Microbiological, morpho-textural, and volatile characterization of Portuguese Queijo de Nisa PDO cheese

Microbiological, morpho-textural, and volatile characterization of Portuguese *Queijo de Nisa* PDO cheese

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ABSTRACT

Queijo de Nisa PDO (Protected Designation of Origin) is a semi-hard cheese obtained from raw ewe's milk clotted with cardoon (*Cynara cardunculus*, L.) steep. The aim of the present study was to characterize the bacterial communities naturally occurring in *Queijo de Nisa* PDO cheese samples through viable counting and metataxonomic analysis. Moreover, physico-chemical and morpho-textural analyses were also performed, together with the analysis of volatile organic compounds (VOCs) through solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS). In the analyzed samples, pH values were comprised between 4.84 and 5.74. As for lactic acid, the concentration varied between 0.83 and 2.10 %. Water activity of samples varied between 0.942 and 0.960. Lightness of the samples ranged from 107.82 to 119.16, whereas hardness ranged between 34.45 N and 126.05 N. As for microbiological analyses, lactococci and lactobacilli counts were up to 9.01 Log CFU g^{-1}. Coagulase-negative cocci (primarily referring to the overall term "catalase-positive" cocci, mainly staphylococci) and enterococci counts were up to 7 Log CFU g^{-1}. Metataxonomic analysis revealed that *Lactococcus lactis* and *Leuconostoc mesenteroides* occurred at high frequency in all the analyzed samples. Moreover, *Lactiplantibacillus plantarum*, *Lactococcus piscium*, and *Lacticaseibacillus zeae* were also detected. The most represented VOCs were carboxylic acids, carbonyl compounds, alcohols, and esters. In the analyzed *Queijo de Nisa* PDO cheese samples, significant relationships between bacteria and VOCs were also evidenced. Of note, research on the microbiological and chemical features, as well as on morpho-textural characteristics, of PDO cheeses can increase the knowledge on the interactions between raw materials, environment, and autochthonous microorganisms, thus enabling protection of biodiversity and tradition.

Keywords: metataxonomic analysis; *Cynara cardunculus* L.; *Lactococcus lactis*; *Leuconostoc mesenteroides*; *Lactococcus piscium*; volatile organic compounds; raw ewe's milk.

1. Introduction

Cheesemaking represents one of the most ancient methods to preserve the high nutritional qualities of milk. Cheese can be obtained by acid-induced coagulation or via enzymatic coagulation of raw or pasteurized milk using animal or vegetable rennet. This latter clotting agent is based on the use of watery extracts from plants, including *Euphorbia*, *Ficus*, *Lactuca*, *Solanum*, *Streblus*, and those commonly referred to as thistles (Liu et al., 2021), these latter including plants of the genera *Carduus*, *Cirsium*, *Cynara*, *Onopordum*, *Scolymus*, *Silybum*, and *Carlina* (Cardinali et al., 2016, 2017). Vegetable clotting agents play a pivotal role in the primary and secondary hydrolysis of milk proteins (Zhao et al., 2019). It is noteworthy that not all the plant extracts produce the same coagulating effect, moreover, vegetable clotting agents could have different effect on cheese quality either during manufacturing or ripening, thus affecting the physico-chemical properties, yield, quality, soluble nitrogen content, type of peptides, and sensory characteristics of the final product (Alexandraki & Moatsou, 2018).

After milk clotting, curd is cut into pieces, to allow whey release, and placed into moulds. Then, a ripening period may occur, depending on the process and the type of cheese. During ripening, curd is subjected to a strong modification that is produced by milk enzymes, and by fermentation carried out by autochthonous microorganisms naturally occurring in raw milk or by starter cultures (Aquilanti et al., 2007).

In the cheese matrix, lactic acid bacteria represent the key microbial group that drives the fermentation through the production of organic acids (mainly lactic and acetic acid), these latter affecting the safety and the sensory attributes of cheese (Aquilanti et al., 2012). Of note, flavor development from milk proteins, carried out by lactic acid bacteria, is based on the proteinase-mediated hydrolysis of casein, with formation of casein-derived peptides (Steele, Broadbent, & Kok, 2013). Moreover, flavor development of cheese in affected by aromatic, sulfur-containing, and branched-chain amino acids, whose production is catalyzed by lyases and aminotransferases (Steele et al., 2013). Hence, cheese sensory traits are strongly influenced by the co-occurring microbial populations interacting with the food matrix and the environment.

In the European Union, the production of cheeses clotted with vegetable rennet is a common practice, especially in the countries located in the Mediterranean biogeographical Region. Among the thistle-curdled cheeses produced in Southern European countries the most renowned are *Pecorino*, *Caciofiore Sardo*, and *Caciofiore della Sibilla* which are produced in Italy, *La Serena* PDO cheese, *Torta del Casar* PDO cheese, *Burgos*, *Manchego* PDO cheese, and *Murcia al Vino* PDO cheese which are produced in Spain, *Serra da Estrela* PDO cheese, *Queijo da Beira Baixa* PDO - *Castelo Branco* cheese, *Queijo de Azeitão* PDO cheese, and *Queijo de Nisa* PDO cheese which are produced in Portugal (Alavi & Momen, 2020).

Queijo de Nisa PDO is a semi-hard cheese that obtained the PDO (Protected Designation of Origin) recognition in the year 1996, registered under Commission Regulation (EC) No 1107/96. *Queijo de Nisa* PDO cheese is obtained from raw ewe's milk clotted with cardoon (*Cynara cardunculus*, L.) steep applied to milk at 25 – 28 °C for 45– 60 min. The ripening process is divided in two phases: the first carried out for 15-18 days at 8-10 °C and 80-90 % relative humidity followed by a second phase carried out for 30-40 days at 10-14 °C and 85-90 % relative humidity. The final product is characterized by a white-yellow colored paste with small holes, and a semi-hard consistency.

