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ABSTRACT
The production of ovine or caprine milk cheeses with thistle rennet is a common practice in the Mediterranean basin. The aim of the present study was to obtain information on bacteria and yeast communities harboured in *Queijo de Azeitão* PDO cheese through viable counting and, for the first time, via metataxonomic analyses. Moreover, solid phase microextraction (SPME) technique was applied to characterize *Queijo de Azeitão* PDO cheese volatile compounds. Nine cheese samples were collected from three different artisan producers located in Portugal. The results of physico-chemical analyses showed significant differences were observed between producers, with mean values ranging from 5.40 ± 0.25 (Producer 1) to 6.00 ± 0.22 (Producer 2). As for TTA, Producer 1 showed the highest mean value attesting at 18.04 ± 6.57 mL of 0.1 M NaOH used to reach pH 8.3. Regarding lactic acid concentration, Producer 1 showed the highest lactic acid mean value attesting at 0.488 ± 0.106 g 100 g^{-1}, whereas, for acetic acid, no significant differences were evidence among producers with values comprised between 0.141 ± 0.021 g 100 g^{-1} and 0.245 ± 0.016 g 100 g^{-1}. No significant differences were observed between overall mean values of the three producers for viable counts of presumptive lactococci, thermophilic cocci, presumptive lactobacilli, thermophilic lactobacilli and total mesophilic aerobes with values in the order of 7-8 log cfu g^{-1}. Moreover, no significant differences were evidenced for viable counts of coagulase-negative cocci, enterococci, *Enterobacteriaceae* and *Pseudomonadaceae*. As for eumycetes, cheeses from Producer 1 showed the lowest average value (2.78 ± 2.42 log cfu g^{-1}) in respect with those detected from the other producers. Concerning microbiota and mycobiota of the analyzed cheeses, the alpha diversity index did not show any significant difference between the three producers in terms of composition and complexity of the microbial population. A simple composition was apparently shared by the three producers, whose cheese manufactures were dominated by the presence of *Leuconostoc mesenteroides* (37% of the relative frequency in average), *Lactococcus lactis* (29%), *Lacticaseibacillus zeae* (4.7%), *Lentilactobacillus kefiri* (4.4%), *Serratia* spp. (3.5%), *Lactiplantibacillus plantarum* (2.7%), and *Lactilactobacillus sakei* (2.5%). The mycobiota composition showed the neat dominance of *Yarrowia lipolytica* (46.7% of the relative frequency in average), followed by *Candida ethanolica* (13.6%), *Kurtzmaniella zeylanoides* (9.4%), *Geotrichum candidum* (8.8%), *Galactomyces geotrichum* (8.7%), *Kluyveromyces lactis* (3.5%), and *Geotrichum silvicola* (2.7%). The volatile profile analysis allowed to identify 24 different compounds: 7 acids, 7 esters, 4 alcohols, 3 ketones, 2 aromatic hydrocarbons, and 1 aldehyde. The most represented volatile organic compounds (VOCs) were 2-butanone, butanoic acid and hexanoic acid. A positive correlation between *Lentilactobacillus kefiri* and hexanoic acid and isopentyl isobutyrate was observed ($P < 0.05$). Whereas *Y. lipolytica* displayed the highest number of positive correlations with 3-methyl-butanal, 2-pentanone and 2-pentanol ($P < 0.05$). To the authors' knowledge, this is the very first detection of *Lentilactobacillus kefiri* in raw ewe's milk cheese coagulated with vegetable rennet.

Keywords: thistle rennet; lactic acid bacteria, *Lentilactobacillus kefiri*; *Yarrowia lipolytica*; volatile organic compounds.

1. Introduction

The production of ovine or caprine milk cheeses with thistle rennet is a common practice in the Mediterranean basin, especially in the Iberian and Italian Peninsulas (Aquilanti et al., 2011; Cardinali et al., 2017). In these latter geographical areas, numerous Designation of Origin (PDO) or Protected Geographical Indications (PGI) cheeses are traditionally manufactured, including *Caciofiore Sardo*, produced in Italy; *La Serena* PDO cheese, *Torta del Casar* PDO cheese, *Queso de Burgos*, *Manchego* PDO cheese, and *Murcia al Vino* PDO cheese, produced in Spain; and *Serra da Estrela* PDO cheese, *Castelo Branco* PDO cheese, and *Queijo de Azeitão* PDO cheese, produced in Portugal (Alavi & Momen, 2020; Cardinali et al., 2017).

