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A comprehensive study on the autochthonous microbiota, volatilome, physico-chemical, and morpho-textural features of Montenegrin *Njeguški* cheese

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Abstract

The present study aims to deepen the knowledge of the microbiota, gross composition, physico-chemical and morpho-textural features, biogenic amines content and volatilome of *Njeguški* cheese, one of the most popular indigenous cheeses produced in Montenegro. Cheese samples were collected in duplicate from three different batches produced by three Montenegrin artisan producers. For the first time, the microbiota of *Njeguški* cheese was investigated using both culture-dependent techniques and metagenomic analysis. Coagulase positive staphylococci viable counts were below the detection limit of the analysis (< 1 log cfu g^{-1}). *Salmonella* spp., *Listeria monocytogenes* and staphylococcal enterotoxins were absent. However, relatively high viable counts of *Enterobacteriaceae*, *Escherichia coli*, Pseudomonadaceae and eumycetes were detected. Metataxonomic analysis revealed a core microbiome composed of *Lactococcus lactis*, *Streptococcus thermophilus*, *Debaryomyces hansenii*, and *Kluyveromyces marxianus*. Furthermore, the detection of opportunistic pathogenic yeasts such as *Magnusiomyces capitatus* and *Wickerhamiella pararugosa*, along with the variable content of biogenic amines, suggests the need for increased attention to hygienic conditions during *Njeguški* cheese production. Significant variability was observed in humidity (ranging from 38.37 to 45.58 %), salt content (ranging from 0.70 to 1.78 %), proteins content (ranging from 21.42 to 25.08 %), ash content (ranging from 2.97 to 4.05 %), hardness, springiness, and color among samples from different producers. Gas chromatography-mass spectrometry analysis showed a well-defined and complex volatilome profile of the *Njeguški* cheese, with alcohols (ethanol, isoamyl alcohol, phenetyl alcol), esters and acetates (ethyl acetate, ethyl butanoate, isoamyl acetate), ketones (acetoin, 2-butanone), and acids (acetic, butanoic, hexanoic acids) being the main chemical groups involved in aroma formation. This research will provide new insights into the still poorly explored identity of *Njeguški* cheese, thus serving as a first baseline for future studies aimed at protecting its tradition.

Keywords: fermented foods, lactic acid bacteria, eumycetes, culture-dependant analysis, metagenomic analysis, quality, safety, authenticity

1. Introduction

The Balkan Peninsula is a region well-known for its variety of traditional dairy products, still manufactured according to ancient traditions, representing a unique treasure and irreplaceable heritage. The Balkan Peninsula is characterized by pastures suitable for breeding cattle, sheep, and goats for milk production and by mountains with rural villages that produce artisanal cheeses (Terzić-Vidojević et al., 2020). In particular, *Njeguški* cheese is one of the most famous and appreciated traditional Montenegrin dairy products. It originates from a village called Njeguši, and its production later expanded to the areas of the Lovćen mountain and partly to the Boka Kotorska Bay (the Adriatic Sea coastline between Herceg Novi and Budva) (Martinovic & Mirecki, 2021; Mirecki et al., 2015). Intriguingly, the herbaceous composition of the pastures and, consequently, the quality of the milk, as well as the ripening conditions of *Njeguški* cheese, are strongly influenced by the collision of different climates from the mountain and Mediterranean areas. This unique combination gives the cheese a piquant aroma, a slightly sour-milky and moderately salty taste, and a pleasant odour (Martinovic & Mirecki, 2021).

Historically, *Njeguški* cheese dates back to the Roman Empire when Rome was provided with a cheese called *Caseus Doclestes*, made in Doclea, an ancient region now known as Montenegro. Hence, it has been suggested that this cheese was a precursor to *Njeguški* cheese (Martinovic & Mirecki, 2021; Mirecki et al., 2015).

Mirecki et al. (2015) were the first to describe the production technology of *Njeguški* cheese, noting that it was originally a hard, full-fat cheese made from raw sheep's milk. However, due to changes in consumer eating habits, *Njeguški* cheese is now mainly manufactured using a mixture of sheep's, cow's, or goat's milk and is marketed as a semi-hard, full-fat cheese. Traditionally, the cheesemaking process is performed manually. The cooled raw milk is filtered through cheesecloth and then heated to about 35°C. Natural rennet from a lamb's stomach is added to the milk. After 30–60 minutes, the cheese curd forms and is cut into pieces about 5 mm in size. The curd is then broken by hand in the whey, which is gradually heated to about 40-45°C. The curd is transferred into wooden or metal molds (20 cm in diameter) and pressed for a total of 24 hours, with the cheese being turned after 12 hours of pressing. A wooden circular board is used for pressing, and a stone is placed on top for additional weight. The cheese is then removed from the mold, placed in a wooden chest, and dry salted for 2 days, with salt being added 2-3 times per day. The optimum maturation period for *Njeguški* cheese is 1 month, and its ripening occurs on wooden shelves in stone cellars. To remove molds from the cheese surface, it is washed with cold, salty water and then dried with a clean cloth. The ripened cheese can also be exposed to smoke or soaked in maize, wheat, or olive oil for further maturation. Ready-to-eat *Njeguški* cheese has the shape of a low cylinder with a height of 3-5 cm, a diameter of 15-25 cm, and a weight of about 1-2.5 kg. The crust is crack-free and has a golden-yellow colour. The cheese texture is homogeneous with a few "cheese eyes" up to 0.5 cm in diameter; it is not brittle and is easy to cut (Martinovic & Mirecki, 2021; Martinovic et al., 2018; Mirecki et al., 2015).

The technology behind this cheese is based on a family tradition that has been passed down through generations, becoming an integral part of the national culture and history of Montenegro (Mirecki et al., 2015). Furthermore, in 2015, Mirecki et al. highlighted that this cheese technology met Montenegrin legal requirements, which were harmonized with EU law concerning protected designation of origin (PDO), protected geographical indication (PGI), and traditional speciality guaranteed (STG), thus emphasizing that the process of protection of origin of *Njeguški* cheese can be initiated. Overall, geographical indication recognitions established by the European Union aim to valorise and protect the specific quality of traditional food products, thereby differentiating them in the market. So far, in Montenegro, only three dairy products have gained national recognition and protection: *Pljevaljski* cheese, *Kolašinski* cheese, and *Durmitorski Skorup*, all under the protected designation of origin (PDO) (Martinovic & Mirecki, 2021).

Despite the great popularity and ancient origin of *Njeguški* cheese, only a few scientific studies have been conducted on this spontaneously fermented cheese. These studies have mainly focused on defining its production technology and basic composition (Mirecki et al., 2015), studying its production using lactic acid bacteria starter cultures (Bojanic Rasovic et al., 2017; Martinovic et al., 2018), and investigating its α-tocopherol content during ripening (Jokanovic et al., 2022). However, deciphering the microbial profile of spontaneously fermented foods significantly contributes to protecting their origin, quality, and technology, as it is well known that the autochthonous microbial population is the most important factor influencing the quality and traditional characteristics of fermented foods, especially dairy products. Indeed, cheese microbiota contribute to the aroma, taste, texture, safety, and nutritional characteristics of the products (Cardinali et al., 2022a,b; Mirecki et al., 2015; O'Sullivan et al., 2013). Therefore, the present study aims to fill the gap of knowledge regarding the microbiota of *Njeguški* cheese through culture-dependent techniques and metagenomic analysis. Furthermore, for the first time, a complete characterization of the cheese in terms of gross composition, physico-chemical and morpho-textural features, biogenic amine content, and volatilome has been conducted. This study will serve as a baseline for further defining biomarkers of quality and authenticity, contributing to the process of protecting the origin of *Njeguški* cheese.