The geographical area of production includes the Portuguese subdistricts of Nisa, Crato, Castelo de Vide, Marvão, Portalegre, Monforte, Arronches, and Alter do Chão. *Queijo de Nisa* PDO cheese has a strong link with its production area, since sheep breeds (*merino* and *saloia*) that provide the raw milk are reared in the regional natural pastures that are covered by oak groves and characterized by a flora rich in Mediterranean vegetable species. Such peculiar environment confers the distinctive characteristics to the milk and, hence, to the cheese. Regarding seasonality, the production of *Queijo de Nisa* PDO cheese is traditionally concentrated in the period from November to June, because of the non-existence of natural conditions for long-term preservation of the milk after milking, which prompts the farmers to produce and ripe cheeses in a cold weather season, as well as the great volume of forage provided by the Mediterranean climate, in spring and early summer (Fragata, Louro Martins, & Vasconcelos, 1999).

To the best of the authors' knowledge no published studies on the bacterial diversity as well as texture and volatilome of *Queijo de Nisa* PDO are available in the scientific literature, except for only one recently published paper investigating the gas holes of this cheese (Dias et al., 2021). Hence, the aim of the present study was to characterize the bacterial communities naturally occurring in ripened *Queijo de Nisa* PDO cheese samples through viable counting and metataxonomic analysis. Moreover, physico-chemical, and morpho-textural analyses were also performed, together with the analysis of volatile organic compounds (VOCs). The cheese microbiota is known to be shaped by the cheese making steps and ripening, which in turn cause a selective pressure on microorganisms. Hence, the qualitative and quantitative investigation of the microbial species dominating in ripened cheeses allow the identification of microbial drivers for quality of *Queijo de Nisa* PDO, thus serving as a first baseline for future studies aimed at protecting its tradition.

2. Materials and methods

2.1. Cheese sampling

Eight samples of *Queijo de Nisa* PDO cheese were collected in the first half of September 2021, soon at the end of the second phase of ripening (overall maturation ~2 months)**,** from four Portuguese artisan producers. In more detail, two

samples from the same production batch were purchased from each producer as follows: producer 1, located in Crato (samples N1 and N2); producer 2, located in Monforte (samples N3 and N4); producer 3, located in Monforte (samples N5 and N6); producer 4, located in Portalegre (samples N7 and N8). All *Queijo de Nisa* PDO cheese samples had the same height (5 cm), diameter (12 cm), and weight (about 200 g). All the four producers were located in the sub-region of Alentejo, at a distance of a few kilometers from each other.

The proximate composition of samples, as reported on the product label, is described in Supplementary Table 1.

All samples were placed into sealed sterile bags (Whirl-Pak® Sample Bag, Merck KGaA, Darmstadt, Germany), shipped to Italy and Poland via express courier under refrigerated conditions, using hand-portable icebox with eutectic plates (Giostyle, Urgnano, Italy), stored at the laboratory at + 4 °C soon after arrival, and analyzed before the expiration date.

A scheme depicting the general manufacturing procedure of *Queijo de Nisa* PDO cheese is shown in Supplementary Figure 1.

2.2. Physico-chemical measurements

The pH of samples was measured using a pHmeter (Hanna Instruments, Padova, Italy) equipped with a HI2031 solid electrode (Hanna Instruments). For each sample, three independent measurements were performed, and the results were reported as mean ± standard deviation.

To measure total titratable acidity (TTA), 10 g of each sample were weighted and blended with 90 mL of deionized water using 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. For each sample, three independent measurements were performed, and the results were reported as mean ± standard deviation.

The concentration (%) of lactic acid was measured using the commercial D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay Kit (Megazyme, Bray, Ireland), in accordance with the manufacturer's instructions. For each sample, three independent measurements were performed, and the results were reported as mean ± standard deviation.

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 performed at the temperature of 25 ± 0°C. For each sample, three independent measurements were performed, and the results were reported as mean ± standard deviation.

Specific volume was calculated using sample weight and volume, which was evaluated as a change after immersing a known weight of the sample 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 g mL^{-1}.

2.3. Morpho-textural analyses

Color parameters were measured with 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. Color 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 evaluated through TPA test (Texture Profile Analysis) in quadruplicate with an AXIS texture analyzer FC200STAV500 (AXIS, Gdansk, Poland) provided with the software "AXIS FM". In more detail, an aluminium 20-mm-diameter cylindrical probe was used in a double compression test (TPA) to penetrate to 50 % depth, at 1 mm s^{-1} speed test. Hardness (*N*) was the force at the maximum deformation, whereas cohesiveness, springiness, chewiness, and resilience were calculated from the peaks. Analysis was carried out in quadruplicate at 25 °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 μL of each dilution was inoculated in duplicate on the following growth media: M17 agar (VWR International, Milan, Italy) for presumptive mesophilic lactococci, with incubation at 22 °C for 48-72 h; M17 agar (VWR) for presumptive thermophilic streptococci, with incubation at 42°C for 48-72 h; MRS Agar (VWR) for presumptive mesophilic lactobacilli, with incubation at 30 °C for 48-72 °C; MSA Agar (VWR) for coagulase-negative cocci (primarily referring to the overall term "catalase-positive" cocci, mainly staphylococci), with incubation at 37 °C for 48 h; Enterococcus Selective Agar (Merck KGAa, Darmstadt, Germany) for enterococci, with incubation at 37 °C for 48 h; VRBGA (VWR) Agar for Enterobacteriaceae, with incubation at 37 °C for 24 h.