Queijo de Azeitão PDO cheese is produced in selected dairy farms located exclusively in Palmela, Sesimbra and Setúbal municipalities, Portugal, according to their regular manufacture disciplinary (Legislative Decree n° 49/86). It is manufactured with raw ovine milk coagulated with vegetable rennet from *Cynara cardunculus* L. According to the Portuguese *Direção-Geral de Agricultura e Desenvolvimento Rural*, the production of *Queijo de Azeitão* PDO cheese has started in the 19th century using milk obtained from Bordaleira sheep breed. After collection, the raw ewe's milk is filtered using a dairy filter cloth (mesh <1 mm) and placed in vats. Then, it is heated to 30-32 °C, added with salt (20 g L^{-1}) and crude aqueous extract obtained from *C. cardunculus* L., prepared by maceration of finely chopped dried flowers (0.5 g L^{-1}) in water (ratio 1:10) for about 24 h at room temperature. After milk clotting (ca. 45-60 min), curd is broken and manually transferred into plastic performed moulds (250 g capacity). Moulded cheeses are then manually pressed and left at 10 °C for 10 days at 93-95 % relative humidity. During this period, cheeses are usually turned upside down manually every day and washed with potable water. Cheese ripening is carried out for approximately 20 days at 10-15 °C and 85-95 % relative humidity. Finally, mature cheeses are washed manually with potable water before packaging in a specific glossy vegetable paper. The product is a ripened semi-soft cheese with butter paste, white or slightly yellowish color and distinctive features, highly valued and easily recognized by consumers. As for other raw milk cheeses, the unique texture, flavor, and aroma of *Queijo de Azeitão* PDO cheese are the result of the interactions among the raw materials, clotting agents, production process, and microbiota. It is therefore known that microbial communities naturally contaminating the raw milk and further selected by process parameters as well as the added ingredients strongly contribute to the definition of the final taste of the product, thus representing a key factor in cheese-making. Hence, the knowledge on the composition of microbial communities lead to a better understanding of the role of different microbial groups in cheese manufacture (Araújo-Rodrigues, Tavaria, dos Santos, Alvarenga & Pintado, 2020).

A crucial step in cheese-making is represented by lactic acid fermentation carried out by lactic acid bacteria, which naturally occur in raw milk or are intentionally added as starter cultures to heat-treated milk. In the early cheese-making process, organic acids produced by lactic acid bacteria from the catabolism of carbohydrates (mainly lactose) contribute to milk coagulation. During cheese ripening, lactic acid bacteria release intracellular enzymes responsible for the hydrolysis of proteins and lipids, thus leading to the production of volatile and nonvolatile flavor compounds (Wilkinson & LaPointe, 2020).

Besides to acidification due to microbial fermentation or direct addition of organic acids, milk coagulation, is commonly promoted by the use of animal or vegetable rennet. Animal rennet consists of milk-clotting enzymes, mainly chymosin (also referred to as rennin) and pepsin, obtained from stomachs of ruminant mammals, including calves, lambs and kids (Alavi & Momen, 2020). In alternative, clotting agents of vegetable origin can also be added to milk, as those prepared from dried flowers of members to the Asteraceae family, commonly referred to as thistles. Milk coagulants of vegetable origin are characterized by a variable content of aspartic proteases, depending on the plant source (Alavi & Momen, 2020), such as *Cynara* (artichoke or cardoon), *Scolymus* (golden thistle or oyster thistle) or *Silybum* (milk thistle or Marian thistle) (Cardinali et al., 2016). Besides to stimulating milk coagulation, the use of thistle rennet can also improve the aromatic and technological properties of cheeses as well as exert an antimicrobial and antioxidant action. Furthermore, cheeses produced with vegetable rennet are more digestible, more flavored than those obtained with animal rennet and meet the requirements of the vegetarian diet (Colombo et al., 2018).

To the authors' knowledge, the microbial diversity of *Queijo de Azeitão* PDO cheese has never been investigated via metataxonomic analyses, before. Accordingly, the aim of the present study was to characterize the bacterial and fungal communities in the *Queijo do Azeitão* cheese PDO cheese, as well as its volatile compounds, in order to identify potential associations of such aspects.

2. Materials and methods

2.1. Cheese production and sampling

Figure 1 shows the flow chart of the cheese-making process of *Queijo de Azeitão* PDO cheese analyzed in the present study.

Nine samples of *Queijo de Azeitão* DOP cheese were collected from three different artisan producers located in Palmela, Portugal, that is one of the three municipalities included in the Legislative Decree nº 49/86. In more detail, three samples from the same production batch were purchased from each producer, namely: Producer 1 (samples Q1, Q2, Q3), Producer 2 (samples Q4, Q5, Q6), and Producer 3 (samples Q7, Q8, Q9). All cheeses were produced during August 2019 and

purchased in September 2019. The ripening time was 20 days for all the samples that were characterized by an average wight of 250 g with a diameter of 10 cm and 5 cm height. Moreover, for all cheeses, the "best before" date was comprised between 10 and 16 December 2019. Cheese samples were transported to the laboratory under refrigerated conditions. After arrival, the samples were stored at +4 °C and analysed before the expiration date.

2.2. Physico-chemical measurements

The pH values of *Queijo de Azeitão* PDO cheese samples were determined with a pHmeter Model 300 (Hanna Instruments, Padova, Italy) equipped with a solid electrode HI2031. Three independent measurements were performed for each sample and mean values ± standard deviations were calculated.
Total titratable acidity (TTA) was determined as described by Nionelli et al. (2014) and expressed as mL of 0.1 M NaOH used to reach pH 8.3. Three independent measurements were performed for each sample and means ± standard deviation were calculated.
The D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit and the Acetic Acid (Acetate Kinase Manual Format) test kit (Megazyme, Bray, Ireland) were used to determine the concentration of lactic acid and acetic acid, respectively. The analyses were carried out in triplicate for each sample and the results were reported as mean values ± standard deviation.

