO cheese are available in the scientific literature.

Accordingly, the aim of the present study was to characterize the bacterial and fungal communities in *Queijo da Beira Baixa* PDO cheese samples produced in Castelo Branco through viable counting and metataxonomic analyses. Physico-chemical and morpho-textural analyses were also carried out, together with the analysis of volatile organic compounds (VOCs).

## 2. Materials and methods

### 2.1. Cheese sampling

Six samples of *Queijo da Beira Baixa* PDO cheese were collected from three artisan producers located in Castelo Branco district. Two samples of each production batch were purchased from the three different producers (producer 1: samples BB1 and BB2; producer 2: samples BB3 and BB4; producer 3: samples BB5 and BB6). All *Queijo da Beira Baixa* PDO cheeses samples had the same height (6 cm), diameter (10 cm), and weight (about 0.55 kg). All samples were transported under refrigerated conditions, stored to the laboratory at + 4 °C and analyzed before the expiration date.

### 2.2. Physico-chemical measurements

The pH was measured with a pHmeter by inserting a HI2031 solid electrode (Hanna Instruments, Padova, Italy) at the core of each sample.

To measure total titratable acidity (TTA), 10 g of each sample were weighted and mixed with 90 mL of deionized water by means of a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) at 260 rpm for 5 min. The TTA was expressed as the total volume (mL) of 0.1 N NaOH solution added to obtain a fixed pH of 8.3.

The concentration of acetic acid and lactic acid was measured using the commercial Acetic Acid Assay Kit (Acetate Kinase Manual Format) (Megazyme, Bray, Ireland) and D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay Kit (Megazyme, Bray, Ireland), respectively, in accordance with the manufacturer's instructions.

Water activity ( $a_w$ ) measurement was carried out using an AquaLab® 3TE analyzer (Decagon Devices, Inc., Pullman, WA, USA). In more detail, approximately 3 g of homogeneous sample was deposited in a plastic cuvette and measurements were taken at a temperature of  $25 \pm 0^\circ\text{C}$ .

For each sample, three independent measurements were performed, and the results were reported as mean  $\pm$  standard deviation.

Specific volume was calculated using sample weight and volume, which was evaluate as a change after immersing the sample of known weight into calibrated cylinder with 0.1 mL accuracy. The measurement was taken five times for each sample probing, and probing was made three times from each cheese sample. Specific volume was calculated as a result of sample weight/sample volume and expressed in  $\text{g mL}^{-1}$ .

### 2.3. Morpho-textural analyses

Colour was measured using a Konica Minolta CR-310 chroma meter (Ramsey, NJ, USA) connected with a Data Processor (DP-301), launched via RS232 serial port to the personal computer. Colour parameters were taken in triplicate, whereas each measurement was taken as a mean of three measurements. Parameters were presented as  $L^*$ ,  $a^*$ ,  $b^*$ , Chroma, and hue.

Cheese texture was determined by TPA test (Texture Profile Analysis) in quadruplicate with an AXIS texture analyser FC200STAV500 (AXIS, Gdansk, Poland) provided with the software "AXIS FM". An aluminium 20-mm-diameter cylindrical probe was used in a double compression test (TPA) to penetrate to 50 % depth, at  $1 \text{ mm s}^{-1}$  speed test. Hardness ( $N$ ) was the force at the maximum deformation while cohesiveness, springiness, chewiness, and resilience were calculated from the peaks. Analysis was carried out in quadruplicate at  $25^\circ\text{C}$  for 20 mm height and 20 mm diameter cylinder taken from the cheese slice of each sample.

### 2.4. Microbiological analyses

Ten grams of each sample were homogenized with 90 mL of sterile peptone water (Oxoid, Basingstoke, UK) in a stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm (Osimani et al., 2009). Serial ten-fold dilutions were prepared and 100  $\mu\text{L}$  of each dilution was inoculated in duplicate on the following growth media: M17 agar (VWR International, Milan, Italy) for presumptive mesophilic lactococci incubated at  $22^\circ\text{C}$  for 48-72 h; M17 agar (VWR) for presumptive thermophilic streptococci incubated at  $42^\circ\text{C}$  for 48-72 h; MRS Agar (VWR) for presumptive mesophilic lactobacilli incubated at  $30^\circ\text{C}$  for 48-72 h; MSA Agar (VWR) for coagulase-negative cocci incubated at  $37^\circ\text{C}$  for 48 h; Enterococcus Selective Agar (Merck KGAA, Darmstadt, Germany) for enterococci incubated at  $37^\circ\text{C}$  for 48 h; VRBGA (VWR) Agar for Enterobacteriaceae incubated at  $37^\circ\text{C}$  for 24 h; RB Agar (VWR) for eumycetes incubated at  $25^\circ\text{C}$  for 48 h.

The results of viable counts were expressed as the Log of cfu (colony-forming units) per gram of each sample and reported as mean value  $\pm$  standard deviation.

## 2.5. DNA extraction and sequencing

E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA) was used for the extraction of total microbial DNA from the cell pellets obtained by the centrifugation of 1 mL of each biological replicate (homogenate at dilution  $10^{-1}$ ) prepared as previously described by Cardinali et al. (2021). The extracted DNAs were checked for quantity and purity by Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) quantified and standardized by using the Qubit ds Kits. For each sample, the DNA extracts obtained from each biological replicate were pooled to reduce the inter-sample variability (Osimani et al., 2021).

## 2.6. Metataxonomic analyses

A metataxonomic approach was applied to analyze the total DNA extracted from the *Queijo da Beira Baxa* PDO cheese samples of the three different producers in order to highlight any differences in microbiota composition. In more detail, the 16S rRNA gene (V3-V4 regions) was amplified using primers and procedures previously described by Klindworth et al. (2013). The 26S rRNA gene was amplified by using the primers NL4R (5'-GGTCCGTGTTTCAAGACGG-3') and LS2-MF (5'-GAGTCGAGTTGTTTGGGAAT-3') following the procedure previously described (Mota-Gutierrez et al., 2019).