For each sample, and for each growth medium, two independent experiments were performed, the results were expressed as the Log of CFU (colony-forming units) per gram of each sample and reported as mean value ± 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. 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 *Queijo de Nisa* PDO cheese samples of four different producers in order to highlight any difference in microbiota composition. The 16S rRNA gene (V3-V4 regions) was amplified using primers and procedures described previously (Klindworth et al., 2013). The Illumina guidelines were used for the PCR products purification, taggering 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 raw read data were deposited in the Sequence Read Archive of NCBI under the bioproject accession number PRJNA822519

2.7. Volatile profile

Solid phase microextraction (SPME) was used to collect the volatile components. In more detail, a 10 mL glass vial was filled with 0.5 g of ground sample and a DVB/PDMS 65 µm fibre (Supelco/Sigma-Aldrich, Milan, Italy) was exposed into the headspace for 45 min at 50°C, as described by Belleggia 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 × 0.25 mm i.d., 0.25 µm film thickness (Phenomenex, Torrance, CA, USA) were used to determine the volatile profile. The operative conditions were those already reported by Foligni et al. (2022). According to Mozzon et al. (2020), the identification of volatile compounds was made by matching the mass spectral data

with NIST/EPA/NIH Mass Spectral Library 2020, and the chromatographic behaviour 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 Honest Significant Difference (HSD) test ($P < 0.05$) was carried out to evaluate differences among producers 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 Kruskall wallis test in R environment. Pairwise Spearman's non-parametric correlations were used to study the relationships between bacteria and VOCs. The correlation plots were visualized in R using the *corrplot* package in R environment (version 4.1.0). 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 carried out on the *Queijo de Nisa* PDO cheese samples are reported in Table 1. Regarding pH, no statistically significant differences were highlighted among the average values of the samples collected from the four producers whose values were comprised between 4.84±0.02 and 5.74 ±0.02.

As for total titratable acidity, samples of producer 1 showed the highest average values, whereas those of producer 4 the lowest. Wide variations were observed among samples whose TTA values ranged between 8.55 ± 1.06 and 31.70 ± 1.13 mL of 0.1N NaOH.

Regarding lactic acid, samples of producer 3 showed the highest average value, whereas those of producer 4 the lowest. In the analyzed samples, lactic acid concentration varied between 0.83 ± 0.14 and 2.10 ± 0.15 %.

Water activity was the highest for samples of producer 1 and the lowest for producer 4, being 0.960 ± 0.007 and 0.942 ± 0.002, respectively. The highest values for specific volume were observed for producer 1 (1.26 ± 0.31g mL^{-1}) and the lowest for producer 2 (1.01 ± 0.15 g mL^{-1}).

3.2. Morpho-textural characterization

The results of morpho-textural analyses carried out on the *Queijo de Nisa* PDO cheese samples are reported in Table 2. The lightness (L*) of the samples varied from 107.82±0.16 to 119.16±0.48, being indistinguishable between producer 1, 2 and 3, whereas cheeses of producer 4 were darker. Cheese samples of producer 1, 2, and 3 revealed greenish tones (a*) varying from -0.11±0.04 to -5.84±0.13, however the samples of producer 4 outstand the trend revealing red tones up to 1.25 ± 0.17. All the cheese samples were towards yellowish tonality (b*) ranging from 24.10±0.14 to 38.98±0.31 being the most pronounced for samples of producer 4. Cheeses of producer 4 differed from the rest of samples and both Chroma and hue values were visibly recognizable as different. The lowest Chroma value was 24.27 ± 0.13 (producer 3), whereas the highest was 38.99 ± 0.31 (producer 4). Hue for cheeses of producers 1, 2 and 3 ranged from 90.2 ± 0.1 to 98.7 ± 0.2 with high diversity among samples, whereas for producer 4 hue was the lowest and very similar among the samples ranging from 88.1 ± 0.3 till 88.6 ± 0.1.

The texture profile analysis (Table 3) revealed huge variations in hardness of the cheese among samples except for producer 4. Hardness ranged between 34.45±2.9 N and 126.05±5.3 N with the highest value for samples of producer 2, where also the highest cohesiveness was observed reaching 0.583±0.129, both those features impacted the chewiness for samples of producer 2 which reached 35.43±4.10. The less force for chew was needed for samples of producer 4, where chewiness showed slight variations among samples, ranging from 3.66±0.39 to 4.19±0.02. The resilience ranged between 0.148±0.001 and 0.209±0.072 being not statistically different among producers and samples.

3.3. Viable counting

The results of microbiological analyses carried out on the *Queijo de Nisa* PDO cheese samples are reported in Table 4. Regarding lactic acid bacteria, no significant differences were observed among the average values of the samples collected from each producer. In more detail, for presumptive lactococci, viable counts were comprised between 7.71 ± 0.08 and 9.01 ± 0.05 Log CFU g^{-1}, whereas counts of presumptive thermophilic cocci ranged between 6.48 ± 0.00 and 8.64 ± 0.21 Log CFU g^{-1}, and presumptive lactobacilli counts were comprised between 7.60 ± 0.08 and 8.94 ± 0.02 Log CFU g^{-1}.

As for coagulase-negative cocci, samples of producer 4 showed the highest average value, whereas those of producer 1 the lowest. In the analyzed samples, viable counts of coagulase-negative cocci were comprised between 3.48 ± 0.01 and 6.80 ± 0.02 Log CFU g^{-1}.