2.3. Microbiological analyses

For the microbial viable counts, 10 g of each sample was homogenized with 90 mL of sterile peptone water (bacteriological peptone, 1 g L^{-1}, Oxoid, Basingstoke, UK) with a stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm (Osimani et al., 2009) and further 10-fold serially diluted.
One-hundred µL of each dilution were inoculated in duplicate on opportune growth media to enumerate: presumptive mesophilic lactococci, presumptive thermophilic streptococci, presumptive mesophilic lactobacilli, presumptive thermophilic lactobacilli, coagulase-negative cocci, total mesophilic aerobes, enterococci, *Enterobacteriaceae, Pseudomonadaceae*, and eumycetes. Growth media and incubation conditions for microbial viable counts are reported in Supplementary Table 1. Viable counts were expressed as means of log colony forming units per gram (cfu g^{-1}) of sample ± standard deviations of duplicate experiments.

2.4. 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 sample homogenate (dilution 10^{-1}) prepared as previously described. 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. Bacteria were studied by amplifying and sequencing the V3-V4 region of the bacterial 16S rRNA gene according to Klindworth et al. (2013), whereas fungi were analyzed by amplifying the D1 domain of the 26S rRNA gene (Mota-Gutierrez et al. 2018). Library preparation and sequencing by MiSeq instrument (Illumina, San Diego, CA) were carried out as previously described by Ferrocino et al. (2017).

2.5. Metataxonomic analyses

After sequencing reads were imported in QIIME2 software (Callahan et al., 2016) and the qiime cutadapt trim-paired script was used for primers removal. Reads were quality filtered, trimmed, denoised, dereplicated; forward and reverse sequences were merged and chimeras were removed by using the qiime2 dada2 denoise-paired method (Callahan, McMurdie & Holmes, 2017) in order to obtain the Amplicon Sequence Variants (ASVs). Sequence variants with less than 10 counts in two samples were excluded from further analysis to increase the confidence of sequence reads and reduce bias by possible sequencing errors. Taxonomy of ASVs were obtained through the qiime feature-classifier by using the search methods against the Greengenes database for the 16S data and by using the 26S rRNA gene database from Mota-Gutierrez et al. (2018). Sequences of each ASVs were manually check by Basic Local Alignment Search Tool (BLAST) to confirm the taxonomic assignment. The ASVs tables displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level, genus was displayed and visualized using Circos software.
The raw read data were deposited in the Sequence Read Archive of NCBI under the bioproject accession number PRJNA706919.

2.6. Volatile profile

1.5 g of ground sample were introduced in a 10-mL vial and closed with a screw cap equipped with an elastomeric septum. The vials were allowed to equilibrate in a heating bath at 40 °C for 5 min (Belleggia et al. 2020). Afterwards, the solid phase microextraction (SPME) fiber (divinylbenzene/carboxen/polydimethylsiloxane, 1 cm, 50/30 µm) from

Supelco/Sigma-Aldrich (Milan, Italy) was exposed into the headspace for 10 min. Volatile profile was analyzed and identified according to Mozzon, Foligni & Mannozzi (2020). Briefly, a Varian 3900 gas chromatograph coupled with a Saturn 2100T ion trap mass detector (Varian Analytical Instruments, Walnut Creek, CA, USA) were used. The injector temperature was set at 250°C; the oven temperature increased from 40°C to 220°C at the rate of 6°C/min and was then maintained for 5 min. Gas flow (He) was used in constant mode at 1.0 mL/min. Full scan MS data were acquired in the mass range of 31–250 amu. The identification of volatile compounds was made according to Maoloni et al. 2021 by matching the mass spectral data with those collected in the NIST/EPA/NIH Mass Spectral Library (National Institute of Standards and Technology, MD, USA) and the Kovats retention Indices (RIs) with those available in the public access database Pubchem. Chemical ionization (methanol) spectral data (parent and base peaks) were also used to confirm the molecular weight of volatile substances.

2.7. Statistical analysis

The Tukey-Kramer's Honest Significant Difference (HSD) test (α=0.05) was carried out to evaluate differences within cheese's samples by one-way analysis of variance (ANOVA) using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC). Alpha and Beta diversity index for microbiota and mycobiota were calculated by the qiime2 diversity script. Differences in microbiota or mycobiota between producers were calculated by Wilcoxon-Mann-Whitney test and results were displayed as box plots. Pairwise Spearman's non-parametric correlations were used to study the relationships between microbial taxa abundance 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.

3. Results

3.1. Physico-chemical characterization

The results of physico-chemical analyses are shown in Table 1.
Regarding pH, significant differences ($P < 0.05$) were observed between producers, with mean values ranging from 5.40 ± 0.25 (Producer 1) to 6.00 ± 0.22 (Producer 2).
Regarding TTA, Producer 1 showed the highest mean value ($P < 0.05$) attesting at 18.04 ± 6.57 mL of 0.1 M NaOH used to reach pH 8.3.
As for lactic acid concentration, Producer 1 showed the highest lactic acid mean value ($P < 0.05$) attesting at 0.488 ± 0.106 g 100 g^{-1}.
Finally, regarding acetic acid, no significant differences were evidence among producers ($P < 0.05$).