The Illumina metagenomic procedure was applied for PCR products purification, tagging, and pooling. Illumina MiSeq platform with V2 chemistry was used to generate 250-bp paired-end reads and the raw *fastq* files obtained were elaborated by QIIME 2 software (Bolyen et al., 2019). The primer sequences were removed by *Cutapter* and DADA2 algorithm was used to denoise the obtained reads by using the *q2-dada2* plugin in QIIME 2 (Callahan et al., 2016). Taxonomy classification was performed against the *SILVA* database by means the QIIME2 feature-classifier. The ASVs with less than five read counts in at least two samples were excluded to increase the confidence of sequence reads.

The taxonomic assignment of the mycobiota dataset was performed against the *SILVA* database implemented in (Mota-Gutierrez et al., 2019). BLASTn suite tool was used to confirm the taxonomic assignment.

The raw read data were deposited in the Sequence Read Archive of NCBI under the bioproject accession number PRJNA822519

## 2.7. Volatilome profile

Headspace Solid-Phase Microextraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS) analysis was used to collect the volatile components from the headspace of a 10 mL glass vial filled with 0.5 g of sample. A DVB/PDMS 65  $\mu\text{m}$  fiber (Supelco/Sigma-Aldrich, Milan, Italy) was exposed into the head space for 45 min at 50 °C, as described by Belleghia et al. (2020). A Trace 1300 gas chromatograph coupled with a ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and equipped with a Zebron ZB-5ms capillary column 30 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness (Phenomenex, Torrance, CA, USA) were used to analyze the volatile profile of samples. As reported by Foligni et al. (2022), the operative parameters were the following: injector temperature 250 °C; oven temperature 40 °C to 220 °C increased at the rate of 6 °C  $\text{min}^{-1}$  and maintained for 5 min; gas flow (He) in constant mode at 1.0 mL  $\text{min}^{-1}$  and full scan MS data were acquired in the mass range of 31–250 atomic mass units (amu). Volatile compounds identification was made according to Mozzoni et al. (2020), by matching the mass spectral data with NIST/EPA/NIH Mass Spectral Library 2020 and the chromatographic behavior with published Kovats retention indices (RIs). An automated spreadsheet was used to simplify the calculation of RIs of the unknown components (Maoloni et al. 2021).

## 2.8. Statistical analysis

The Tukey-Kramer's Honest Significant Difference (HSD) test ( $\alpha=0.05$ ) was used to evaluate differences within cheese samples by one-way analysis of variance (ANOVA) using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC).

Alpha diversity indices were calculated through the diversity script of QIIME2. Differences between alpha diversity parameters and ASVs frequency were analyzed by non-parametric Kruskal wallis test in R environment. Pairwise Spearman's non-parametric correlations were used to study the relationships between fungi and bacteria and between microbes and metabolites. The correlation plots were visualized in R using the *corrplot* package in R environment. A *P* value of 0.05 or lower was considered as statistically significant.

## Results

### 3.1. Physico-chemical characterization

The results of physico-chemical analyses carried out on the analyzed cheese samples are reported in Table 1.

In detail, pH values ranged between  $4.72\pm 0.15$  and  $5.85\pm 0.02$ , with samples of producer 3 showing statistically higher average pH value than those detected in samples of producers 1 and 2.

As for TTA, the detected values were comprised between  $12.30\pm 0.42$  and  $23.35\pm 1.77$  mL of 0.1 NaOH, with samples of producer 3 showing the lowest average value.

Regarding acetic acid, the values ranged between  $0.09\pm 0.02$  and  $0.34\pm 0.03$  g 100 g<sup>-1</sup>, with no statistically significant differences among producers.

Finally, lactic acid values were comprised between  $0.64\pm 0.00$  and  $1.95\pm 0.16$  g 100 g<sup>-1</sup>, with samples of producer 3 showing the average lowest value.

Water activity ranged between  $0.928\pm 0.04$  and  $0.961\pm 0.05$ , with samples of producer 3 revealing the lowest average value.

As for specific volume, the detected values ranged from  $1.09\pm 0.08$  to  $1.32\pm 0.02$  g mL<sup>-1</sup> with samples of producer 3 showing the lowest average value.

### 3.2. Morpho-textural characterization

The results of color analyses on the cheese samples are reported in Table 2, whereas results of texture profile are reported in Table 3.

The lightness (L\*) of the samples varied from  $79.35\pm 0.94$  to  $88.02\pm 0.36$  being the highest for producer 1. The analyzed samples revealed greenish tones (a\*) varying from  $-2.71\pm 0.12$  to  $-4.87\pm 0.06$  and the most intense green tones were detected in samples of producer 2. The yellowish tonality (b\*) ranged from  $15.30\pm 0.10$  to  $25.94\pm 0.29$ , being more pronounced for samples of producer 2. Chroma varied from  $15.58\pm 0.10$  to  $26.39\pm 0.29$  with the highest values for samples of producer 2, whereas hue, in range from  $96.87\pm 0.4$  to  $101.9\pm 0.2$ , was the lowest for samples of producer 3. Two-way ANOVA confirmed strong interaction between producer and batch at  $P < 0.01$  and  $P < 0.001$ .

Texture profile analysis reveal variations in hardness and springiness of the cheese samples between producers. Hardness ranged between  $38.3\pm 9.6$  N and  $68.55\pm 7.5$  N with the highest average value for samples of producer 2 which was correlated with springiness. This latter parameter ranged from  $0.198\pm 0.034$  to  $0.356 \pm 0.117$  with the lowest average value for samples of producer. Cohesiveness, chewiness, and resilience ranged between  $0.199\pm 0.004$  and  $0.282\pm 0.005$ ,  $2.3\pm 0.1$  and  $4.9\pm 3.0$ ,  $0.088\pm 0.006$  and  $0.152\pm 0.109$ , respectively, with no significantly statistical differences among producers and batches.