Regarding enterococci, the highest average value was detected in samples of producer 3, whereas the lowest was detected in samples of producer 1. The counts of enterococci ranged between 3.86 ± 0.36 and 6.91 ± 0.07 Log CFU g^{-1}.

Enterobacteriaceae were only detected in samples of producer 4 with values comprised between 2.48 ± 0.00 and 4.86 ± 0.02 Log CFU g^{-1}.

3.4. Microbiota composition

The data analysis showed that microbiota composition of cheeses was associated with the different four producers (Figure 1, Supplementary Table 2). In more detail, *Lactococcus lactis* (relative frequency from 28 to 61%) and *Leuconostoc mesenteroides* (relative frequency from 2 to 15%) were detected at relatively high frequency in all the analyzed samples. Whereas *Lactiplantibacillus plantarum* showed the highest relative frequency in samples of producer 1 (43%) and producer 4 (49%). Samples of producer 2 were characterized by the high relative frequency of *Lactococcus piscium* (28%) and *Serratia* (16%). Whereas only in samples of producer 3 was detected *Lactococcus garvieae* (4%) as a minor ASVs (Figure 1). *Lacticaseibacillus zeae* was detected at 5 % of the relative frequency in samples from producer 1 and producer 2, whereas the same lactic acid bacterium was detected at 2 and 0.2 % of the relative frequency in samples from producer 3 and producer 4, respectively. *Corynebacterium* was present at low relative frequency (4%) only in samples of producer 4. *Staphylococcus* species were present in all the samples at low relative frequency except in samples of producer 2, where no staphylococci were detected (Figure 1).

Overall, samples of producer 1 showed the lowest value of species heterogeneity. Nevertheless, no significant differences between the cheeses microbiota and alpha diversity index were observed (data not shown).

3.5. Volatile profile

The average percentages of the classes of VOCs found in *Queijo de Nisa* PDO cheese samples are reported in Figure 2, whereas the detailed identification of VOCS is reported in Table 5.

The most represented compounds detected in the *Queijo de Nisa* PDO cheese samples were carboxylic acids (2-methyl propanoic, butanoic, 2-methyl butanoic, 3-methyl butanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, dodecanoic) (31-50% of total volatiles), followed by carbonyl compounds (2-butanone, 3-methyl butanal, 3-hydroxybutan-2-one, 2-heptanone, benzaldehyde, phenylacetaldehyde, 2-nonanone, dodecanal) (5-24% of total volatiles), alcohols (ethanol, 2-propanol, 3-methyl-butanol) (1-24% of total volatiles), and esters (isobutyl acetate, 2-butyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate) (1-4% of total volatiles).

Of note, butanoic, octanoic and nonanoic represented the most volatile fatty acids, whereas 2-butanone (butterscotch odour) was the most abundant methyl ketone. Finally, the ester group was mainly represented by the ethyl hexanoate.

3.6. Correlation analysis

The significant relationships between bacteria and VOCs in *Queijo de Nisa* PDO cheese samples are reported in Figure 3. In more detail, the correlation analysis showed a positive relationship between the concentration of ethanol and propan-2-ol and the presence of *Carnobacterium, Gluconacetobacter, L. piscium, Pseudomonas fragi* and *Serratia* ($P < 0.05$). *L. piscium, P. fragi*, and *Gluconacetobacter* showed a negative correlation with isobutyl acetate, 2−heptanone and dodecane ($P < 0.05$). The presence of *Staphylococcus* was positively correlated with isobutyl acetate, ethyl hexanoate, 2-nonanone, and dodecane; whereas *Staphylococcus* showed a negative correlation with ethanol, propan-2-ol, 2-butanone, and butanoic acid 3−methyl (P values < 0.05).

Enterobacteriaceae showed only positive correlations with decanoic acid ethyl ester and dodecanoic acid ethyl ester ($P < 0.05$). *L. plantarum* was negatively correlated with ethanol and propan−2−ol, and positively correlated with isobutyl acetate, 2−heptanone, benzaldehyde, heptanoic acid, 2−nonanone, and dodecane ($P < 0.05$). *Latilactobacillus sakei* showed a negative correlation with benzaldehyde ($P < 0.05$). The presence of *Ln. mesenteroides* was positively correlated with propan−2−ol, and negatively correlated with isobutyl acetate, 2−nonanone, and dodecane ($P < 0.05$).

4. Discussion

Studies dealing with microbiological and physico-chemical characteristics of traditional fermented foods are pivotal for preserving their peculiar traits that define cheese quality and authenticity (Gobbetti et al., 2018).

In the present study *Queijo de Nisa* PDO cheeses produced in the summer grazing period at four artisan producers located in the subregion of Alto Alentejo in Portugal were subjected to the characterization of the main microbiological, physico-chemical and morpho-textural traits. Artisan cheeses, such as that herein investigated, are food matrices, whose complexity reflects the complexity of the production environment, especially in mountain regions (Turri et al., 2021). Microbial communities are acknowledged to deeply affect the quality of artisan cheeses, together with several environmental and processing variables, including pasture grazing, raw milk quality and treatment, cheese-making and ripening conditions, season, etc. Therefore, a great variation of artisan cheeses produced from different pastures, raw milk batches, seasons, etc., is expected and often greatly appreciated by consumers, due to the uniqueness of these cheeses (Turri et al., 2021).