3.2. Viable counting

The results of viable counting are shown in Table 2.
No significant differences ($P < 0.05$) were observed between overall mean values of the three producers for viable counts of presumptive lactococci, thermophilic cocci, presumptive lactobacilli, thermophilic lactobacilli and total mesophilic aerobes with values in the order of 7-8 log cfu g^{-1}. Moreover, no significant differences ($P < 0.05$) were evidenced for viable counts of coagulase-negative cocci, enterococci, *Enterobacteriaceae* and *Pseudomonadaceae*.
As for eumycetes, cheeses from Producer 1 showed the lowest average value (2.78 ± 2.42 log cfu g^{-1}) in respect with those detected from the other producers ($P < 0.05$).

3.3. Microbiota and mycobiota composition

Concerning microbiota and mycobiota of the analyzed cheeses, the alpha diversity index did not show any significant difference between the three producers in terms of composition and complexity of the microbial population (Supplementary table 1). Indeed, a simple composition was apparently shared by the three producers (Figure 2), whose cheese manufactures were dominated by the presence of *Leuconostoc mesenteroides* (37% of the relative frequency in average), *Lactococcus lactis* (29%), *Lacticaseibacillus zeae* (basonym *Lactobacillus zeae*) (4.7%), *Lentilactobacillus kefiri* (basonym *Lactobacillus kefiri*) (4.4%), *Serratia* spp. (3.5%), *Lactiplantibacillus plantarum* (basonym *Lactobacillus plantarum* subsp. *plantarum*) (2.7%), and *Lactilactobacillus sakei* (basonym *Lactobacillus sakei* subsp. *sakei*) (2.5%). A minor fraction of ASVs belonging to lactic acid bacteria (<1%) as well as spoilage taxa were also observed ~~was also observed among cheeses~~ (Table 3) ~~including *Levilactobacillus brevis* (basonym *Lactobacillus brevis*), *Leuconostoc gelidum*, *Lactococcus garvieae*, and *Weissella confusa*. In addition, several spoilage taxa were observed, including *Fusobacterium* spp., *Corynebacterium* spp., *Rahnella* spp., *Brochothrix* spp., *Psychrobacter* spp., and *Acinetobacter johnsonii*.~~
By plotting the weighted unifrac distance matrix, a separation of the samples based on the batch of production was observed (Figure 3, Anosim P<0.05). A few ASVs were found to drive the separation of the samples (Figure 4). In more detail, Producer 1 was characterized by the presence of *Lentilactobacillus kefiri~~, Leuconostoc gelidum~~* and *Serratia* spp.,

whereas Producer 2 was dominated by f *W. confusa* and Producer 3 was characterized by the presence of *Lacticaseibacillus zeae* ~~and *Streptococcus parauberis*~~ (Figure 4, $P<0.05$).
The mycobiota composition (Figure 5) showed the neat dominance of *Yarrowia lipolytica* (46.7% of the relative frequency in average), followed by *Candida ethanolica* (13.6%), *Kurtzmaniella zeylanoides* (9.4%), *Geotrichum candidum* (8.8%), *Galactomyces geotrichum* (8.7%), *Kluyveromyces lactis* (3.5%), and *Geotrichum silvicola* (2.7%)~~. Several minor ASVs were also observed, including *Saccharomyces cerevisiae, Kazachstania unispora, Debaryomyces hansenii, Starmerella orientalis, Pichia norvegensis, Cladosporium crousii,* and members of the genus *Candida* ~~(Table 3). By plotting the weighted unifrac distance matrix, no clear separation of the samples based on the mycobiota composition was observed (Figure 6, Anosim $P>0.05$), notwithstanding, compared with the other samples, cheeses from Producer 1 were characterized by the dominance of *Candida sake* and *Cladosporium crousii* (Figure 4, $P<0.05$).
The analysis of the co-occurrence and co-exclusion showed 15 negative correlation and 25 positive relationship (Figure 7, FDR <0.05) and within the core-ASVs showed a positive correlation of *Leuconostoc mesenteroides* with *Candida ethanolica* and *Kazachstania unispora*. *Lactococcus lactis* showed multiple negative correlations with *D. hansenii, Kluyveromyces lactis* and *Malassezia*. *Lentilactobacillus kefiri* showed a co-occurrence with *Cladosporium crousii* and *Malassezia*. ~~*Leuconostoc gelidum* showed multiple positive correlations with *Cladosporium crousii, D. hansenii, Malassezia*, and *Saturnispora mendoncae*.~~ Finally, *Candida ethanolica* showed a positive relation with *A. johnsonii* and *Leuconostoc mesenteroides* (Figure 7, FDR <0.05).

3.4. Volatile profile

The volatile profile analysis (Table 4) allowed to identify 24 different compounds: 7 acids (acetic acid, isobutyric acid, butanoic acid, 3-methylbutanoic acid, 2-methylbutanoic acid, 4-Methyl-2-oxovaleric acid, and hexanoic acid), 7 esters (butyl acetate, ethyl butyrate, propyl butyrate, ethyl exanoate, isopentyl isobutyrate, 2-nonanone, and ethyl octanoate), 4 alcohols (n-propanol, 2-pentanol, 3-Methyl-1-butanol, and 2-heptanol), 3 ketones (2-butanone, 2-pentanone, and 2-heptanone), 2 aromatic hydrocarbons (benzene and toluene), 1 aldehyde (3-methyl-butanal). The most represented volatile organic compounds (VOCs) were 2-butanone, butanoic acid and hexanoic acid. Significant differences were observed only for isobutyric acid.
By plotting the correlation between ASVs and VOCs a positive correlation between *Lentilactobacillus kefiri* and hexanoic acid and isopentyl isobutyrate was observed. Regarding the correlation among mycobiota and volatilome profiles *Y. lipolytica* displayed the highest number of positive correlations with 3-methyl-butanal, 2-pentanone and 2-pentanol. For *Kurtzmaniella zeylanoides* a positive relationship with ethyl butyrate was observed, whereas *Kluyveromyces lactis* was associated with 2−heptanol (Figure 8, FDR <0.05).