### 3.3. Viable counts

The results of viable counts carried out on the analyzed cheese samples are reported in Table 4.

Regarding presumptive lactococci, the detected values ranged between  $6.32\pm 0.02$  and  $9.07\pm 0.05$  Log cfu g<sup>-1</sup>, with no statistically significant differences among samples.

As for presumptive thermophilic cocci, values comprised between  $6.18\pm 0.00$  and  $8.98\pm 0.01$  Log cfu g<sup>-1</sup> were detected among samples, with counts in samples of producer 1 showing the highest average values and those in samples of producer 3 the lowest.

For presumptive lactobacilli, the counts ranged from  $7.64\pm 0.01$  to  $9.35\pm 0.01$  Log cfu g<sup>-1</sup>, with counts in samples of producer 1 showing the highest average value and those in samples of producer 3 the lowest.

Regarding coagulase-negative cocci, counts were between  $3.68\pm 0.05$  and  $7.01\pm 0.01$  Log cfu g<sup>-1</sup>, with average counts in samples of producer 2 showing the lowest values.

Concerning enterococci, the detected values ranged from  $4.78\pm 0.02$  to  $6.26\pm 0.02$  Log cfu g<sup>-1</sup>, with no statistically significant differences among the samples of the three producers.

Enterobacteriaceae showed viable counts comprised between  $< 1$  and  $4.07\pm 0.44$  Log cfu g<sup>-1</sup>, with samples of producer 2 showing the lowest average value and samples of producer 3 the highest.

Finally, counts of eumycetes showed values between  $< 1$  and  $4.74\pm 0.06$  Log cfu g<sup>-1</sup>, with samples of producer 2 showing the lowest average value.

### 3.4. Microbiota and mycobiota composition

The results of metataxonomic analysis of bacteria are reported in Figure 1. In more detail, the metataxonomic analysis and alpha diversity index did not show significant differences among producers. In all the analyzed samples *Lactococcus lactis* was present at high relative frequency (ranging from 43% to 87%), with the highest relative frequency in samples of producer 3. Moreover, other minor amplicon sequence variants (ASVs) were detected in all the samples, i.e., *Lactiplantibacillus plantarum* (ranging from 4% to 14%) and *Loigolactobacillus coryniformis* (ranging from 5% to 9%). Samples of producer 1 were characterized by the presence of *Lactococcus piscium* (10%), whereas *Streptococcus thermophilus* was present at low relative frequency (5%) only in samples of producer 2. *Staphylococcus* spp. was detected in samples of producer 1 and 3; moreover, *Lacticaseibacillus zae* showed the highest relative frequency in samples of producer 1 and the lowest in samples of producer 3.

The results of metataxonomic analysis of eumycetes are reported in Figure 2. Although the metataxonomic analyses did not show significant differences between the cheeses mycobiota associated with the different three producers, some differences were detected. In details, cheeses of producer 1 showed the highest presence of *Candida sake* (44%), *Geotrichum* (8%), and *Ustilago* (21%) species. Cheeses of producer 2 were characterized by the high prevalence of *Cladosporium variabile* (24%), and *Starmerella* (56%). Producer 3 showed the highest relative frequency of *Cladosporium variabile* (24%), *Debaryomyces hansenii* (17%) and *Pichia kluyveri* (21%), and *Starmerella* (16%). By performing a co-occurrence co-exclusion analysis between the microbiota and mycobiota (Figure 3) a positive correlation between *Leuconostoc mesenteroides*, *Candida davisiana*, and *Metschnikowia fructicola* was observed; whereas a strong negative correlation with *Apiotrichum loubieri* was observed. *Streptococcus thermophilus* showed only positive correlations, in particular with *Clavispora lusitanae*, *M. fructicola*, *Quambalaria cyanescens*, and *Starmerella*. *Acinetobacter* spp. showed negative correlations with *C. variabile*, *D. hansenii*, *P. kluyveri*, *Protomyces inouyei*, *Starmerella*, and *Symbiotaphrina*. The presence of *L. piscium* was negatively correlated with the presence of *D. hansenii*, *P. kluyveri*, and *Vishniacozyma victoriae*, but positively correlated to *Geotrichum*. The presence of *L. plantarum* was correlated with *Penicillium* spp.

### 3.5. Volatilome profile

Volatile compounds detected in the headspace of the analyzed cheese samples are reported in Table 5.

In more detail, carboxylic acids (2-methyl propanoic, butanoic, 2-methyl butanoic, 3-methyl butanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids) dominated the headspace of cheese samples (61% of total volatiles), followed by esters (isobutyl acetate, 2-butyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate) (20% of total volatiles), carbonyl compounds (2-butanone, 3-methyl butanal, 3-hydroxybutan-2-one, 2-heptanone, benzaldehyde, phenylacetaldehyde, 2-nonanone, dodecanal) (12% of total volatiles), and alcohols (ethanol, 2-propanol, 3-methyl-butanol) (5% of total volatiles).

Higher levels of 2-butanone, 3-methylbutanal, and ethyl hexanoate were observed in samples of producer 3.

The results of the correlation analysis between the ASVs and VOCs of *Queijo da Beira Baixa* PDO cheese samples (Figure 4) showed that the presence of *Acinetobacter* sp. was negatively associated with and isobutyl acetate, propanoic acid-2-methyl, and butanoic acid-3-methyl ( $P$  value < 0.05). Whereas 2-butanone was negatively correlated with *C. davisiana*, *Geotrichum*, *Metschnikowia*, *Latilactobacillus sakei*, and *Serratia*. The high presence of octanoic acid showed a strong positive correlation with *C. sake* and a strong negative correlation with *Ogataea boidinii*. *D. hansenii* showed a positive correlation with propanoic acid-2-methyl, butanoic acid-3-methyl, and ethyl hexanoate and a negative correlation with ethanol, heptanoic acid, and dodecanoic acid. *Streptococcus* showed negative correlations with isobutyl acetate, acetoin, propanoic acid 2-methyl, butanoic acid 3-methyl, and benzaldehyde. *L. lactis* displayed a negative correlation with ethanol and heptanoic acid and a positive relationship with methyl ester of propanoic and butanoic acids.