2. Materials and methods

2.1. Sampling

Eighteen *Njeguški* cheese wheels were produced and collected in duplicate from three different batches (Batch I, II, III) of three Montenegrin artisan producers (Producers A, B, and C) between May and July 2023. The samples were placed into sterile vacuum-sealed bags, shipped to Italy via express courier under refrigerated conditions using an icebox, and stored in the laboratory at 4°C upon arrival until analysis. All cheeses were produced with the following ingredients: 70 % sheep's milk, 30 % cow's milk, marine salt, and animal rennet. The ripening time for all samples was approximately 30 days. Each cheese had an average weight of 1 kg, with a diameter of about 14 cm and a height of 3.5 cm. The scheme depicting the general manufacturing procedure of *Njeguški* cheese is shown in Supplementary Figure 1.

2.2 Microbiological analyses

Ten g of each sample were weighted and homogenized with 90 mL of sterile peptone (1 g L^{-1}) (Oxoid, Milan, Italy) water using a Stomacher 400 Circulator apparatus (VWR International PBI) at 260 rpm for 5 min. The microbiological viable counts were performed on the same solution after tenfold serial dilutions. De Man Rogosa and Sharpe (MRS) agar and M17 agar (VWR Prolabo Chemicals, Leuven, Belgium) medium supplemented with cycloheximide (250 mg L^{-1}) were used for enumeration of presumptive lactobacilli and lactococci with incubation for 48-72 h at 37 °C. Chromogenic Coliform Agar (CCA) medium (VWR, Leuven, Belgium) was used for the enumeration of *Escherichia coli* with incubation at 37 °C for 24 h. Violet Red Bile Glucose Agar (VRBGA) (VWR Prolabo Chemicals) was used for enumeration of Enterobacteriaceae with incubation for 24 h at 37 °C. Coagulase positive staphylococci were enumerated according to UNI EN ISO 6888-2:2021. Pseudomonas Agar Base (PAB) (VWR Prolabo Chemicals) added with cetrimide-fucidin-cephalosporin (CFC) selective supplement (VWR International, Milan, Italy) was used for enumeration of Pseudomonadaceae with incubation for 24–48 h at 30 °C. Rose Bengal Chloramphenicol Agar (VWR Prolabo Chemicals) was used for enumeration of eumycetes with incubation for 72 h at 25 °C. The results of two biological and three technical replicates were expressed as the log of colony-forming units (cfu) per gram of sample and reported as mean ± standard deviation.

Finally, a miniVIDAS apparatus (bioMérieux, Marcy l'Etoile, France) was used to assess the presence/absence of *Listeria monocytogenes* and *Salmonella* spp. using the enzyme-linked fluorescent assay (ELFA) method, in accordance with the AFNOR BIO 12/11–03/04 and AFNOR BIO 12/16–09/05 standard methods, respectively (Haouet et al., 2017).

2.3 Detection of staphylococcal enterotoxins

The detection of staphylococcal enterotoxins was performed according to UNI EN ISO 19020:2017 using a two steps protocol based on extraction/concentration and immuno-enzymatic detection carried out using the VIDAS® equipment with Staph enterotoxin II (SET2) kit (bioMérieux, Marcy-l'´Etoile, France), as described by Cesaro et al. (2022).

2.4 Microbial DNA extraction, sequencing, and bioinformatics

Aliquots of 1 mL were collected from the first dilution (10^{-1}) of each cheese sample and centrifuged at 14,000 rpm for 10 min. The supernatants were discarded, and the pellets were treated for total microbial DNA extraction using the E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions.

A total of 18 DNA samples (6 for each producer) were quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized to 5 ng μL^{-1}. Two µl of each DNA sample was amplified for microbiota analysis by using the primers and conditions for the amplification of the V3-V4 region of the 16S rRNA gene as described by Klindworth et al. (2013). The mycobiota was studied by the amplification of the D1-D2 domain of the 26S rRNA gene according to Mota-Gutierrez, Ferrocino, Rantsiou, & Cocolin (2019). Pair-end sequencing (2X250bp) was performed with a MiSeq Illumina instrument (Illumina, San Diego, CA, USA) using V2 chemistry according to the manufacturer's instructions. Raw reads were analyzed by using the Quantitative Insights Into Microbial Ecology (QIIME2) (Bolyen et al., 2019). Primers and adapters were first trimmed by using Cutadapter and then quality filtered using the DADA2 algorithm (Callahan et al., 2016). Low-quality bases, chimeric sequences, and sequences shorter than 300 bp were filtered out by using the dada2 denoise-paired plug in of QIIME2. Amplicon Sequence Variants (ASVs) generated by DADA2 were rarefied at the lowest sequences per sample and used for taxonomic assignment using the QIIME feature-classifier plugin against the Greengenes 16S rRNA gene database (version 13_5) for the microbiota, and the manually built database for the mycobiota (Mota-Gutierrez et al., 2019). Taxonomy assignment at the highest taxonomic resolution reached for 16S rRNA gene and 26S rRNA gene was confirmed by double checking on BLAST suite tools. The raw read data generated by sequencing were deposited in the NCBI Sequence Read Archive (SRA) under the Bioproject Accession Number PRJNA1133970.

2.5 Physico-chemical analysis

The pH value was determined using a pH meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy), which was inserted at the core of cheeses.

The water activity (a_w) of the cheeses was measured according to the ISO 21807:2004 method using an AwTherm apparatus (Rotronic, Bassersdorf, Switzerland).

The moisture/dry matter was determined by the gravimetric method (AOAC Official Method 950.46).

The salt (sodium chloride) content was determined through ion chromatography analysis. Briefly, 2 ± 0.1 g of sample was weighted, added with 20 mL of water, mixed for 20 min (orbital mixer KS 501Digital, IKA® Werke, Staufen, Germany), centrifuged at 800 rpm for 5 min (Rotanta 460 R, Hettich GmbH & Co. KG, Tuttlingen, Germany), filtered through a 0.45 µm syringe filter and analyzed by ion chromatography (ICS 5000 Dionex, ThermoFisher Scientific, Milan, Italy). The chromatography conditions were: Dionex IonPack CS12A 4x250 mm column and Dionex IonPack CG12A 4x50 mm precolumn (Thermo Fisher Scientific, Milan, Italy), 50 µL injection, 1 mL min^{-1} flow, 100 mA SRS and 20 mM methanesulphonic acid as mobile phase.

The proximate composition of the samples was determined as follows: protein (%), assessed by Kjeldahl method (AOAC, 981.10); fat (%), assessed by Soxhlet extraction (AOAC, 991.36); ash (%), determined in a convection oven (AOAC, 920.153); finally, carbohydrate (CHO) content was calculated by difference (Cardinali et al., 2022a).

Saturated and unsaturated fatty acids were determined through gas chromatography with flame ionization detector (FID) analysis according to Jarukas et al. (2021).

The analyses were carried out in triplicate for each biological replicate, and the results were reported as mean values ± standard deviation.

2.6 Biogenic amines content

Biogenic amines content was determined through High-performance liquid chromatography-UV-visible detection method according to Altissimi et al. (2017). Results for each biological replicate were expressed as mean ± standard deviation.

2.7 Morpho-textural analyses

The colour of the cheeses was determined using a Chroma Meter CR-200 (Minolta, Osaka, Japan) with a D65 illuminant. Color was determined on 2 cm thick slices according to the CIE *L*a*b** system (*L**, lightness; *a**, redness/greenness; *b**, blueness/yellowness). Images of the cheeses were obtained by slicing them longitudinally (7 mm thickness) and imaging the cross sections with a scanner (ENVY 6200 Series, HP, Palo Alto, CA, USA) (Osimani et al., 2023).