4. Discussion

Thistle curdled cheeses have recently attracted the attention of consumers and the market. Notwithstanding, most of these cheeses are still produced by small dairy plants, often following traditional recipes. Due to their unique sensory characteristics, many of them are included in the Registry of the Protected Geographical Indications (PGI) and Designation of Origin (POD) products (Alavi & Momen, 2020). At this regard, although carried out on a limited number of samples, the present study on the microbial ecology and associated volatile metabolites of *Queijo de Azeitão* PDO cheese contributes to understand its identity characteristics and added value.
In the present investigation, pH values of *Queijo de Azeitão* PDO cheese manufactures were in accordance with those reported for other Portuguese cheeses such as *Serpa* PDO cheese (Roseiro, Wilbey & Barbosa, 2003; Alvarenga, Silva, Garcia & Sousa, 2008; Dos Santos, Benito, de Guía Córdoba, Alvarenga & de Herrera, 2017; Dos Santos et al., 2018; Araújo-Rodrigues, Tavaria, dos Santos, Alvarenga & Pintado, 2020), *Serra da Estrela* PDO cheese (Macedo, Tavares & Malcata, 2004; Fogeiro, Barracosa, Oliveira, & Wessel, 2020). It is known that the pH of cheese is affected by the metabolic activity of lactic acid bacteria, being the latter responsible for the production of organic acids from lactose. In cheese, the pH level strongly influences ripening due to its impact on both proteolysis and enzymes of vegetable rennet (*Cynara cardunculus*), the latter expressing their maximum activity at pH 5.1 (Heimgartner, Pieltzake,Geersen, Brodeluis, da Silva & Pais, 1999; Roa, López & Mendiola, 1999). However, as already reported by Sanjuán, Millán, Saavedra, Carmona, Gómez & Fernández-Salguero (2002) the contribution of vegetable rennet in the definition of pH of cheese is still controversial.
The viable counts of total mesophilic aerobes were slightly lower than those reported by Mimoso, Firme and Carreira (1992) for *Queijo de Azeitão* PDO cheese, but comparable with those reported for other thistle-coagulated cheeses such as *Serpa* PDO cheese (Barbosa, 2000), *Castelo Branco* PDO cheese (Freitas & Malcata, 2000), *Caciofiore della Sibilla* cheese (Cardinali et al., 2017) and *Caciotta* cheese (Aquilanti, Santarelli, Babini, Osimani & Clementi, 2013).
As for coagulase-negative cocci, members of this microbial group are frequently isolated from fermented foods including ewe's or goat's milk cheese (Irlinger et al., 2008; Coton et al., 2012). These microorganisms generally produce proteolytic and lipolytic enzymes, which significantly contribute to the flavor and aroma definition in the end products (Iacumin, Comi, Cantoni & Cocolin, 2006; Aquilanti et al., 2007). The loads of coagulase-negative cocci revealed in the present study were in accordance with those reported in *Évora* PDO cheese manufactured with vegetable rennet (Freitas &

Malcata, 2000). Differently, *Serra de Estrela* PDO cheese showed lower values after 35 days of ripening, whereas higher coagulase-negative cocci counts were reported for *Caciofiore della Sibilla* cheese and *La Serena* PDO cheese at the 21st and the 60th day of ripening, respectively (Cardinali et al., 2017; Freitas & Malcata, 2000), thus suggesting a high variability of this microbiological parameter.

Enterococci are part of the natural gastrointestinal microflora of humans and animals and are frequently identified in fermented foods, sometimes as a consequence of cross-contaminations. The counts of enterococci in the present study were lower than those reported for raw ewe's milk cheeses clotted with vegetable rennet as *Serpa* PDO cheese (Freitas & Malcata, 2000), *Serra de Estrela* PDO cheese (Dahl, Tavaria & Xavier Malcata, 2000; Macedo, Tavares & Malcata, 2004; Tavaria & Malcata, 2000) and *Caciofiore della Sibilla* cheese (Cardinali et al., 2017).

Enterobacteriaceae are part of the natural microbiota of raw milk and their presence in the end product gives an indication of the poor hygiene conditions applied during cheese processing (Newkirk, Hedberg & Bender, 2011, Coton et al., 2012). The counts of *Enterobacteriaceae* detected in the present study were in accordance with those reported for other cheeses as *Castelo Branco* PDO cheese (Freitas & Malcata, 2000) and *Serpa* PDO cheese (Roseiro & Barbosa, 1996); whereas they were lower than those reported for *Queijo de Azeitão* PDO cheese (Mimoso, Firme & Carreira, 1992), *Serra de Estrela* PDO cheese (Dahl, Tavaria & Xavier Malcata 2000; Macedo, Tavares & Malcata, 2004; Tavaria & Malcata, 2000) and *Caciofiore della Sibilla* cheese (Cardinali et al., 2017). Conversely, *Enterobacteriaceae* counts were higher than those reported for *Caciotta* cheese (Aquilanti et., 2011) after 21 days of ripening or for raw ewes' milk cheeses manufactured with crude aqueous extract of *C. cardunculus* L. (Galán, Cabezas & Fernández-Salguero, 2012).