## 4. Discussion

To the authors' knowledge, this study represents the first attempt to disclose the microbial diversity of *Queijo da Beira Baixa* PDO cheese. Moreover, only one published study actually deals with the volatile organic compounds of this thistle-curdled PDO cheese (Ferreira et al., 2009), and a few data on physico-chemical characterization of *Queijo da Beira Baixa* PDO cheese are available (Freitas & Malcata, 2000). Hence, the present study allows a step forward in the knowledge of Portuguese cheeses to be performed.

In the present study, pH values detected in the analyzed samples were generally in accordance with those reported by Freitas & Malcata (2000) for Castelo Branco cheese. Moreover, pH values recorded in the cheese samples analyzed in the present study were in accordance with those detected by Cardinali et al. (2017) in the Italian thistle-curdled cheese *Caciofiore della Sibilla* and by Ordiales et al. (2013) in *Torta del Casar*, a Spanish PDO cheese coagulated with *C. cardunculus*, attesting at about 5 at the end of ripening. pH values recorded in the analyzed *Queijo da Beira Baixa* PDO cheese samples were slightly lower than those detected in the Portuguese raw-milk thistle-curdled cheese *Queijo de Azeitão* PDO by Cardinali et al. (2021), that attested up to 6. In cheese, pH is the result of the metabolic activity of lactic acid bacteria that, during ripening, produce lactic acid (homofermentative species) and other organic acids (heterofermentative species). In cheese, pH values and organic acids play a key role in defining texture and safety of the end product (Pamuksuz, Bulduk, & Ozturk, 2020; Wemmenhove, Wells-Bennik, & Zwietering, 2021). As reported by Pamuksuz et al. (2020), pH value of cheese strongly affects its hardness, elasticity, adhesiveness, and cohesiveness by altering the electrostatic interactions; indeed, the more the cheese pH is similar to the isoelectric point of caseins (4.8) the more the electrostatic repulsion between caseins decreases, thus producing a harder and crumbly cheese.

As for the lactic acid content, the detected values reflected the intense metabolic activity of the viable lactic acid bacteria populations. The lactic acid content recorded in the samples under study were notably higher than those reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO, that attested up to 0.488 g 100 g<sup>-1</sup>. Of note, the lowest counts of lactobacilli and thermophilic cocci detected in samples of producer 3 could reflect in the lower amount of lactic acid detected in the same samples. As for acetic acid, the amount of this organic acid was in accordance with the values



reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO, thus attesting the occurrence of lactic acid bacteria with heterofermentative metabolism.

In the analyzed samples, the detected  $a_w$  values were high (0.941-0.956), such feature may affect the stability of the product (Tapia, Alzamora, & Chirife, 2020) and prone the mould development.

Specific volume is a suitable parameter that reveals the porosity of the food matrix. In the present study, the porosity of the samples was almost neglected, as confirmed by the external appearance evaluation of the analyzed *Queijo da Beira Baixa* PDO cheese samples. The observation was confirmed by specific volume scores mainly close to 1.00.

The porosity of the casein matrix is strongly correlated with a particular activity of the microbial community of the cheese, mainly with the CO<sub>2</sub> production of yeasts under anaerobic conditions. In the analyzed samples, low porosity suggested that the microbial profile shifted towards organic acids excretion. Porosity also influences the colour and the texture of the sample, thus contributing to tonal change and hardness variation. The *Queijo da Beira Baixa* PDO cheese samples were characterized by high lightness with green and yellow tonalities intensively pronounced. According to Balthasar et al. (2017), ewe's milk is characterized by almost twice amount of fat comparing to cow's milk, which results in tonality changes towards yellow during casein curd maturing. Also, the high concentration of vitamin A in ewe's milk compared to cow's milk, which occurs because  $\beta$ -carotene is converted to retinol, results in greenish tonalities due to a lack of red carotenoids (Balthasar et al., 2017). In the present study, all colour parameters depended on the cheese producer, which suggests a strong influence of the origin of the milk, and, more specifically, of the amount and type of feed consumed by the sheep. The same characteristic was observed for texture parameters which are related to porosity. The detected hardness and springiness were dependent on producer and were well also correlated with specific volume and TTA of the samples. The lowest porosity of the producer 2 samples resulted in the highest hardness and lowest springiness of the sample.

The viable counts carried out on the *Queijo da Beira Baixa* PDO cheese samples allowed active microbial populations, including lactic acid bacteria, coagulase negative cocci, enterococci, Enterobacteriaceae, and eumycetes, to be disclosed. Lactic acid bacteria represent the key microorganisms in cheesemaking. In the cheese curd, their activity leads to the formation of lactic acid, as main metabolite, together with other organic acids (e.g., acetic acid), CO<sub>2</sub>, and ethanol. Lactic acid bacteria also play a pivotal role in the definition of safety and sensory features of the cheese. Indeed, as reported by Steele, Broadbent, & Kok (2013), enzymatic protein and peptide breakdown, exerted by lactic acid bacteria during cheese ripening, lead to the formation of aroma compounds derived from the catabolism of aromatic, sulfur-containing, and branched-chain amino acids. Furthermore, as reported by Favaro, Barretto Penna, & Todorov (2015), lactic acid bacteria produce natural antimicrobial compounds commonly referred to as bacteriocins. Indeed, bacteriocin-producing strains of *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Lactococcus* have already been isolated from dairy products, as Cheddar, goat milk cheese, white-brined cheese, medium-hard or hard cheeses, and fresh cheese (Favaro et al., 2015).