To the best of the authors' knowledge, the microbiota of *Queijo de Nisa* PDO cheese has never been thoroughly assessed. At this regard, to date the viable counts of lactobacilli, lactococci, and enterococci as well as the antibiotic resistance traits

of a few *Enterococcus* spp. strains isolated from *Queijo de Nisa* PDO cheeses collected at 2 cheesemaking units have just been investigated (Bastião Rocha et al., 2022); similarly, no data are currently available on physico-chemical and morpho textural traits of this thistle-curdled cheese.

For pH, it is noteworthy that the acidification of curd through the metabolic activity of lactic acid bacteria produces a safe and stable cheese throughout time. Moreover, the pH of cheese has a direct impact on its functional properties; indeed, low pH increases the amount of soluble calcium in the cheese and decreases the ability of cheese to melt and stretch (McMahon, Paulson, & Oberg, 2005). The pH values detected in the analyzed *Queijo de Nisa* PDO cheese samples were in accordance with those detected by Cardinali et al. (2021) in *Queijo de Azeitão* PDO cheese, a Portuguese thistle-curdled cheese whose values ranged between 5.1 and 6.1. The detected pH values were also similar with those measured by Cardinali et al. (2017) in *Caciofiore della Sibilla*, an Italian cheese clotted with thistle rennet from *Carlina acanthifolia* All. subsp. *acanthifolia*, and with those reported by Ordiales et al. (2013) for the traditional Spanish cheese *Torta del Casar* clotted using *C. cardunculus* L. rennet.

Regarding the TTA, this parameter is used to evaluate the free acidity in food and is expressed as the quantity of the dissociated and undissociated carboxyl functional groups in lactic acid solutions (Feng, Xiang, Bian, & Li, 2020), whereas lactic acid concentration value expresses the total amount of monomeric lactic acid, oligomers, and lactide with the corresponding water of hydrolysis (Feng et al., 2020). Lactic acid is the result of microbial fermentation of lactose contained in the milk and in the curd after clotting. The content of this organic acid is higher in cheese characterized by lactic acid bacteria with homolactic fermentation (e.g., *L. lactis*) (Kuda et al., 2014). The amount of lactic acid detected in the samples under study was notably higher than the amount reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO cheese, whose average values were up to 0.488 %, thus likely reflecting the high metabolic activity of the autochthonous lactic acid bacteria population.

Water activity is defined as index of the free water that is available to contribute to water vapour pressure over the product surface. The method of manufacturing cheese impacts the initial water content in curd, whereas salt and proteolysis restrict the mobile phase of water (Hickey, Guinee, Hou, & Wilkinson, 2013). The lowest a_w was observed for samples of producer 4 which correlates with the blandest texture of those samples, being a result of progressing proteolysis. During cheese ripening, microorganisms thrive as immobilized cells, metabolizing substrates in the matrix to produce products triggered by enzymatic reactions. Local rates of diffusion limitation, both in the matrix and in bacterial colonies, may be responsible for modulating the metabolic and enzymatic activity of microorganisms during ripening (Floury, Jeanson, Madec, & Lortal, 2013).

In cheese manufacturing, color and texture are direct results of the ripening process, however progressing proteolysis liquefies the matrix, thus leading to transition from solid to gummy texture. The use of the cardoon, *C. cardunculus L.*,

as a main coagulant in *Queijo de Nisa* PDO cheese manufacturing is a requirement for obtaining the status of PDO; however, the is no standardized method of cardoon extract preparation. Moreover, cardoon flowers are picked up locally, which may cause uncontrolled diversity in content of cardosins in cardoon flower due to different stages of plant maturity. Of note, the enzymatic activity of extracts of cardoon flowers was previously correlated with specific and technologic features mostly in ewes' cheeses, mainly as the effect of proteolytic enzymes (Gomes et al., 2018). While the cheese making process benefits in quantitative and qualitative differences on α- and β-casein proteolysis, the diverse activity of coagulants impacts on biochemical, textural, and sensory properties of the cheese (Pino, Prados, Galán, McSweeney, & Fernández-Salguero, 2009). The geographical production area of *Queijo de Nisa* PDO includes large part of the country where differences in climate zones and, hence, in plants characteristics are observed. Gomes et al. (2018) studied the characteristics of cardoon flower from Alentejo as coagulant for cheese making and observed up to five different ecotypes of *C. cardunculus L.* flowers with diversified clotting activity. Hence, these findings may explain the significant different texture features observed for *Queijo de Nisa* PDO samples from Portalegre (producer 4). Indeed, those samples showed signs of high proteolysis that impacted on texture profile; the same samples also showed reddish tonalities in color. The overall activity of used cardoon coagulant could have also impacted on the microbial profile of resulting cheese as the highest Enterobacteriaceae counts were observed in cheeses of producer 4.

Regarding bacterial communities, the primary substrate used in cheese-making, being raw milk (not analyzed in the present study), is notoriously characterized by a neatly lower biodiversity and less pronounced aroma than the final product (McSweeney, 2004). From this raw ingredient, hundreds of cheeses with distinctive taste, texture, flavor, and aroma can be obtained, due to a unique combination of microbiological, biochemical, and technological factors (Wouters et al., 2002). Apart from organic acids production from lactose, which occurs at the very early stage of cheese fermentation, the main biochemical changes in cheese occur over the ripening process, being: (i) proteolysis, lipolysis, metabolism of residual lactose (primary processes); and (ii) amino acid and fatty acid metabolism (secondary processes). Both primary and secondary processes are mainly driven by microbiological changes occurring during ripening, including the lysis of starter lactic acid bacteria and the overgrowth of secondary non-starter lactic acid bacteria (McSweeney, 2004; Marilley & Casey, 2004). Thus, the lactic acid bacteria dominating along ripening are those most affecting taste and quality of cheeses (Antonsson et al., 2003). Hence, the investigation of the microbiota thriving in the final cheeses is of utmost importance for the understanding of the interrelation between cheese microbiological components of quality traits of the final products.