For each cheese, cylindrical specimens (height: 15 mm, diameter: 20 mm) were obtained, and then subjected to uniaxial compression with a CT3-4500 texture analyzer (Brookfield Engineering Laboratories Inc., Middleboro MA, USA) equipped with a 36 mm diameter cylindrical probe (mod. TA-AACC36) at 1.5 mm s^{-1} applying a non-destructive deformation (40 %) (Osimani et al., 2023).

Specimens were positioned between the load cell and the fixture base table of the instrument, and a 4500 g load cell was used.

2.8 HS-SPME-GC/MS analysis of volatile components

Headspace volatiles from each sample were analyzed by headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography–mass spectrometry (GC-MS) (HS-SPME-GC/MS), using a 7890 Agilent GC system coupled to an Agilent 5975 (Agilent Technologies, Santa Clara, California, USA) inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler (Gerstel, Mülheim, Germany), as described by Reale et al (2016) with some modifications. Briefly, 2 g of samples were placed into a 20 mL headspace vial, and 5 µL of 3-octanol (internal standard, 100 mg/L standard solution) was added. The vial was placed in a thermostatic block (40 °C) on a stirrer, the fiber was inserted and maintained in the sample headspace for 30 min, then removed and immediately inserted into the GC/MS injector for the desorption of compounds. The extraction was performed automatically by the multipurpose sampler of the GC/MS system. A silica fiber, coated with 75 µm of Carboxen/Polydimethylsiloxane (CAR/PDMS (Supelco, Bellefonte, PA, USA) was used for analysis. the operating conditions were as follows: HP-Innowax capillary column (Agilent Technologies, 30 m × 0.25 mm ID, film thickness 0.32 µm), gas carrier was helium (flow 1.5 mL/min), and SPME injections were splitless (straight glass line, 0.75 mm ID) at 240 °C for 20 min, during which time thermal desorption of the analytes from the fiber occurred. The oven parameters were as follows: initial temperature of 40 °C held for 3 min, followed by an increase to 240 °C at a rate of 5 °C/min, and then held for 0 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240 °C, 150 °C, 230 °C and 200 °C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70 eV electron energy in the range 31–500 amu. VOCs identification was achieved by comparing mass spectra with the Nist library (NIST 20) and by matching the retention indices (RI) calculated according to the equation of Van Den Dool and Kratz (1963) and based on a series of alkanes. The data are expressed like relative peak area

(RAP) with respect to internal standard. Blank experiments were carried out in two different modalities: blank of the fiber and blank of the empty vial. All the analyses were performed in duplicate for each biological replicate and the results expressed as mean value of four replicates ± standard deviation.

2.9 Statistical analysis

The statistical analysis of microbiological, physico-chemical, colour, and texture data was performed to determine differences among cheese samples using the JMP v11.0.0 software (SAS Institute Inc., Cary, NC). To this end, the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used by one-way analysis of variance (ANOVA). To evaluate the relationship between cheeses and biogenic amines, a Principal Component Analysis (PCA) was performed using JMP v11.0.0 software (SAS Institute Inc., Cary, NC).

ASV tables and taxonomic classifications were submitted to MicrobiomeAnalyst (Chong et al., 2020) to calculate alpha and beta diversity based on Shannon and Bray-Curtis indices, respectively. Anosim statistical test was used to find differences in microbial composition in R environment. Differences in microbiota and mycobiota among producers were also calculated by Wilcoxon-Mann-Whitney test and results were displayed as box plots.

To evaluate how the different cheeses were distributed according to the detected volatile organic compounds, PCA was performed using Tanagra 1.4 software.

3. Results

3.1 Microbial counts

The results of the viable counts are reported in Table 1. Average counts of presumptive lactobacilli ranged from 8.29 ± 0.25 (producer C) to 8.57 ± 0.31 log cfu g^{-1} (producer B), with no statistically significant differences among producers. For presumptive lactococci, average viable counts ranged from 8.37 ± 0.27 (producer C) to 9.04 ± 0.17 log cfu g^{-1} (producer A), with samples from producer C showing the lowest values. For Enterobacteriaceae average counts ranged from 4.99 ± 0.58 (producer A) to 5.25 ± 0.44 log cfu g^{-1} (producer C), with no statistically significant differences among producers. Similarly, no statistically significant differences were seen among producers for *E. coli*, with average viable counts ranging from 3.61 ± 0.86 (producer A) to 3.89 ± 0.61 log cfu g^{-1} (producer C). Regarding Pseudomonadaceae, average counts ranged from 4.80 ± 0.16 (producer C) to 5.03 ± 0.27 log cfu g^{-1} (producer A), with no statistically significant differences among producers. Average counts of yeasts ranged from 6.39 ± 0.22 (producer C) to 6.53 ± 0.29 log cfu g^{-1} (producer A), with no statistically significant differences among producers. No statistically significant differences were observed among producers for mold presence with average viable counts ranging from 4.54 ± 0.15 (producer C) to 4.63 ± 0.26 log cfu g^{-1} (producer A).

Finally, coagulase positive staphylococci viable counts were below the detection limit of the analysis (< 1 log cfu g^{-1}). *L. monocytogenes*, *Salmonella* spp., and staphylococcal enterotoxins were never detected.

3.2 Microbiota and mycobiota composition

A total of 1,142,654 denoised reads (63,481 reads on average per sample) for bacteria and 2,441,952 (135,664 reads on average per sample) for fungi were analyzed, with a coverage greater than 99 %. No statistically significant differences ($p > 0.05$) were observed in alpha diversity (Shannon index) for both microbiota and mycobiota among *Njeguški* cheese samples from different producers (A, B, and C) (Supplementary Figure 2). By contrast, significant differences (p < 0.05) emerged in beta-diversity (Figure 1). The composition of the bacterial biota of *Njeguški* cheese samples is shown in

Figure 2 (panel a) and Supplementary Table 1. Moreover, the ASVs of bacterial taxa showing statistically significant differences among the cheese samples are reported in Figure 2 (panel b). Overall, the bacterial biota of *Njeguški* cheese samples was dominated by lactic acid bacteria, namely *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp. *Lactococcus lactis* was significantly more abundant in cheeses from producer B (approximately 50 % of the relative frequency) and was found at relative frequencies between 30–40 % in cheeses from producers A and C. Instead, cheeses from producer A showed higher relative frequencies of *Lactobacillus* spp. (approx. 30 %) compared to producers B and C (3 % and 9 %, respectively). All cheese samples from producer C showed a higher incidence of Bifidobacteriaceae (approx. 4.50 % of the relative frequency) compared to the other samples (approx. 1 % and 0.12 % in producer A and B, respectively). *Lactococcus garvieae*, *Lacticaseibacillus zeae*, and *Enterococcus* spp. were also detected in cheese samples from the three different producers with relative frequencies ranging from 0.23 % to 2.72 %. Notably, cheese samples from producer B showed a higher presence of Enterobacteriaceae (approx. 3.70 % of the relative frequency) compared to the cheeses from the other producers. A minor fraction of ASVs belonged to other lactic acid bacteria taxa as well as to spoilage microorganisms.

The composition of the fungal biota of *Njeguški* cheese samples is shown in Figure 3 (panel a) and Supplementary Table 2. Moreover, the ASVs of fungal taxa with statistically significant differences among the cheese samples are reported in Figure 3 (panel b). In more detail, *Debaryomyces hansenii* showed the highest relative frequency in all cheese producers. A clear predominance of this species was revealed in cheeses from producer B (approx. 75.60 % of the relative frequency), whereas significantly lower values were observed in cheeses from producer A and C (43 % and 30 % of the relative frequency, respectively). *Galactomyces* spp. and *Kluyveromyces marxianus* were also revealed as dominant taxa in all cheese samples, particularly those from producers A and C. Cheeses from producer B were characterized by a higher incidence of *Kazachstania unispora* (approx. 9 % of the relative frequency), *Magnusiomyces capitatus* (approx. 10 % of the relative frequency), and

Wickerhamiella pararugosa (approx. 21.50 % of the relative frequency) compared to the other samples. Conversely, *Torulaspora delbrueckii* was predominant in samples from producer A (approx. 16 % of the relative frequency). *Saccharomyces cerevisiae* and *Geotrichum* spp. were equally distributed in all cheese samples. Beyond the microorganisms listed above, minority taxa were sporadically detected at very low relative frequencies, including *Candida sake*, *Geotrichum fragans*, *Kurtzmaniella zeylanoides*, *Pichia* spp., *Starmerella apicola*, *Torulaspora quercuum*, *Trichosporon* spp., and *Wickerhamomyces anomalus*.