In the present study, the viable counts of lactococci were in accordance with those reported by Macedo et al. (2004) in *Serra da Estrela* PDO cheese, that ranged between 7 and 9 Log cfu g<sup>-1</sup>. In the same study, counts of lactobacilli attested up to 9 Log cfu g<sup>-1</sup>, being similar to those detected in the *Queijo da Beira Baixa* PDO cheese samples analyzed in the present study. Counts of lactococci and lactobacilli detected in the *Queijo da Beira Baixa* PDO cheese samples were also similar with those detected in *Queijo de Azeitão* PDO cheese, whose counts were up to 7.6 and 8.7 Log cfu g<sup>-1</sup> for lactococci and lactobacilli, respectively (Cardinali et al., 2021). Lactic acid bacteria counts were also in accordance with those detected by Ordiales et al. (2013) in *Torta del Casar*, that attested up to 9 Log cfu g<sup>-1</sup>. As for thermophilic cocci, the counts detected in the present study were in accordance with those reported by Cardinali et al. (2017, 2021) in *Caciopfiore della Sibilla* cheese and in *Queijo de Azeitão* PDO cheese, both attesting up to 7 Log cfu g<sup>-1</sup> at the end of ripening.

Regarding coagulase-negative cocci, the proteolytic and lipolytic activity of this microbial group notably impacts on the final flavour and aroma of cheese (Ruaro, Andrighetto, Torriani, & Lombardi, 2013; Aquilanti et al., 2007). Of note, coagulase-negative cocci are salt and acid tolerant microorganisms that have frequently been detected in ewe's or goat's cheeses, in smear cheeses, and in raw milk cheeses produced in Northern Italy (Irlinger, 2008; Ruaro et al., 2013). In the present study, counts of coagulase-negative cocci were similar with those reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO cheese and with those reported by Freitas & Malcata (2000) for *Evora* PDO coagulated with vegetable rennet, thus suggesting the wide presence of this microbial group in Portuguese thistle-curdled cheeses.

As for enterococci, the presence of this microbial group is characteristic of raw milk cheeses (Ordiales et al., 2013). As reported by Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst (2006), proteolysis, lipolysis, and citrate breakdown by enterococci have a key role in the formation of the typical taste and flavour of traditional cheeses produced in the Mediterranean countries. Indeed, enterococci have already been detected by Gonçalves et al. (2018) in *Serpa* PDO cheese and by Cardinali et al. (2021) in *Queijo de Azeitão* PDO, with attesting up to 5 Log cfu g<sup>-1</sup>.

The presence of Enterobacteriaceae in cheese reflects a lack of acidification of the curd as well as improper hygiene practices during manufacturing (Tabla et al., 2016). Of note, Enterobacteriaceae can be present in milk and cheese through faecal contamination; however, members belonging to this bacterial family are not acid resistant, and their growth in cheese is usually inhibited by organic acids produced by lactic acid bacteria (Tabla et al., 2016). Interestingly, the samples of *Queijo da Beira Baixa* PDO cheese that presented the highest counts of Enterobacteriaceae were those of producer 3,

which showed the highest pH values and the lowest organic acids amounts, thus suggesting a lack of acidification of the curd during ripening.

Finally, eumycetes (yeasts and moulds) represent important microorganisms that are involved in cheese ripening. The physico-chemical conditions as, high salt concentration, low temperature, water activity, and pH, occurring in cheese during ripening, represent an optimal environment for eumycetes growth (Merchán et al., 2022). Moreover, some eumycetes species show specific competitive advantages in cheese, being them able to metabolize lactose and galactose as well as succinic, lactic, and citric acids (Merchán et al., 2022). Eumycete metabolism of lactate leads to the increase of cheese pH, whereas the proteolytic and lipolytic activities of such microorganisms positively contribute to the formation of cheese aroma (Merchán et al., 2022). In the present study, counts of eumycetes were generally comparable with those reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO, that ranged between 2.7 and 5.9 Log cfu g<sup>-1</sup>. Similarly, Ordiales et al. (2013) reported values of yeasts and moulds up to 4.2 Log cfu g<sup>-1</sup> in *Torta del Casar* PDO cheese.

Metataxonomic analysis highlighted the prevalence of a homogeneous same microbiota strongly characterized by the presence of lactic acid bacteria among which *L. lactis* represented the key species. Moreover, a few lactic acid bacteria species occurred at various relative frequencies among samples. To the authors' knowledge, no studies dealing with metataxonomic characterization of microorganisms in *Queijo da Beira Baixa* PDO cheese are available in the scientific literature, hence, data will be compared with those reported by similar studies dealing with thistle-curdled cheeses produced in Mediterranean countries.

*L. lactis* represents the predominant lactic acid bacterium in dairy products (Cavanagh, Fitzgerald, & McAuliffe, 2015). Indeed, such species represents a large part of the dairy environment house microbiota, and it largely colonizes the teat surface (Gobbetti et al., 2018). Interestingly, the evolution of this bacterial species from the plant to the milk environment is due to genome modification, including acquisition of genes for protein and lactose utilization (Cavanagh et al., 2015). The massive presence of *L. lactis* in all the analyzed samples is not surprising *per se*, since its presence in Portuguese (thistle-curdled) cheeses (e.g., *Queijo de Azeitão* PDO cheese and *Serra da Estrela* PDO cheese) has already been reported by many authors (Cardinali et al., 2021; Macedo, Tavares, & Malcata, 2003, 2004; Tavararia, Silva-Ferreira, & Malcata, 2006). *L. lactis* is among the main species that are responsible for lactic acid production in the cheese curd; moreover, it is also able to use milk citrate as a secondary energy source. This lactic acid bacterium is also responsible for many other activities in cheese, since it harbours genes involved in casein breakdown, bacteriophage resistance, bacteriocins and exopolysaccharides productions (Kazou, 2022). At this regard, *L. lactis* possesses cell-envelope proteinase which is a key-enzyme in the cheese system. Indeed, such enzyme plays a pivotal role in secondary proteolysis since it is active on the chymosin-generated peptides, thus promoting amino acid production mediated by lactococcal peptidases and, hence, formation of volatile aroma compounds in cheese (Exterkate, Slangen, & Siezen, 2001). Moreover, *L. lactis* can also improve the safety of cheese, being such species able to produce bacteriocins (e.g., nisin), that are active against foodborne pathogens (e.g., *Listeria monocytogenes*) (Kondrotiene et al., 2018). Finally, Korcz & Varga (2021) reported that exopolysaccharides produced by *L. lactis* strains can be able to affect textural and melting properties of cheese. Of note, *L. lactis* strains isolated from *Serra da Estrela* PDO cheese also exhibited a strong esterase activity, thus suggesting a further release of flavour compounds during the late stage of cheese ripening (Macedo et al., 2003).