As for *Pseudomonadaceae*, this family encompasses spoilage bacteria of dairy products, so far detected in raw milk and raw milk cheeses (Cousin, Jay & Vasavada, 2001; Leriche et al., 2004; Morales, Fernandez-Garcia & Nunez, 2005). *Pseudomonas* spp. can produce heat-stable extracellular enzymes such as lipases and proteases that are responsible for cheese alterations (Herrera, 2001). In the presence study, even if presumptive *Pseudomonadaceae* were detected by viable counting, their presence was not highlighted by metataxonomic analysis.

Regarding lactic acid bacteria, it is known that such a microbial group of pro-technological bacteria strongly influences the rheological and sensory properties of cheese, also playing an important role for the inhibition of foodborne pathogens (Ortolani, Yamazi, Moraes, Viçosa & Nero, 2010). Lactic acid bacteria have been reported to predominate in artisan cheeses manufactured with raw ewe's milk coagulated with vegetable rennet as *Serra de Estrela* PDO cheese (Dahl, Tavaria & Xavier Malcata 2000), *Évora* PDO cheese*, Serpa* PDO cheese (Reis & Malcata, 2011), *Caciofiore della Sibilla* cheese (Cardinali et al., 2017), *Caciotta* cheese (Aquilanti et., 2011) and *Torta del Casar* PDO cheese (Ordiales et al., 2013). The average load of lactic acid bacteria in the *Queijo de Azeitão* PDO cheese samples analyzed in the present study agrees with the values reported by Mimoso, Firme and Carreira (1992) for the same type of cheese and with those reported for other Portuguese cheeses (Macedo & Malcata, 1997; Potes & Marinho, 1998; Barbosa 2000; Roseiro, Wilbey & Barbosa, 2003). The lack of significant differences among lactic acid bacteria counts recorded in the analyzed samples attests that, although produced by different manufacturers, the production techniques allowed to obtain similar final products with a specific identity. It is noteworthy that, regarding lactic acid bacteria, there are no growth media able to sufficiently select for the growth of the sole lactococci or lactobacilli, thus limiting the information obtained by classical methods. Hence, the use of culture-independent methods in combination with agar media appears particularly convenient (Vera et al., 2009).

As already demonstrated by several authors, the use of a polyphasic approach for the disclosure of microbial populations in food matrices allows a clearer picture of the microbial species and their relative abundance to be obtained (Cardinali et al., 2017). During the last decade, the use of "next-generation" sequencing techniques has revolutionized the sequencing of DNA; among the most reliable methods, Illumina sequencing provides sound information on the relative abundances of microbial taxa.

The prevalence of *Leuconostoc mesenteroides* in *Queijo de Azeitão* PDO cheeses analyzed in the present study is not surprising since the presence of this species has previously been described in raw ewe's milk cheese and raw cow's milk cheese manufactured with thistle rennet from *C. cardunculus* L., including *Caciotta* cheese (Aquilanti et., 2011), *Serra de Estrela* PDO cheese, *La Serena* PDO cheese (Macedo, Tavares & Malcata, 2004) and *Évora* PDO cheese (Reis & Malcata, 2011), thus confirming the adaptation of this genus to cheeses obtained with vegetable coagulants. Generally, leuconostocs are known to produce aromatic compounds, thus contributing to cheese flavor definition (Van Mastrigt, Egas, Abee & Smid, 2019).

Lactococcus lactis represents one of the major lactic acid bacteria species in the dairy industry. It produces lactic acid from lactose and flavor compounds from the proteolysis of milk-proteins (Cavanagh, Fitzgerald & McAuliffe, 2015). In the present study, the highest relative abundance of *Lactococcus lactis* was detected in samples from Producer 1 that were characterized by the lowest pH values and the highest amount of lactic acid. Interestingly, a study in model Portuguese cheeses carried out by Pereira, Gomes, Gomes & Malcata (2008) highlighted that, in such cheeses, wild strains of *Lactococcus lactis* are responsible for the production of medium- and small-sized peptides as well as free amino acids with high impact on flavor. Furthermore, *Lactococcus lactis* is able to produce exopolysaccharides (EPS) that strongly influence the texture development of cheese (Surber, Schäper, Wefers, Rohm & Jaros, 2020). Of note, the occurrence of a protocooperation between *Lactococcus lactis* and *Kluyveromyces lactis* has been reported by Mangia, Garau, Murgia, Bennani & Deiana (2014), thus suggesting the occurrence of similar complex interaction among those two species in *Queijo de Azeitão* PDO cheese.