3.3 Physico-chemical characterization

The results of physico-chemical and proximate composition analyses carried out on the cheese samples under study are reported in Table 2.

In more detail, pH average values ranged between 4.38 ± 0.32 (producer C) and 4.80 ± 0.31 (producer B), whereas water activity (a_w) average values ranged from 0.97 ± 0.01 (producer A and C) to 0.98 ± 0.01 (producer B). No differences were observed for pH and water activity average values, irrespective of the producer.

Concerning humidity, average values ranged between 38.37 ± 1.66 % (producer C) and 45.58 ± 3.03 % (producer A), with average counts of samples from producer C showing the lowest values.

The proximate composition analysis revealed no statistically significant differences among the carbohydrate, lipids, and total saturated fatty acids content of the samples from the three producers. In detail, carbohydrate average content ranged from 1.93 ± 0.91 (producer B) to 2.37 ± 0.49 % (producer C), lipids average content ranged from 27.94 ± 1.09 (producer A) to 30.11 ± 1.07 % (producer C), whereas total saturated fatty acids average content ranged from 18.70 ± 1.32 (producer A) to 19.59 ± 0.27 % (producer B). However, for protein, salt, and ash significant differences were observed among producers, with samples of producer C showing the highest content and samples of producer A showing the lowest content. In detail, proteins average values were comprised between

21.42 ± 1.70 (producer A) and 25.08 ± 1.36 % (producer C), salt average content was comprised between 0.70 ± 0.12 (producer A) and 1.78 ± 0.36 % (producer C), and ash average content was comprised between 2.97 ± 0.28 (producer A) and 4.05 ± 0.87 % (producer C).

3.4 Biogenic amines content

The results of biogenic amines analyses carried out on the *Njeguški* cheese samples are reported in Table 3. The analyses revealed a significant variation in biogenic amines content of the cheese among samples except for spermine that was below the detection limit of the analysis for all of the samples (< 1 mg kg^{-1}). Only samples from batches II and III of producer A showed tryptamine levels > 1 mg kg^{-1}, whereas batch I from producer A was characterized by the presence of only spermidine and tyramine. 2-phenylethylamine was detected only in batch I from producer B. Overall, significantly higher levels of biogenic amines as cadaverine, histamine, putrescine, and spermidine was found in samples from producer C.

To better understand the differences between the analyzed *Njeguški* cheese samples, a PCA of the biogenic amines detected was carried out, and the results are shown in Figure 4. The two principal components explained 74.60 % of the total variance of the data. PC1 accounted for 51.70 % of the variability, whereas PC2 accounted for 22.90 % of the total variability. Producers B and C demonstrated clear distinctions from each other and with respect to producer A. Specifically, producer B has positive relationship with PHE (negative loading in PC1), whereas producer C has positive relationships with CAD, PUT and HIS (positive loadings in PC1).

3.5 Morpho-textural characterization

The results of colour analyses carried out on the *Njeguški* cheese samples are reported in Table 4. Cheese colour evaluation revealed no statistically significant differences in L^* parameter (lightness) among producers ranging from 76.54 (producer C) to 80.65 (producer A), whereas for the *a** parameter (redness/greenness), the average value (-1.19) of samples from producer C was the lowest. Finally, concerning *b** parameter (blueness/yellowness), the average value (17.27) of samples from producer A was the lowest.

The texture profile analyses (Table 5) showed significant differences in the hardness of the cheese ranging from 4.27 (producer A) to 15.05 N (producer C), with the average value of samples from producer C being the highest. As for cohesiveness, no statistically significant differences were observed among samples, with values ranging from 0.84 (producer B) to 0.90 (producer C). Springiness' average values ranged from 1.53 (producer A) to 1.75 (producer C), with the average value of samples from producer C being the highest.

3.6 Volatile organic composition

The HS-SPME-GC/MS analysis allowed us to identify 37 volatile organic compounds in Montenegrin *Njeguški* cheese (Table 6). The compounds with RAP < 1 % were discarded from further statistical and graphical analyses. The volatile components belonged to seven classes, including ketones (6), aldehydes (1), alcohols (6), esters and acetates (11), acids (9), terpenes (2), sulfur compounds (2). Samples were mainly characterized by acids, alcohols, ketones, esters and acetates while terpenes, sulfur compounds and aldehydes were found in traces.

Among acids, acetic, butanoic, 3-methylbutanoic and hexanoic acids were found in the highest amounts in all the samples, while octanoic, propanoic and isobutanoic acids were found in smaller amounts in the samples. Pentanoic and decanoic acids were found in traces.

Among alcohols, isoamyl alcohols, ethanol, and phenylethyl alcohol were detected in all the samples in the highest amounts, while 2,3-butanediol, 2-butanol and isobutanol were found in smaller amounts and only in a few samples.

Among ketones, the most representative were acetoin, 2-butanone and acetone, found in all the samples. Minor amounts were found of 2-pentanone, 2-heptanone, 2-nonanone mainly in the samples from producer B and C.

The predominant esters and acetates in almost all samples were ethyl acetate, ethyl butanoate, isoamyl acetate, pentyl butanoate, ethyl hexanoate and ethyl octanoate.

Traces of terpenes (i.e., limonene, 4-carene, ®-pinene) were only found in the samples of producer B and C. Among sulfur compounds, traces of dimethyl disulfide were found only in the samples from producer A, while methionol was found only in the samples from producer A and B.

To better understand the differences among the cheese samples, PCA was applied to the volatile compounds detected in the cheese from the different producers (A, B, C) and batches (I, II, III) (Figure 5). The analysis of the Principal Component Analysis (PCA) of the volatile organic compounds detected in the cheeses showed that samples from producers A and B were more similar respect to the samples from producer C. In fact, based on the volatile profiles, samples of producer A and B were clearly separated from those obtained from producer C. The first two principal components (PC) explained 55.17 % of the total variance. Samples from producers A and B were characterized by ketones (2-butane, acetoin), alcohols (isoamyl alcohol, phenylethyl alcohol) and esters and acetates (ethyl acetate), while samples from producer C differed mainly in acids (acetic, butanoic and hexanoic acids).

4. Discussion

4.1 Microbial populations

To the best of the authors' knowledge, the microbiota of *Njeguški* cheese has never been deeply investigated.

Concerning viable counts, a general lack of significant differences among lactic acid bacteria (in terms of presumptive lactobacilli and lactococci), Enterobacteriaceae, *Escherichia coli*, Pseudomonadaceae, yeasts and molds loads recorded in the analyzed *Njeguški* cheese samples. These results suggest that, although manufactured from different producers in Montenegro, the production technology allows similar products to be obtained.