Regarding *L. plantarum* detected in the samples under study, Cardinali et al. (2021) already reported the presence of this species in *Queijo de Azeitão* PDO cheese; moreover, Macedo, Tavares, & Malcata (2003b) reported the occurrence of the same lactic acid bacterium in *Serra da Estrela* PDO cheese. Interestingly, Macedo et al. (2003b) also purified and characterized an intracellular aminopeptidase produced by *L. plantarum*, thus suggesting a role in flavour formation during the late stage of cheese ripening. Of note, Macedo et al. (2004) observed that the co-occurrence of *L. lactis* and *L. plantarum* in *Serra da Estrela* PDO cheese seemed to exert high control of Enterobacteriaceae growth as well as increase in proteolysis.

To the authors' knowledge, no previous studies that report the presence of *L. coryniformis* in Portuguese cheeses are available in the available scientific literature. *L. coryniformis* has already been isolated from other cheeses as goat's milk cheese, *Castelmagno* cheese, *Feta* cheese, and Turkish *Kaşar* cheese (Aydemir, Harth, Weckx, Dervişoğlu, & De Vuyst, 2015; Martin et al., 2005; Oberg, McMahon, Culumber, McAuliffe, & Oberg, 2022). Interestingly, *L. coryniformis* strains isolated by Martin et al. (2005) from Spanish raw goat's milk cheese showed high survival and adhesion rates after transit through an in vitro gastrointestinal model, thus suggesting a potential probiotic activity. Moreover, the same lactic acid bacteria showed the production of the bacteriocin reuterin and of cobalamin, thus suggesting potential beneficial effects for the consumers.

Regarding the occurrence of *L. piscium*, to the authors' knowledge, the isolation or the detection of this lactic acid bacteria species in cheese is extremely rare, although this lactic acid bacteria species has already been detected in milk and at the beginning of ripening of *Montasio* cheese (Carraro et al., 2011). *L. piscium* is a psychrotrophic lactococcus, first isolated from rainbow trout (Williams, Fryer, & Collins, 1990), that is typically responsible for meat spoilage (Rahkila, Nieminen, Johansson, Säde, & Björkroth, 2012). As reported by Rahkila et al. (2012), *L. piscium* strains isolated from meat showed a weak acid production from galactose as well as variable acid production from gluconate and lactose. Rahkila et al. (2012) also observed arginine hydrolysis and variable acetoin production carried out by some *L. piscium* isolates. Based on the abovementioned activities, it is likely that *L. piscium* could have contributed to a weak acid production and flavour compound production (e.g., acetoin) in the analyzed *Queijo da Beira Baixa* PDO cheese samples. Interestingly, Saraoui et al (2016) described the inhibitory activity carried out by a *L. piscium* strain towards *L. monocytogenes*, due to a cell-

to-cell contact-dependent mechanism. Of note, although many studies confirmed the alterative role of *L. piscium* in meat, other studies showed that it does not have detrimental effect on the sensory properties of seafood (Saraoui, Leroi, Björkroth, & Pilet, 2016). Hence, further research is needed to better clarify the real role of *L. piscium* in dairy products. *S. thermophilus* is considered one of the most important species of lactic acid bacteria in the dairy industry. Operational taxonomic units of *Streptococcus* have already been detected by Cardinali et al. (2017) in Italian raw ewes' milk cheese clotted with *Carlina acanthifolia* All. subsp. *Acanthifolia*; however, to the authors' knowledge no studies reporting the presence of *S. thermophilus* in Portuguese cheeses are available in the scientific literature for further comparison of data. It is noteworthy that the adaptation of *S. thermophilus* to cheese is mainly due to the acquisition of genes originating from other dairy species, such as *L. lactis* and *Lactobacillus delbrueckii* (Iyer, Tomar, Maheswari, & Singh, 2010). Due to its fast acidification of milk, *S. thermophilus* is commonly used for the production of Swiss cheese, Brick cheese, *Parmesan*, *Provolone*, *Mozzarella*, *Ragusano* PDO, and *Asiago* PDO. Together with lactic acid, *S. thermophilus* also produces formate, acetoin, diacetyl, acetaldehyde, and acetate, thus affecting the final sensory characteristics of cheese (Iyer, Tomar, Maheswari, & Singh, 2010). *S. thermophilus* possesses efficient proteolytic enzymes, as extracellular proteases, that hydrolyse casein, and intracellular peptidases that hydrolyse casein-derived peptides (Hols et al., 2005). Of note, *S. thermophilus* is also able to produce exopolysaccharides mainly consisting of polymers of galactose, glucose and rhamnose monomers, thus affecting the texture of cheese (Iyer, Tomar, Maheswari, & Singh, 2010). Finally, *S. thermophilus* can produce bacteriocins as, thermophilin 347, thermophilin A, and thermophilin T that are effective against a broad number of pathogenic or spoilage bacteria (e.g., *Clostridium tyrobutyricum* in hard cheese) (Iyer, Tomar, Maheswari, & Singh, 2010; Mathot, Beliard, & Thuault, 2003).