In the present study, high counts of presumptive mesophilic and thermophilic lactococci, as well as lactobacilli, were detected in all samples. The counts of mesophilic lactococci herein detected were in accordance with those reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO cheese, that attested up to 7.6 Log CFU g^{-1}, and with those reported by

Dahl, Tavaria, & Malcata (2000) for *Serra da Estrela* PDO cheese, that were between 7 and 8 Log CFU g^{-1}. Due to their fast acidification, lactococci represent one of the dominant lactic acid bacteria groups in cheese and are usually used as starter cultures (Widyastuti & Febrisiantosa, 2014).

In cheese, thermophilic streptococci are rapid acidifier whose activity is at maximum during the beginning of cheese manufacturing. Of note, despite the high counts of presumptive thermophilic streptococci detected in the analyzed samples, neither *Streptococcus thermophilus* nor other thermophilic streptococci were detected through the metataxonomic analysis. Hence, it is likely that the applied incubation temperature (42°C) of M17 medium was not sufficiently selective to inhibit the growth of lactobacilli (e.g., *L. plantarum*). Interestingly, as suggested by Veselá et al. (2019), in contrast to the standard method for enumeration of thermophilic streptococci, the incubation temperature should be increased to 45 °C to preclude *L. plantarum* growth and to enumerate *S. thermophilus* on M17 agar medium.

As for lactobacilli, their presence has already been reported by Macedo et al. (2000) in *Serra da Estrela* PDO cheese and by Cardinali et al. (2021; 2022) in *Queijo de Azeitão* PDO cheese and in *Queijo da Beira Baixa* PDO cheese, with counts up to 8 Log CFU g^{-1}. As reported by Gobbetti, De Angelis, Di Cagno, Mancini, & Fox (2015), mesophilic lactobacilli represent the most important microorganisms of the non-starter lactic acid bacteria microbiota of cheese. Such microorganisms originate from the raw milk and the dairy environment and are responsible for proteolysis and flavor formation (Gobbetti et al., 2015). Of note, typical thermophilic dairy lactobacilli (e.g., *Lactobacillus delbrueckii* and *Lactobacillus helveticus*) were never detected in the cheese samples under study.

Regarding coagulase-negative cocci, species belonging to this microbial group contribute to the development of sensory characteristics of cheese due to proteolysis and lipolysis carried out by microbial enzymes (Ruaro, Andrighetto, Torriani, & Lombardi, 2013). Of note, coagulase-negative cocci are easily recovered from ewe's or goat's cheeses and represent a group of salt and acid tolerant microorganisms (Ruaro et al., 2013), thus explaining their adaptation to cheese. Counts of coagulase-negative cocci detected in the analyzed samples were in accordance with those reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO cheese, that attested up to 6 Log CFU g^{-1}, and with those reported by Freitas & Malcata (2000) for *Evora* PDO cheese clotted with vegetable rennet, that attested up to 5 Log CFU g^{-1}.

In cheese, enterococci have a controversial role (Bastião Rocha et al., 2022). Indeed, such microbial group, that encompasses microorganisms that are also included within lactic acid bacteria, can perform multiple functions. On the one hand, enterococci are known to be important in developing aroma and flavor in cheese, on the other hand, these microorganisms are indicators of poor hygiene during cheese production (Bastião Rocha et al., 2022). The counts of enterococci detected in the present study were in accordance with those reported by Bastião Rocha et al. (2022) for *Queijo de Nisa* PDO cheese, that attested between 4 and 8 Log CFU g^{-1}. Interestingly, in the same cheese, Bastião Rocha et al. (2022) also highlighted the presence of antibiotic-resistant strains of *Enterococcus durans*, *Enterococcus faecium*, and

Enterococcus faecalis, thus confirming the sanitary importance of these lactic acid bacteria in food. Counts of enterococci detected in the present study were also in accordance with those reported by Cardinali et al. (2021) for *Queijo de Azeitão* PDO cheese that were up to 6.7 Log CFU g^{-1}, thus confirming the adaptation of such microbial group to Portuguese cheeses.

Regarding Enterobacteriaceae, only samples of producer 4 showed the presence of counts attesting about 4 Log CFU g^{-1}. It is known that, in cheese, bacterial species belonging to the Enterobacteriaceae family are easily inhibited by organic acids produced by lactic acid bacteria during fermentation. The occurrence of high Enterobacteriaceae counts in samples of *Queijo de Nisa* PDO of producer 4 suggests a lack of lactate production by lactic acid bacteria. Indeed, in samples of producer 4, the average amount of this organic acid was the lowest among samples. Interestingly, Westling et al. (2016) observed an increased intensity of the sensory characteristics bitter, metallic, pungent, manure, and ammonia in cheese containing high levels of Enterobacteriaceae. Of note, in samples of producer 4 high levels of butanoic acid were detected, with this compound often associated with the putrid flavor note.

The metataxonomic analysis showed that only a few lactic acid bacteria were detected with high relative frequency, thus representing the dominant microbiota shared among samples.