High counts of presumptive lactobacilli and lactococci were found in all samples, and the data are in accordance with the study by Martinovic et al. (2018) aimed at monitoring the microbial population in experimental *Njeguški* cheese during ripening, showing maximum number of presumptive lactococci in 14-day-old cheeses up to 9 log cfu g^{-1}, and the maximum number of presumptive lactobacilli in 7-day-old cheese up to about 8 log cfu g^{-1}. Other studies on semi-hard raw sheep's cheeses from Mediterranean countries reported similar values. In detail, Schirone et al. (2013) studying twelve different *Pecorino* cheeses from the Abruzzo region (central Italy) and Cardinali et al. (2022a) studying *Queijo da Baixa* PDO cheese (Portugal) reported viable counts of lactobacilli up to 9 log cfu g^{-1}. In the latter study, counts of lactococci ranged from 6.32 to 9.07 log cfu g^{-1}, which were similar to those detected in the *Njeguški* cheese samples analyzed in the present study. Moreover, presumptive lactobacilli and lactococci counts were in line with those reported by Cardinali et al. (2022b) in the Portoguese *Queijo de Nisa* PDO cheese with values ranging from 7.60 to 8.84 log cfu g^{-1} and 7.66 to 9.01 log cfu g^{-1}, respectively. These data were quite expected, as the main role of lactic acid bacteria during cheese manufacturing and ripening is well-known. Lactic acid bacteria naturally occur in milk and become predominant during cheese manufacturing thanks to their ability to ferment carbohydrates forming organic acids (mainly lactic and acetic acids), ethanol and CO_2, through homo- or heterofermentative metabolism. Besides organic acids, lactic acid bacteria produce other metabolites (e.g., hydrogen peroxide, antifungal peptides, and bacteriocins) with inhibitory effect against the growth of spoilage and pathogenic bacteria, thus increasing food safety and prolonging the shelf-life of the cheese. The biosynthetic activity of lactic acid bacteria also plays a key role that ensures the following effects: the development of a wide range of volatile compounds (e.g., organic acids, heterocyclic compounds, aldehydes, ketones, etc.), textural changes (e.g., exopolysaccharides production that increase the product viscosity), and production of valuable nutritional compounds (such as vitamins, polyphenols, polysaccharides, and polyunsaturated fatty acids) that enhance cheese nutritional profile. Lactic acid bacteria metabolism also consists of the hydrolyzing activity of polymers that improve digestibility and bioavailability of the products and influence the aromatic profile of products (e.g. amino acids) (Garofalo et al., 2022; Terzić-Vidojević et al., 2020). Moreover, some lactic acid bacteria strains are recognized for their probiotic functions that promote human health (Garofalo et al., 2022; Terzić-Vidojević et al., 2020).

The detection of Enterobacteriaceae in raw milk cheese is common since they are part of the indigenous microbiota of raw milk, generally due to faecal contamination of raw milk, and they are also associated with poor hygienic conditions applied during cheese production (Cardinali et al., 2021, 2022a,b; Rampanti et al., 2023a). The counts of Enterobacteriaceae detected in the present study are in line with those reported by Cardinali et al. (2021) for a Portuguese raw ewe's cheese called *Queijo de Azeitão* PDO, ranging from 4.50 to 5.25 log cfu g^{-1}, whereas they were slightly higher than those reported by Tabla et al. (2016) for a Spanish semi hard raw ewe's milk cheese at 30 days of ripening, which attested at about 4 log cfu g^{-1}. Although members of the Enterobacteriaceae family are inhibited by an acidic environment such as that found in the cheese samples under study (pH 4.38-4.80), the occurrence of high loads of about 5 log cfu g^{-1} in the final products may indicate an initial high contamination in raw milk or the need for a longer ripening time as demonstrated by Tabla et al. (2016). Indeed, these authors observed a slow decline of Enterobacteriaceae counts up to 60 days of ripening in Spanish semi hard raw ewe's milk cheese to about 2 log cfu g^{-1}. Enterobacteriaceae are hygiene indicators and are of great concern because they may include potential pathogenic bacteria responsible for raw milk cheese-related illnesses (Rampanti et al., 2023a; Tabla et al., 2016). Moreover, Enterobacteriaceae can produce gas whose presence is considered a defect (e.g., texture with fissures or eyes, or gas within the packaging) (Tabla et al., 2016).

Among Enterobacteriaceae, the occurrence of viable counts of *Escherichia coli* ranging from 3.61 to 3.89 log cfu g^{-1} is of concern since this bacterial species represents a hygiene indicator that may potentially include pathogenic serotypes.

Pseudomonadaceae comprises bacterial members that mainly act as spoilage agents in food rich in proteins and fat, due to their production and secretion of heat-stable lipases and proteases. These enzymes are often responsible for cheese alterations. Pseudomonads are frequently detected in raw milk and raw milk cheeses, as also found in *Queijo de Azeitão* PDO cheese, showing Pseudomonadaceae counts ranging from 4.63 to 6.03 log cfu g^{-1} (Cardinali et al., 2021).

Eumycetes represent an important part of the cheese's microbial population, specifically in artisanal cheeses, influencing sensory characteristics such as appearance, flavor, aroma, and texture of the products during the ripening process (Bintsis, 2021; Cardinali et al., 2021, 2022a,b; Fröhlich-Wyder et al., 2018; Rampanti et al., 2023a,b; Ropars et al., 2012). Yeasts are capable of metabolizing the lactate produced by lactic acid bacteria and producing NH_3 from amino acids, being responsible for the rising of the pH on cheese surface (deacidification process) (Fröhlich-Wyder et al., 2018). Yeasts and molds may also cause cheese spoilage in terms of off-flavour, early blowing, and discolouration of the cheese (Fröhlich-Wyder et al., 2018). However, no visible alterations were detected in the *Njeguški* cheeses under study. Yeasts grow well in acidic environments and are salt-tolerant (Bintsis, 2021; Fröhlich-Wyder et al., 2018), thus justifying the high viable counts detected in the *Njeguški* cheese under study. The yeasts viable counts found in the present study are in line with those detected in Spanish raw milk semi-hard cheeses reaching up to 7 log cfu g^{-1} at the third week of maturation (Bintsis, 2021). To the author's knowledge, to date, eumycetes community of *Njeguški* cheese has never been investigated; hence, no data on eumycetes counts are available in the scientific literature for further comparison of the results. Therefore, the present study represents a significant advancement in the knowledge of the microbial population occurring in this dairy product.

For disclosure of the major and minor taxa occurring in *Njeguški* cheese, the metagenomic analysis of the microbiota and mycobiota has been applied. In detail, metataxonomic analysis showed the prevalence of a core microbiota in the *Njeguški* cheese samples composed of *Lc. lactis*, *Str. thermophilus*, *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., irrespective of the producer.

The dominance of the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus* was quite expected due to the high lactic acid bacteria viable counts recorded. Among these, *Lc. lactis* is considered a key lactic acid bacteria species in dairy products manufacture since it is involved in milk acidification, the hydrolysis of milk proteins, and the production of aroma compounds such as aldehydes, ketones, amino acids, and sulphur compounds. Moreover, strains of *Lc. lactis* are able to produce bacteriocins and exopolisaccharides (EPS), thus improving both the safety and the texture of cheeses (Terzić-Vidojević et al., 2020). *Lc. lactis* was already isolated from Montenegrin soft (*Bijeli* and *Masni*) and hard (*Njeguški*) cheeses and several strains have been biochemically characterized and used in *Njeguški* pilot scale productions (Martinovic et al., 2018; Rasovic et al., 2017). Other studies reported *Lc. lactis* as a predominant bacterium in other semi-hard cheeses produced with raw sheep's milk either in the Balkan area such as Croatian *Istrian*, *Krcki*, and *Paski* cheeses (Terzić-Vidojević et al., 2020) as well as in the Mediterranean area, including Italian artisanal cheeses (Biolcati et al., 2020), Portuguese cheeses (Cardinali et al., 2021, 2022a,b; Rampanti et al., 2023b), French cheeses as *Tomme d'Orchies* cheese (Ceugniez et al., 2017) and Savoyard raw milk cheeses (Lecaudé et al., 2024), as well as Spanish *San Simón da Costa* cheese (Terzić-Vidojević et al., 2020).