The occurrence of *L. zae* in thistle-curdled cheese has already been reported by Aquilanti et al. (2013) in *Caciotta* cheese clotted with aqueous extract of *C. cardunculus* and, more recently, by Cardinali et al. (2021) in *Queijo de Azeitão* PDO cheese. *L. zae* is a facultatively heterofermentative species that, due to its high proteolytic activity that leads to the production of free amino acids, can be responsible for the formation of aroma compound in cheese (Terzić-Vidojević et al., 2020).

Regarding mycobiota, *Starmerella* has already been detected by Cardinali et al. (2021) in *Queijo de Azeitão* PDO cheese, although at very low relative abundance. To the authors' knowledge, a scarcity of information regarding the occurrence of *Starmerella* in cheese is available in the scientific literature for further comparison of data. Interestingly, species of *Starmerella* have been shown to effectively produce sophorolipids (a group of extracellular biosurfactants) from dairy wastewater (Jiménez-Peñalver, Rodríguez, Daverey, Font, & Gea, 2019). At this regard, Hipólito et al. (2020) reported that sophorolipids produced by *Starmerella* species showed to be effective in controlling unwanted foodborne fungi, as *Aspergillus flavus*, *Aspergillus melleus*, *Aspergillus ochraceus*, *Aspergillus parasiticus*, *Aspergillus niger*, *Fusarium oxysporum*, *Botrytis cinerea*, and *Rhizopus* spp., thus enhancing the safety of the end product.

As for *C. sake*, the presence of this yeast species in cheese has already been reported by Cardinali et al. (2021) in *Queijo de Azeitão* PDO as well as by other authors in Greek PDO cheeses, Brie cheese, *Robiola di Roccaverano* cheese, and French cheeses (Michailidou et al., 2021; Dugat-Bony et al., 2016; Bonetta, Bonetta, Carraro, Rantsiou, & Cocolin, 2008; Viljoen, Khoury, & Hattingh, 2003). In dairy product, *C. sake* exerts lipolytic activity, thus contributing to flavor development in the end product (Narvhus, & Gadaga, 2003).

In the present study, *C. variabile* was detected at high relative frequencies in samples of producer 2 and producer 3. The presence of this fungal genus has already been reported by Cardinali et al. (2021) in *Queijo de Azeitão* PDO cheese and by Panelli, Buffoni, Bonacina, & Feligini (2012) in *Taleggio* cheese. The genus *Cladosporium* encompasses airborne fungi that include species (e.g., *Cladosporium cladosporioides*) that pose safety issues for human health (Arteau, Labrie, & Roy, 2010). It is likely that, in the analyzed samples, the presence of *C. variabile* could be of environmental origin, being this mold commonly associated with airborne mycobiota in the dairy environment (Masotti et al., 2019).

*Ustilago* represents another fungal genus that has already been found in the aerosols at dairy farms (Mbareche, Veillette, Bilodeau, & Duchaine, 2019). Moreover, Xing et al. (2020) reported high relative abundance of *Ustilago* in the rumen fluid of ewes. Hence, the massive occurrence of this airborne mold in the analyzed samples of producer 1 can be related to environmental contamination of milk or cheese at dairy plant.

*D. hansenii* represents one of the most detected yeast species in cheese (Del Bove et al., 2009). The dominance of *D. hansenii* among the cheese mycobiota has already been reported in Portuguese cheeses (Cardinali et al. 2021; Gonçalves Dos Santos, Benito, de Guía Córdoba, Alvarenga, & Ruiz-Moyano Seco de Herrera, 2017), ewe's milk cheeses, as well as in Cheddar, Gouda, blue cheese, smear cheese, and buffalo *Mozzarella* (Del Bove et al., 2009). In the *Queijo da Beira Baixa* PDO cheese samples analyzed in the present study, *D. hansenii* was unexpectedly detected at high relative abundance only in samples of producer 3, whereas very low relative abundances of such yeast were detected in samples of producer 1 and producer 2. In cheese, *D. hansenii* is essential for deacidification during ripening, moreover it is involved in the metabolism of amino acids (Tilocca et al., 2020; Bockelmann, Willems, Neve, & Heller, 2005). Indeed, as reported by Zhang et al. (2021), the consumption of isoleucine, leucine, and phenylalanine by *D. hansenii* leads to the production of 2- and 3-methyl-butanal, 3-methyl-1-butanol, and 2-phenylethanol, respectively, thus strongly affecting the volatile compounds of cheese. Of note, the lipolytic activities exerted by this pro-technologic yeast during ripening enhances cheese flavor (Khattab, Guirguis, Tawfik, & Farag, 2019).

Finally, *P. kluyveri* was detected at high relative abundance only in samples of producer 3; whereas it constituted a minor fraction of the mycobiota in samples of producer 1 and producer 2. To the authors' knowledge, the occurrence of *P.*

*kluyveri* in cheese is rare, being this yeast species mostly associated with fermented vegetables as cocoa beans and olives (Mendoza Salazar, Martínez Álvarez, Ardila Castañeda, Lizarazo Medina, 2022; Parafati, Palmeri, Pitino, & Restuccia, 2022). Of note, *P. kluyveri* together with other *Pichia* species can exert spoilage activity of yoghurt, fermented milk, and cheese, thus resulting particularly unwanted (Abu-Khalaf, & Masoud, 2022; Milanović et al., 2021; Osimani, Garofalo, Harasym, & Aquilanti, 2022). Notwithstanding, in the analyzed samples, no sign of spoilage was observed.

In the present study, HS-SPME-GC/MS analysis allowed an increase of the knowledge on the major and minor volatile compounds occurring in *Queijo da Beira Baixa* PDO cheese to be obtained.