As for the detection of *Lacticaseibacillus zeae,* this species has previously been detected in *Caciotta* cheese manufactured with raw cow's milk and aqueous extract of *C. cardunculus* (Aquilanti, Santarelli, Babini, Osimani & Clementi, 2013). *Lacticaseibacillus zeae* is characterized by high proteolytic activity for the presence of catalytic domains encoding for three different proteinase genes (Terzić-Vidojević et al., 2020), thus likely contributing to the production of free amino acids which are responsible for definition of flavour of the product.
Members belonging to the *Lactiplantibacillus plantarum* group have already been identified in other Portuguese cheeses manufactured with raw milk and thistle rennet such as *Évora* PDO cheese (Freitas & Malcata, 2000) and *Serra de Estrela* PDO cheese (Tavaria, Tavares, Silva-Ferreira & Malcata, 2006; Pereira et al., 2010). Moreover, *Lactococcus lactis* spp. lactis, *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* were the most important species contributing to free amino acid profile and carboxylic acid content of *Serra de Estrela* PDO cheese (Pereira et al., 2010), thus suggesting the same contribution in the analyzed *Queijo de Azeitão* PDO cheeses.
Regarding *Lentilactobacillus kefiri* detected in the cheeses analyzed in the present study, to the authors' knowledge, this is the very first detection of such lactic acid bacteria in raw ewe's milk cheese coagulated with vegetable rennet. To date, *Lentilactobacillus kefiri* has usually been detected in kefir beverages (Garofalo et al., 2015; Slattery, Cotter & O'Toole, 2019) and has showed a good inhibitory activity against human foodborne pathogens (Kim et al., 2018). Recently, Maoloni et al. (2020) have also detected *Lentilactobacillus kefiri* in Gioddu, an Italian fermented milk. The presence of *Lentilactobacillus kefiri* in *Queijo de Azeitão* PDO cheese deserves a further in depth, since in kefir, this species, together with *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, and *Lentilactobacillus parakefiri*, contributes to the production of health-promoting EPS with acknowledged antioxidant, antitumor, antimicrobial, cholesterol reducing and immune-modulating properties (Maoloni et al., 2020). It is also worth noticing that, in kefir, the production of bioactive compounds exerted by *Lentilactobacillus kefiri* is boosted by the symbiotic activity with yeasts, thus suggesting a similar behaviour also in *Queijo de Azeitão* PDO cheese. It is noteworthy that *Lentilactobacillus kefiri* strains can also inhibit the growth of some foodborne pathogens, as *Pseudomonas aeruginosa*, *Salmonella* Enteritidis, *Listeria monocytogenes*, and *Bacillus cereus*, thus contributing to improve the safety of the product. Interestingly, in the present study, high loads of *Pseudomonadaceae* have been detected, thus suggesting the need for further investigation. Interestingly, *Lentilactobacillus kefiri* showed a positive correlation with the presence of hexanoic acid, this latter compound already detected by Nambou et al. (2014) in kefir beverages produced using *Lentilactobacillus kefiri* as starter culture. The detection of *Lentilactobacillus kefiri* in *Queijo de Azeitão* PDO cheese represents an absolute novelty, which suggests that this cheese could have still unknown beneficial effects on the health of consumers.
Although a few ASVs related to spoilage microorganism were sporadically detected (e.g., *Brochothrix* spp., *Serratia* spp., etc.), according to the results of Illumina sequencing, no major foodborne pathogens (e.g., *Listeria monocytogenes* or *Salmonella* spp.) were detected in all the analyzed samples, thus suggesting that good hygiene practices were adopted during production.
Finally, eumycetes are widely distributed in the dairy environment and they play an important role in flavour development during cheese ripening. Moreover, these microorganisms produce aromatic compounds, proteolytic, lipolytic enzymes and bioactive molecules able to inhibit spoilage microorganisms during cheese ripening (Beresford, Fitszimons, Brennan & Cogan, 2001; Jacques & Casaregola, 2008; Panelli, Brambati, Bonacina & Feligini 2013; Padilla, Belloch, López-Díez, Flores & Manzanares, 2014; Cardoso et al., 2015). The counts of eumycetes in *Queijo de Azeitão* PDO cheeses (Producers 2 and 3) were in accordance with those reported for *Serpa* and *Serra de Estrela* PDO cheeses (Dahl, Tavaria & Xavier Malcata 2000; Roseiro, Wilbey & Barbosa, 2003), whereas they were lower than those reported for *Évora* PDO cheese (Reis & Malcata, 2011) and *Caciofiore della Sibilla* cheese (Cardinali et al., 2016). Based on the available scientific literature, the most frequently detected yeast species in raw milk cheeses are *D. hansenii*, *Galactomyces* spp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Pichia* spp., *Candida* spp. and *Y. lipolytica* (Banjara, Suhr & Hallen-Adams, 2015; Binetti, Carrasco, Reinheimer & Suárez, 2013; Ceugniez, Drider, Jacques & Coucheney, 2015; Dos Santos, Benito, de Guía Córdoba, Alvarenga & de Herrera, 2017; Ordiales et al., 2013). In the present study, the ascomycetous yeast *Y. lipolytica* (anamorph *Candida lipolytica*) was found to prevail. This yeast is usually present on the cheese surface and in the internal cheese paste (Fleet 1990; Beresford & Williams, 2004) where it produces important metabolites for cheese flavour (Barth & Gaillardin, 1997). Of note, a study by Carreira, Paloma & Loureiro (1998), carried out in Portuguese ewes' cheese made from raw milk, provided evidence that *Yarrowia lipolytica* can also be the causative agent of the browning defect that could likely be related to tyrosine metabolism.
As for *G. candidum* (teleomorph *Galactomyces candidus*), this yeast represents a common species found in mold-ripened, smear-ripened, and acid-coagulated cheeses. *G. candidum* is important in cheese-making for its contribution to the appearance, taste and aroma of cheese. However, sometimes *G. candidum* is defined as spoilage yeast of fermented milks and fresh cheeses. Regarding vegetable clotted cheeses, yeasts belonging to the genus *Galactomyces* have already been detected in *Serpa* PDO cheese (Araújo-Rodrigues et al. 2020) as well as in *Caciofiore della Sibilla* cheese (Cardinali et al., 2017).
Interestingly, regarding *Kluyveromyces lactis*, the association of 2-heptanol with the presence of such yeast species in cheese has already been reported by Padilla, Belloch, López-Díez, Flores and Manzanares (2014) in ewes' and goats' cheeses coagulated with *C. cardunculus*, thus suggesting a potential interaction between this yeast species and the raw materials (thistle rennet and raw milk).