Str. thermophilus is a thermophilic lactic acid bacteria with great economic value for the dairy industry. Thanks to its rapid acidification of milk, it is extensively used as a starter culture for the manufacture of several dairy products (Grizon et al., 2023). It is the only species among the genus *Streptococcus* that has obtained the Generally Recognized as Safe (GRAS) status and the Qualified Presumption of Safety QPS) status. *Str. thermophilus* is also a highly proteolytic bacterium thus playing a central role in flavor development and texture formation of cheese by liberating peptides and free amino acids that undergo secondary metabolism (Grizon et al., 2023).

Intriguingly, Bifidobacteriaceae have also been detected with high frequency in all cheese samples from producer C. This family includes Gram-positive, anaerobic and facultative anaerobic, non-motile and non-spore forming bacteria, which are part of the human and other mammals gut microbiota with potential probiotic or health promoting effects on the host (Hanifi et al., 2021).

Alegria et al. (2012) reported the first detection of Bifidobacteriaceae in traditional Polish cheese. Later, Marino et al. (2017) and Mohamed et al. (2022) found the presence of Bifidobacteriaceae in Italian brined cheeses and in Egyptian cheeses, respectively, despite the high salinity of such environments. Indeed, Bifidobacteriaceae cannot survive NaCl concentrations higher than 5%, thus suggesting the presence of strains adapted to high salinities (Marino et al., 2017; Mohamed et al., 2022). Of note, the highest frequency of Bifidobacteriaceae has been found in *Njeguški* cheese samples from producer C which were characterized by the highest salt content, although below 5 %.

Lc. garvieae is considered the only pathogenic species of its genus. It is responsible for lactococcosis, a septicemic process, that was first found in rainbow trout in Japan. It is also responsible for mastitis in cows and is considered an emerging zoonotic pathogen (Abdelfataha & Mahboubb, 2018). However, *Lc. garviae* has been reported as part of the autochthonous microbiota of different artisanal dairy products manufactured from raw milk (Fernández et al., 2010). Moreover, it has been suggested the use of *Lc. garvieae* as adjunct cultures in cheese production since its metabolic activity may contribute to the final sensory features and safety of the products (Fernández et al., 2010). Intriguingly, *Lc. garvieae* isolated from raw milk and dairy products can produce bacteriocins, antimicrobial peptides that inhibit the growth of closely related species, called garviecin L1-5, garvicin ML, garvieacin Q, garvicin A, and garvicin KS (Abdelfataha & Mahboubb, 2018). Specifically, a strain of *Lc. garvieae* isolated from raw cow's milk was active against the growth of pathogenic *St. aureus* in artificially contaminated cheese during refrigerated storage (Abdelfataha & Mahboubb, 2018), indicating a possible contribution of this species in biopreservation of *Njeguški* cheese. However, due to the abovementioned health risks, it is suggested the use of isolated bacteriocins instead of *Lc. garvieae* strains inoculated in milk.

Lcb. zeae is a mesophilic, facultatively heterofermentative, lactic acid bacteria with the capacity to metabolise citrate in acetate, lactate and ethanol and it is characterized by a high proteolytic activity (Skeie et al., 2008; Terzić-Vidojević et al., 2020), suggesting its contribution in defining the aroma profile of cheese. *Lcb. zeae* is a species very close to *Lcb. casei* and it has already been found with 9

% of frequency in *Grana Padano* PDO cheese (da Silva Duarte et al., 2021), and in Portuguese cheeses as *Queijo de Azeitão* PDO cheese (Cardinali et al., 2021), *Queijo de Nisa* PDO cheese (Cardinali et al., 2022a), *Queijo de Beira Baixa* PDO cheese (Cardinali et al., 2022b).

Enterococcus spp. have been detected at low relative frequencies in all the samples. Enterococci are part of the non-starter lactic acid bacteria (NSLAB) typically associated with raw milk cheeses contributing to cheese flavor and texture (Terzić-Vidojević et al., 2020). It is also common that many strains of enterococci are able to secrete bacteriocins called enterocins, which have activity against pathogens and spoilage bacteria (Terzić-Vidojević et al., 2020). Furthermore, *Enterococcus* spp. may act as probiotics modulating the immune system through the induction of cytokine secretion by epithelial cells in a strain-specific manner (Terzić-Vidojević et al., 2020).

Enterobacteriaceae have been revealed by metataxonomic analysis, thus confirming their detection through culturable methods.

Concerning mycobiota, the dominant species *D. hansenii* is the prevailing yeast species in several cheese type, as hard, semi-hard, soft, white brined, mould surface ripened, bacteria surface ripened, and blue-veined cheese (Bintsis, 2021). *D. hansenii* normally colonizes cheese surface since it shows poor growth in the absence of oxygen (Frölich-Wyder et al., 2018). Its dominance in this food matrix is mainly due to its high halotolerance (it can survive up to 20-24 % (w w^{-1})), together with its ability to grow on lactose as well as lactate as carbon sources, and at low pH (Bintsis, 2021; Frölich-Wyder et al., 2018). *D. hansenii* impacts texture and aroma of the cheese thanks to its proteolytic and lipolytic activities, although the degree of intensity is strain specific. Furthermore, this yeast species is able to produce volatile molecules such as branched-chain aldehydes and alcohols that contribute to cheese flavour (Frölich-Wyder et al., 2018).

K. marxianus is frequently isolated from dairy products thanks to its ability to metabolize lactose as carbon sources producing CO_2 and ethanol, whereas it can use lactate after lactose is depleted (Bintsis, 2021; Frölich-Wyder et al., 2018). *K. marxianus* is a respiro-fermentative, fast-growing thermotolerant species that tolerates low pH values. During maturation, *K. marxianus* strongly influences the final texture and flavor of the cheese due to its proteolytic, lipolytic, and esterase activity producing esters (fruity aroma) and acetaldehyde (Bintsis, 2021; Frölich-Wyder et al., 2018). *Galactomyces* spp. is the teleomorphic genus of *Geotricum* spp.; the filamentous yeast-like species *Geotrichum candidum* is the only species widely used as a starter culture or adjunct culture in the dairy industry for the production of felted-looking cheese or cheese ripening (Frölich-Wyder et al., 2018; Pottier et al., 2008). In the present study, *G. candidum* has not been specifically detected, whereas the contaminant species *M. capitatus* (synonym *Geotricum capitatum*) has been found at about 10 % of the relative frequency in cheeses from producer B. Among the genus *Magnusiomyces*, *M. capitatus* is the most important clinical species since it is an emerging opportunistic yeast with thermophilic nature responsible for systemic infections such as fungemia, endocarditis, and pulmonary infections (Zhu et al., 2022). Moreover, cheeses from producer B were characterized by high relative frequency of *Wickerhamiella pararugosa* (synonym *Candida pararugosa*) (about 21.50 %), an emerging and rare pathogenic yeast identified from different organs and biological fluids of humans and animals, responsible for invasive candidemia associated with high morbidity and mortality mainly in immunocompromised patients (Kumar et al., 2022).

K. unispora is a lactose-negative yeast previously found along the ripening process of traditional Spanish and French semi-hard ewes' and goats' cheeses (Padilla et al., 2014).

T. delbrueckii has been reported as the most frequent yeast in *Canastra* cheese, a Brazilian semi-hard cheese produced from raw cow's milk inoculated with commercial rennet and pingo, which is a natural starter derived from the cheese whey from the previous day (Bintsis, 2021). However, *T. delbrueckii* is well-known as the most attractive non-*Saccharomyces* yeast species typically associated with winemaking to produce higher levels of alcohols, ethyl and acetate esters during the initial steps of the process, compared to *S. cerevisiae*. Thanks to these biochemical properties and its resistance to osmotic and freezing stresses, *T. delbrueckii* is considered a promising yeast for biotechnological exploitation in a wide range of industries (Fernandes et al., 2022; Silva-Sousa, 2022).