In the analyzed samples, the most represented volatile fatty acids were hexanoic, butanoic, and the branched-chain 3-methyl- and 2-methylbutanoic acids. Due to their low perception thresholds, short-chain fatty acids are considered important contributors to the flavour profile in a wide variety of cheeses (Delgado et al., 2011). Lipolysis occurring in cheese originates most of linear acids. Moreover, microbial fermentation could also significantly contribute to the butyric acid level. The microbial activity also generates the branched-chain fatty acids 2-methylpropanoic (isobutyric), 3-methylbutanoic (isovaleric), and 2-methylbutanoic acids, through the metabolism of the amino acids valine, leucine, and isoleucine respectively (McSweeney et al., 2000). At this regard, positive correlations between methyl ester of propanoic and butanoic acids and *L. lactis* were observed. Of note, these compounds were already correlated with *L. lactis* metabolism during ripening of *Toma*-like cheese (Ruggirello et al., 2018).

Carboxylic acids such as, methyl ketones, aldehydes, and esters are also precursors of other sensory compounds (Collins et al., 2003). In more detail, 2-butanone, with a butterscotch odour, was the most abundant methyl ketone in the headspace of the analyzed *Queijo da Beira Baixa* PDO cheese samples; such compound has previously been identified as main odorant in Cheddar cheese (Arora et al., 1995). Fruity, floral, and musty notes have already been associated with other methyl ketones, such as 2-heptanone and 2-nonanone, hence, their presence in cheese can positively affect the final aroma. Aldehydes, such as 3-methylbutanal, are quickly reduced to alcohols or oxidised to the corresponding acids. In the present study, the most abundant aldehydes (phenylacetaldehyde and benzaldehyde) could have originated from the microbial metabolism of tryptophan and phenylalanine, as suggested by Zheng et al. (2021).

Esterification reactions between fatty acids and primary and secondary alcohols derived from lactose fermentation or from amino acid catabolism generate esters, thus enhancing specific fruity and floral notes of cheese. In the present study, the ester fraction was mainly constituted by ethyl esters of fatty acids. Among these, the ethyl ester of octanoic and decanoic acids were the most abundant compounds.

As reported by Ferreira et al. (2009), high amounts of alcohols were already observed in *Queijo da Beira Baixa* PDO cheese. It is noteworthy that ethanol, produced from lactose metabolism by heterofermentative lactic acid bacteria, has a limited direct role in the aroma of cheeses, but it contributes to the formation of esters. Of note, negative correlation between ethanol and *L. piscium* were observed in the analyzed *Queijo da Beira Baixa* PDO cheese samples.

The occurrence of branched-chain alcohol 3-methyl-1-butanol detected in the analyzed samples could originate from reduction of 3-methylbutanal, which in turn derives from the catabolism of leucine (Carbonell et al., 2002). Ferreira et al. (2009) suggested that that presence of this compound in *Queijo da Beira Baixa* PDO cheese could be favoured by the high proteolytic activity of the vegetable rennet.

Finally, secondary alcohols (2-propanol) detected in the analyzed samples could be formed by enzymatic reduction (alcohol dehydrogenase) of the corresponding methyl ketones (acetone).

## 5. Conclusions

The in-depth microbiological and volatile characterization of a PDO cheese can contribute to depict a clear picture of its identity, thus increasing its link with the place of manufacturing. In the present study, the microbial diversity of *Queijo da Beira Baixa* PDO cheese was disclosed for the first time. Of note, *L. lactis* confirmed to be the key lactic acid bacteria species in fermented dairy products. Moreover, the analyzed cheese samples showed to be a source of still undisclosed microbial diversity, harboring rarely detected species, as *L. piscium* and *L. coryniformis*, that were found at unexpectedly relevant relative abundance. As for yeasts, species that are rarely detected in cheese (e.g., *Starmerella*, *C. variable*, and *P. kluyveri*) were found at surprising high relative abundances. Further research is needed to better clarify the role of these microbial taxa in *Queijo da Beira Baixa* PDO cheese, as well as the eventual contribution of the thistle curdle on their origin.

## CRedit authorship contribution statement

**Federica Cardinali**: Investigation, Formal analysis, Writing - Original Draft. **Roberta Foligni**: Investigation, Formal analysis, Writing - Original Draft. **Ilario Ferrocino**: Investigation, Formal analysis, Writing - Original Draft. **Joanna Harasym**: Investigation, Formal analysis, Writing - Original Draft. **Agnieszka Orkusz**: Investigation, Formal analysis. **Irene Franciosa**: Investigation, Formal analysis. **Vesna Milanović**: Formal analysis, Resources. **Cristiana Garofalo**: Formal analysis, Resources. **Cinzia Mannozi**: Formal analysis. **Massimo Mozzon**: Formal analysis, Resources. **Luca Cocolin**: Writing - original draft. **Andrea Osimani**: Conceptualization, Writing - Review & Editing, Supervision, Resources. **Lucia Aquilanti**: Review & Editing, Resources.

## 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.

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## FIGURE CAPTIONS

**Fig. 1.** Circular ideogram showing the microbiota distribution (>1% of the relative frequency in at least 2 samples) among cheeses.

ASVs and samples are connected with a ribbon, and its thickness is proportional to the abundance of an ASV in the connected sample. The outer circle displays the proportion of each ASV in a given sample and vice versa.

**Fig. 2.** Circular ideogram showing the mycobiota distribution (>1% of the relative frequency in at least 2 samples) among cheeses.

ASVs and samples are connected with a ribbon, and its thickness is proportional to the abundance of an ASV in the connected sample. The outer circle displays the proportion of each ASV in a given sample and vice versa.

**Fig. 3.** Significant co-occurrence and co-exclusion relationships between bacterial and fungal ASVs.

The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red) between bacterial and fungal ASVs. Only significant correlations ( $P < 0.05$ ) are shown.

**Fig. 4.** Significant relationships between microbial taxa and VOCs.

The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red). Only significant correlations ( $P < 0.05$ ) are shown.