In more detail, *Lc. lactis* represented the most prevalent lactic acid bacterium in all samples, irrespective of the producer. *Lc. lactis*, which includes four subspecies (namely *lactis*, *cremoris*, *hordniae*, and *tructae*), is a mesophilic lactic acid bacterium that in cheese is principally involved in lactic acid production from lactose (Kazou, 2022). Moreover, *Lc. lactis* converts milk proteins into aroma compounds, thus representing a species of paramount significance in both industrial and artisanal dairy fermentations (Kazou, 2022). In cheese, *Lc. lactis* can produce small peptides by caseins hydrolysis and it can further degrade peptides into free amino acids through microbial peptidases (Bourdat-Deschamps, Le Bars, Yvon, & Chapot-Chartier, 2004). Although *Lc. lactis* is generally considered to have negligible lipolytic activity, Macedo, Tavares, & Malcata (2003) observed a strong esterase activity in *Lc. lactis* strains isolated from the Portuguese *Serra da Estrela* PDO cheese. Moreover, Román Naranjo, Callanan, Thierry, & McAuliffe (2020) recently reported a high esterolytic activity in *Lc. lactis* strains of environmental origin. The high prevalence of *Lc. lactis* in naturally fermented Portuguese cheeses has also been reported by Gonçalves et al. (2018) in *Serpa* PDO cheese produced with raw ovine milk and cardoon flower rennet, and by Cardinali et al. (2021) in *Queijo de Azeitão* PDO cheese.

As for *Ln. mesenteroides*, this heterofermentative mesophilic lactic acid bacterium is responsible for the production of aroma compounds in cheese through catabolism of free amino acids, mainly realized via aminotransferase activity (Pedersen, Vogensen, & Ardö, 2016; Endo, Maeno, & Liu, 2022). As reviewed by Gobbetti et al. (2018), in cheese, the growth of *Ln. mesenteroides* depends on the proteolytic activity exerted by *Lc. lactis*, which produces small peptides and essential free amino acids. Interestingly, *Ln. mesenteroides* can produce carbon dioxide, with subsequent formation of

"eyes" (Zheng et al., 2021), as those present in *Queijo de Nisa* PDO cheese. *Ln. mesenteroides* has already been detected by Macedo, Vieira, Poças, & Malcata (2000) in *Serra da Estrela* PDO cheese, and by Cardinali et al. (2021) among the major bacterial taxa occurring in *Queijo de Azeitão* PDO cheese. Moreover, this lactic acid bacterium, has already been isolated in *Pico* cheese produced in the Azores Island (Pico, Portugal) using raw cow's milk (Domingos-Lopes, Stanton, Ross, Dapkevicius, & Silva, 2017).

L. plantarum, detected at high relative frequency in samples of producer 1 and 4, and, with a lesser extent, also in samples of producer 2 and 3, has already been detected by Cardinali et al. (2017) in thistle rennet, thus suggesting vegetable rennet as the possible origin of this lactic acid bacterium in the analyzed *Queijo de Nisa* PDO samples. As reported by Mugampoza, Gkatzionis, Linforth, & Dodd (2019), in cheese, *L. plantarum* can interact with *Lc. lactis* to produce lactic acid, diacetyl, and acetoin, thus affecting the final aroma of the product. Interestingly, esterase and lipase from *L. plantarum* have already been characterized by Esteban-Torres, Mancheño, de las Rivas, & Muñoz (2014), thus highlighting the existence of a lipolytic activity carried out by this lactic acid bacterium in cheese. The occurrence of *L. plantarum* in Portuguese cheeses has already been reported by Macedo et al. (2003) in *Serra da Estrela* PDO cheese, and, as minority taxon, by Cardinali et al. (2021) in *Queijo de Azeitão* PDO cheese. Of note, Ribeiro, Stanton, Yang, Ross, & Silva (2018) reported that *L. plantarum* strains isolated from the Azorean *Pico* cheese were able to adhere to intestinal human cells, and to prevent their colonization by *Escherichia coli*, thus suggesting potential probiotic activity of *L. plantarum* strains (Ribeiro et al., 2018).

As for *L. piscium*, it is known that this psychrotrophic lactic acid bacterium can exert spoilage activity, depending on the strain and the food matrix (Saraoui, Leroi, Björkroth, Pilet, 2016). Although the occurrence of *L. piscium* in cheese is uncommon, this microorganism has already been detected by Carraro et al. (2011) in raw milk, and, by the same authors, during the early stage of maturation of *Montasio* cheese. As reported by Saraoui et al. (2016), *L. piscium* is unable to growth at pH below 4.8, this feature could help to explain the occurrence of this microorganism in the analyzed samples, whose pH values at the end of ripening were always above 4.8.

Regarding the presence of *L. zeae*, this lactic acid bacterium has already been detected in Portuguese thistle-curdled cheeses as *Queijo de Azeitão* PDO (Cardinali et al., 2021) and *Queijo da Beira Baixa* PDO (Cardinali et al., 2022). *L. zeae* is a facultatively heterofermentative microorganism whose metabolism produces aroma compounds originated by proteolysis (Terzić-Vidojević et al., 2020).