S. cerevisiae is a lactose-negative yeast, ascosporogenous, capable of anaerobic or semi-anaerobic fermentation of sugar to produce ethanol and carbon dioxide. *S. cerevisiae* is commonly found on the surface of mould-ripened cheeses. It metabolizes hexoses, lactic acid, and other organic acids, with an optimum pH for growth between 4.50 and 6.50 (Frank & Hassan, 2011).

Of note, among the minority taxa found in cheeses under study, *K. zeylanoides* (synonym *Candida zeylanoides*) and *W. anomalus* have been already isolated at the end of the ripening stage of the Italian *Fossa* cheese with interesting biotechnological properties (Biagiotti et al., 2018). *K. zeylanoides* has also been isolated from artisanal semi-hard Portuguese ewe's cheese (Bintsis, 2021).

S. apicola (synonym *Candida apicola*) is not a common species in fermented dairy products, although *Starmerella* spp. has already been found among the minority species in Portoguese cheeses *Queijo de Azeitão* PDO, *Queijo da Baixa* PDO, and *Queijo Serra da Estrela* PDO (Cardinali et al., 2022a,b; Rampanti et al., 2023b). Intriguingly, this species produces extracellular glycolipids called sophorolipids that are promising biosurfactants active against food spoilage and pathogen fungi (Hipólito et al., 2020), thus indicating a possible biotechnological role of *S. apicola* in enhancing cheese safety.

Overall, as reviewed by Bintsis (2021), artisanal cheeses possess a great diversity of yeast species belonging to several genera, such as those found in the present study and ascribed to *Candida*, *Pichia*, *Torulaspora*, *Trichosporon*, *Debaryomyces*, *Geotricum*, *Kluyveromyces*, *Kazachstania*, *Saccharomyces*, thus confirming the fungal richness of *Njeguški* cheese.

4.2 Physico-chemical characterization

The pH values recorded in the samples under study were generally in accordance with those reported by Martinovic et al. (2018) for laboratory-cheese production of *Njeguški* manufactured with different starter cultures at 21 days of ripening and ranging from pH 4.50 to 4.80 and are in line with those of the Greek sheep's semi-hard *Sfela* PDO cheese which was attested at pH 4.76 (Danezis et al., 2020).

In contrast, pH values detected in the cheese samples analyzed in the present study were lower than those detected in hard and semi-hard Italian *Pecorino* cheese made with pure ewes' milk (5.10-5.50) (Bansal & Veena, 2024; Schirone et al., 2012) and lower than those found in semi-hard raw ewe's milk cheese from Spain ripened for 30 days (5.15) (Tabla et al., 2016). During cheese manufacturing, pH reduction is mainly due to the metabolism of lactic acid bacteria that produce organic acids from fermentation of lactose. From manufacturing to ripening, the proper acidification induced by lactic acid bacteria affects the stability and the quality of final product in terms of safety, sensory profile, rennet coagulation, activity on other enzymes that influence aroma and quality of the cheese, proteolysis, whey syneresis, salt absorption, moisture as well as texture of the cheese (Bansal & Nagaraj, 2022; Bansal & Veena, 2024; Cardinali et al., 2022a,b; Garofalo et al., 2022; Rampanti et al., 2023a,b).

In the samples under study, the very high a_w values detected (0.97-0.98) may affect the stability of the cheese. Indeed, this parameter together with pH and humidity, represents a pivotal factor that preserves cheese against microbial growth and spoilage (Rampanti et al., 2023b). To the authors' knowledge, a lack of data regarding a_w of *Njeguški* cheese is currently available in the scientific literature for further comparison of data.

Humidity of food is a fundamental parameter that significantly impacts the quality, safety, and shelf-life of food products. The humidity content of foods strongly influences microbial growth, enzymatic activity, and chemical reactions. Overall, the humidity content of *Njeguški* cheese samples is in line with data reviewed by Teneva-Angelova (2018) for the same cheese and corresponding to moisture values ranging between 41-54 %. In detail, humidity content from producer B is in accordance with the data reported by Mirecki et al. (2015) for traditional *Njeguški* cheese ripened 40-50 days and corresponding to 42.07 ± 4.28 %, whereas samples from producer A and C showed higher (45.58 ± 3.03 %) and lower (38.37 ± 1.66 %) mean values than those reported by Mirecki et al. (2015), respectively. In particular, the latter samples show moisture content closest to hard cheeses that is generally below 40 % due to the pressure applied during the manufacturing process aimed at forcing the drainage of the whey (Bintsis 2021; Terzić-Vidojević et al., 2020). Overall, moisture content of cheeses under study is in accordance with raw ewe's hard and semi-hard cheeses from Greece with moisture percentages ranging from 37.10 % (*Ladotyri Mytilinis*) to 49.40 % (*Batzos*) (Danezis et al., 2020).

The salt content of the cheeses under study is lower than the data reviewed by Teneva-Angelova (2018) for the same cheese and ranging from 1.90-2.30 %. In more detail, the average value of cheeses from producer C (1.78 ± 0.36 %) was slightly lower than the salt average values of *Njeguški*-type cheese produced with ewe milk ripened at 60 days (2.02 ± 0.28 %) (Jokanovic et al., 2022), and those of traditional *Njeguški* cheese of 40-50 days ripening (1.94 ± 0.56 %) (Mirecki et al., 2015). On the other hand, much lower salt content values were found for cheeses from producer B and A showing values closest to NaCl content of semi-hard raw ewe's milk cheese from Spain ripened 30 days which was attested at 1.18 ± 0.07 % (Tabla et al., 2016).

Dairy products are generally consumed because of their nutritional and high-protein content. Indeed, beside their functional properties, proteins in cheese are among the main macronutrients necessary for the human body's metabolism (Diaz-Bustamante et al., 2023). The mean values of the total protein content of the cheeses under study were in line with the mean value reported by Teneva-Angelova (2018) and by Mirecki et al. (2015) ranging from 21 to 25 %. In detail, the cheeses from producer C showed the highest values, with an average value of protein content closest to those reported by Jokanovic et al. (2022) for *Njeguški*-type cheese produced with ewe milk at 45-60 days of ripening (25.45 ± 0.79 %-25.98 ± 0.49 %). The protein content of the *Njeguški* cheeses under study was also in accordance with that reported by Danezis et al. (2020) in Greek hard raw ewe's milk *Kefalograviera* cheese that exhibited 24.10 % of protein content.

Concerning lipids, the average values of all the cheeses under study were generally in line with the values reported by Mirecki et al. (2015) for traditional *Njeguški* cheese ripened 40-50 days (29.97 ± 3.02 %) and by Jokanovic et al. (2022) for *Njeguški*-type cheese produced with ewe milk until 60 days of ripening (30.24 ± 3.17 %). Furthermore, the lipid content of the *Njeguški* cheeses analyzed was slightly higher than those reported by Tabla et al. (2016) for Spanish semi-hard raw ewe's milk cheese at 30 days of ripening and attested at 26.80 ± 0.60 %, but it falls within the fat value for the semi-hard *Ladotyri Mytilinis* PDO cheese from Greece attested up to 35.30 % (Danezis et al., 2020). The lipid amount and composition are parameters that influence the most the nutritional composition as well as the color, texture and flavor of cheese (Inmaculada González-Martín et al., 2020). Cheese is particularly rich in saturated fatty acids (SFAs), which include a heterogenous group of fatty acids that contain only carbon-to-carbon single bonds. SFAs are categorized as short-chain (4-6 carbon atoms), medium-chain (8-12 carbon atoms), long-chain (14-20 carbon atoms), and very long-chain (22 or more carbon atoms). Depending on the length of the carbon chains, different effect of SFA on human health have been shown (Inmaculada González-Martín et al., 2020; Astrup et al., 2020; Paszczyk and Łuczyńska, 2020). Overall, diets with high concentrations of SFA are associated with cardiovascular disease, obesity, and cancers (Inmaculada González-Martín et al., 2020; Paszczyk, 2022; Paszczyk & Łuczyńska, 2020). Moreover, for authenticity purposes the chemical composition and fatty acid profile of traditional cheeses are used as potential tracers. This issue is challenging due to the influence of multiple factors such as feeding system of the animals, variation in production methods, different breeds, and others (Danezis et al., 2020; Margalho et al., 2021; Paszczyk, 2022). No previous data on SFAs content as well as carbohydrates and ash content of *Njeguški* cheese are available for further comparison with the results.