As general consideration, it is important to underline that, given the well-known difficulties in associating viable counts of microbial groups with metagenomics profiles, the identification of isolates obtained through culture-dependent methods is pivotal for a proper comparison of microbial diversity.
In the present study, SPME technique allowed for the first time to detect the major and minor volatile compounds in *Queijo de Azeitão* PDO cheese.
It is noteworthy that, the cheese flavour is influenced by milk enzymes, rennet and microbial enzymes naturally deriving from the occurring microbiota. To date, more than 600 volatile organic compounds (VOCs) have been identified in different types of cheese and many of them have been related to specific and peculiar flavour notes (Curioni & Bosset, 2002; Molimard & Spinnler, 1996; Sablé & Cottenceau. 1999). In more detail, the role of lactic acid bacteria, especially of autochthonous species, is very important for the development of cheese's profile together with moulds, yeasts and non-pathogenic adventitious microorganisms (as those detected in the present study) that naturally occur in the raw materials (vegetable rennet and raw milk).
VOCs are formed during the cheese's ripening by the enzymatic degradation of volatile and branched-chain amino acids; in particular, amino acid transamination is catalysed by aminotransferases of lactococci (Yvon, Thirouin, Rijnen, Fromentier & Gripon, 1997; Tanous, Gori, Rijnen, Chambellon & Yvon, 2005). Cheese volatile profile is influenced by the nature of milk as well as by milk pasteurization treatment, which is known to reduce the amount of volatile compounds, thus explaining the stronger flavor and aroma of raw milk cheeses in respect with those produced using pasteurized milk (Barron et al., 2005).
Ketones are typical VOCs from dairy products and are commonly associated with the aroma of surface-mould ripened and blue veined cheeses (Curioni & Bosset, 2002).
Sensory descriptors such as butter, cheese, chemical, chocolate, ethereal, and gaseous are associated with the presence of 2-butanone that has previously been identified also in *Cheddar* (Arora, Cormier & Lee, 1995) and *Bryndza* cheeses (Štefániková, Árvay, Miškeje, & Kačániová 2020).
Butanoic acid, typically associated with strong, cheese, sweaty odours, has previously been identified in ewe´s cheeses as the *Oscypek* PDO cheese, *Canestrato Pugliese* PDO cheese, *Fiore Sardo* PDO cheese, *Torta del Casar* PDO cheese, *Terrincho* PDO cheese, *Roncal* PDO cheese, *Manchego* PDO cheese and *Pecorino Romano* PDO cheese (Barron et al., 2005; Massouras, Pappa & Mallatou, 2006; Majcher, Goderska, Pikul & Jeleń, 2011; Sádecká, Kolek, Pangallo, Valík, & Kuchta 2014; Delgado-Martínez, Carrapiso, Contador, & Rosario Ramírez, 2019).
Finally, hexanoic acid, commonly associated with waxy, soapy, goaty, and sweaty odours (Ferreira, Pinho & Sampaio, 2009) has previously been identified in raw milk *Feta* PDO and *Piacentinu Ennese* PDO cheeses (Horne et al., 2005).

5. Conclusions

Raw milk cheeses coagulated with thistle rennet represent a source of still undisclosed microbial diversity and the present study contributed to increase the knowledge on microbial species naturally harboured by these food products. In the present study, besides to lactic acid bacteria species acknowledged to contribute to cheese fermentation and ripening, further unexpected bacterial species were detected. In particular, *Lentilactobacillus kefiri* was found in all *Queijo de Azeitão* PDO cheese manufactures analyzed, irrespective of the producer. In addition, this species, commonly associated with kefir beverage, was positively correlated with hexanoic acid and isopentyl isobutyrate, thus suggesting its direct contribution with the release of these VOCs.
As far as eumycetes are concerned, major and minor fungal species were detected, with *Y. lipolytica* representing the dominant yeast species. Whereas other minority yeast species were associated with the presence of volatile compounds.
Further studies considering other producers could help to improve the characterization of *Queijo de Azeitão* PDO cheese identity. Moreover, the study of microbial dynamics occurring during production is also needed to better understand the contribution of dominant and minority species in cheese flavor formation.

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