Corynebacterium species are commonly detected on the surface smear-ripened cheeses as *Chaumes*, *Gryere*, *Tilsit*, *Limburger*, and *Reblochon* (Bockelmann, Willems, Neve, & Heller, 2005; Milani et al., 2019; Schröder, & Tauch, 2010). *Corynebacterium* has already been detected by Cardinali et al. (2021) among the minority taxa in *Queijo de Azeitão* PDO cheese, and by Milani et al. (2019) in Parmesan cheese. As reported by Nogueira, Lacorte, de Oliveira Paciulli, & Ferreira

485 Rodrigues (2021), *Corynebacterium* is a highly proteolytic microorganism that can be responsible for the production of
486 sulfur and ammonia compounds in cheese.
487 VOCs strongly characterize cheese identity and authenticity, and their identification could be relevant for the study of
488 traditional products. Of note, the correlation analysis allowed positive and negative correlations between the bacterial
489 taxa and the VOCs detected in the analyzed *Queijo de Nisa* PDO samples to be disclosed.
490 Among the detected VOCs, short chain acids have an important role in the overall aroma of cheese due to their low
491 perception thresholds (Delgado, González-Crespo, Cava, & Ramírez, 2011). Linear acids are largely generated from
492 lipolysis; moreover, microbial activity can contribute to the butyric acid level and to the formation of the branched-chain
493 fatty acids as 2-methylpropanoic (isobutyric), 3-methylbutanoic (isovaleric), and 2-methylbutanoic, through
494 metabolization of valine, leucine, and isoleucine (McSweeney, & Sousa, 2000).
495 In cheese, the presence of methyl ketones represents a positive feature, due to their fruity and musty notes. Ketones are
496 formed by enzymatic oxidation of free fatty acids to β-ketoacids and their consequent decarboxylation to methyl ketones
497 (Senoussi et al., 2022). The enzymatic reduction (through alcohol dehydrogenase) of methyl ketones produces the
498 corresponding secondary alcohols (e.g., 2-propanol). It is noteworthy that, among the known subspecies, *Lc. lactis* subsp.
499 *lactis* produces diacetyl and acetoin which are desirable flavour notes (e.g., creamy and buttery) in ripened cheeses
500 (Fusieger, Martins, de Freitas, Nero, & Fernandes de Carvalho, 2020). Of note, such compounds belong to ketones whose
501 presence has massively been detected in the analyzed samples, thus highlighting a great contribution of *Lc. lactis* in aroma
502 definition. As reported by Fusieger et al. (2020), ketones represent essential compounds of the volatilome of many dairy
503 products, including *Camembert*, *Cheddar*, and *Emmental*. As for 2-butanone, Pedersen et al. (2016) reported a high
504 content of this compound in cheeses containing *Ln. mesenteroides*, thus confirming the impact of this species on the
505 volatile characteristics of cheese. Regarding the correlation between *L. plantarum* and 2-heptanone, Lang, Wen, Wu, Pan,
506 & Wang (2022) recently observed the accumulation of such ketone in yoghurt obtained by a mixed culture of
507 *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *L. plantarum*, this latter used as adjunct culture. Moreover,
508 the presence of 2-nonanone was detected by Wang, Fang, Wu, Min, & Yang (2018) in *Cheddar* cheese with *L. plantarum*
509 used as adjunct culture, thus suggesting the contribution of this bacterium in the formation of such compound.
510 It is noteworthy that *Lc. lactis* cell lysis contributes to the formation of free amino acids during cheese ripening by
511 promoting the catabolism of aromatic amino acids and methionine and boosting the development of sulphur compounds
512 and benzaldehyde (Bourdat-Deschamps et al., 2004). Of note, benzaldehyde, that affects cheese flavour with sweet and
513 strong almond odour notes, was detected in all the analyzed *Queijo de Nisa* PDO cheese samples.
514 Regarding esters, it is noteworthy that lactose metabolism by heterofermentative lactic acid bacteria produces ethanol
515 that, although not directly involved in the aroma of cheese, has a role in the formation of esters. As for the positive

association of ethanol with *Carnobacterium*, Picon, López-Pérez, Torres, Garde, & Nuñez (2019) already reported the contribution of this lactic acid bacterium in ethanol production in raw goats' milk cheeses. Regarding the association of ethanol with *L. piscium*, no previous investigations dealing with the production of ethanol by this microorganism in cheese are reported in the scientific literature, although it is known that heterofermentative species of lactic acid bacteria can produce alcohols in cheese (Picon et al., 2019).

5. Conclusions

Research on the microbiological and chemical features, as well as on morpho-textural characteristics of PDO cheeses can increase the knowledge on the interactions between raw materials, environment, and autochthonous microorganisms, thus enabling protection of biodiversity and tradition. In the present study, the *Queijo de Nisa* PDO cheeses manufactured by 4 Portuguese producers revealed the presence of a few key pro-technological species. In more detail, *Lc. lactis* and *Ln. mesenteroides* showed to be the most represented species in all the cheese samples. Moreover, a few lactic acid bacteria species characterized the samples, depending on the producer. Such findings highlighted the establishment of a stable and uniform microbiota that could be representative of the *Queijo de Nisa* PDO cheese, with differences in the prevalence of co-occurring species that could be related to artisan production practices and to the dairy environments. In all the analyzed samples, carboxylic acids, carbonyl compounds, alcohols, and esters represented the most detected classes of volatile compounds, thus suggesting common flavor and aroma traits among the *Queijo de Nisa* PDO cheese manufactures.

Of note, wide variations in some of the physico-chemical parameters of cheeses coming from the same batch suggested a lack of standardization of the production process likely due to the artisanal nature of the products.

There are limitations in this study that might be addressed in future research. The primary limitation is represented by the relatively low number of samples collected from the four *Queijo de Nisa* PDO cheese producers. Hence, a wider sampling campaign might be planned, also in view of the investigation of the effect of environmental variables, such as grazing pasture and production season, *Queijo de Nisa* PDO cheese traits. Enumeration/detection of pathogenic microorganisms, monitoring of the microbial evolution from raw milk to ripened cheeses, and isolation of pro-technological strains, are also recommended to better elucidate the interrelation between cheese quality and raw materials, production environment, and conditions for cheese-making and ripening.

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