4.3 Biogenic amines content

Variable levels of biogenic amines were observed in the cheese samples under study, thus suggesting the presence of microorganisms with decarboxylation or amination activities. Indeed, biogenic amines are aliphatic, heterocyclic, or aromatic organic nitrogenous molecules with low molecular weight deriving from specific amino acids that after the elimination of the *a*-caboxyl group give the corresponding amines: histamine derives from histidine, tyramine from tyrosine, tryptamine from tryptophan, putrescine from ornithine, cadaverine from lysine, and 2-phenylethylamine from phenylalanine (O'Sullivan et al., 2013). Alternatively, amines as putrescine are also produced through deamination of agmatine-by-agmatine deaminase in the genera *Enterococcus* and *Lactobacillus* (O'Sullivan et al., 2013). The production of decarboxylases is primarily associated with Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae, *Clostridium* spp., and lactic acid bacteria (O'Sullivan et al., 2013; Schirone et al., 2022), and secondarily to yeasts ascribed to *Yarrowia lipolytica* and *D. hansenii*. The latter play a marginal role in the production of biogenic amines in semi-hard and hard cheeses. Furthermore, *Y. lipolytica* and *D. hansenii* together with *G. candidum* have been found able to degrade biogenic amines (Frölich-Wyder et al., 2018).

Biogenic amines in food are associated with food quality and human health since they are precursors of carcinogens and they can exhibit toxic effects on both the vascular and nervous systems causing several symptoms as headache, heart palpitations, respiratory distress, localized inflammation, nausea, vomiting, and hypo/hypertension (O'Sullivan et al., 2013; Schirone et al., 2013).

These hazardous substances are considered quality markers often associated with several protein-rich foods as cheese, fish, and dry sausage as well as in wine and beer manufactured under poor or uncontrolled hygiene conditions (O'Sullivan et al., 2013; Schirone et al., 2013). Specifically, cheese is correlated with histamine poisoning and tyramine toxicity (Schirone et al., 2013). The EU Regulation No 2073/2005 establishes histamine limits for fish species associated with a high amount of histidine between 100 mg kg^{-1} and 200 mg kg^{-1}, for fishery products subjected to enzyme maturation treatment in brine between 200 mg kg^{-1} and 400 mg kg^{-1}, and for fish sauce produced by fermentation of fishery products at 400 mg kg^{-1}, while the US Food and Drug Administration indicates the limit of histamine at 50 mg kg^{-1} as harmful to health (Belleggia et al., 2022; Schirone et al., 2022). Although there is no regulation on histamine and tyramine content in most foodstuffs, Schirone et al. (2013; 2022) reported the accepted limit of histamine as 100 mg kg^{-1} and of tyramine as 800 mg kg^{-1} in fermented food products. Overall, a great variability of biogenic amines levels has been found in literature among different types of cheeses, and it is attributed to several factors that influence biogenic amines accumulation in dairy products as the use of starter cultures, milk pasteurization, microbial quality of raw milk, type of rennet, pH, temperature during maturation and storage, NaCl concentration, ripening and post-ripening technological process, overall manufacturing process and the sanitation procedures used (Linares et al., 2012; Schirone et al., 2013; 2022). Of note, the European Food Safety Authority (EFSA) Opinion (EFSA, 2011) reported a maximum of histamine and tyramine in hard cheese corresponding to 457 and to 1450 mg kg^{-1}, respectively (Schirone et al., 2022). In the present study, only samples from producer C showed histamine content higher than 100 mg kg^{-1} (Schirone et al., 2013; 2022) but below 457 mg kg^{-1} (EFSA, 2011), while tyramine content was widely below the levels above-mentioned (EFSA, 2011; Schirone et al., 2013; 2022) although a synergistic toxicity of tyramine and histamine on intestinal cell cultures has been recently reported (Schirone et al., 2022).

Concerning cadaverine and putrescine, a maximum tolerable amount of 180 and 540 mg kg^{-1}, respectively, has been proposed in cheese (Rauscher-Gabernig et al., 2012). Again, *Njeguški* cheese samples from producer C showed higher levels of both of these biogenic amines than the tolerance levels proposed.

Overall, the level of biogenic amines found in the present study was similar to or lower than that reported by Manca et al. (2020) for 37 samples of *Fiore Sardo* PDO cheese produced in Sardinia (Italy) from raw sheep's milk, showing the tyramine as the main biogenic amine with maximum level of 820 mg kg^{-1}, followed by putrescine with a mean value of 210 mg $kg^{-1}$and by cadaverine, histamine, ß-phenylethylamine, and tryptamine at concentrations lower than 100 mg kg^{-1}.

4.4 Morpho-textural characterization

In the CIE *L*a*b** system, the *a** parameter is used to indicate the green–red opponent colors (<0 toward green and >0 toward red), while *b** parameter denote the blue–yellow opponent colors (<0 toward blue and >0 toward yellow) (Rampanti et al., 2024). All the cheese samples were characterized by high lightness with yellow tonalities pronounced. Specifically, a higher yellowness and higher greenness have been found in samples from producers C that were also characterized by lower humidity, higher salt, proteins and ash content. Cheese yellowness is generally correlated to carotenoids, that are fat-soluble pigments ranging from red to yellow, available with variable levels in milk depending on kind of forages used for cows' and ewes' diets, while the greenish tonalities are due to the lack of red carotenoids (Cardinali et al., 2022a; Rampanti et al., 2023a). Overall, as reported by Rampanti et al. (2023b) different factors, such as origin of the milk, lipid content, pasture location, the amount and type of feed for sheep and cows, the grazing seasons, and ripening process, affect the color of the cheeses.

Concerning the morpho-textural traits, hardness is a parameter that indicates the maximum force experienced during the initial compression of the samples (Belleggia et al., 2024). The cohesiveness is a parameter that establishes the extend to which a sample maintains its integrity when underwent to a second deformation compared to its resistance to a first deformation (Rampanti et al., 2024). Springiness is the index of elastic recovery, indicating how quickly a deformed sample goes back to the initial state as soon as the force causing the deformation stops (Rampanti et al., 2024). The texture profile analyses of the cheese samples under study showed that samples from producer C had higher hardness and springiness compared to those from producer B and A. Samples from producer C showed the lowest moisture, thus probably explaining the highest level of hardness. Beside the composition, these data may depend also on ripening process of the samples since the proteolysis occurring during the ripening can partially melt the cheese matrix thus influencing the texture of the product (Cardinali et al., 2022b).

4.5 Volatile organic composition

To the authors' knowledge, this is the first study on the characterization of volatile organic compounds in Montenegrin *Njeguški* cheese. In general, all the cheeses analyzed were characterized by similar classes of volatile organic compounds, although their relative percentages differed. HS-SPME-GC/MS analysis identified major and minor volatile components; in particular, the volatile fraction of cheeses was dominated mainly by alcohols, esters and acetates, aldehydes and acids, while only traces of terpenes, sulfides and aldehydes were found.

Ketones were present in high amounts in all samples from the three producers, where 2-butanone, acetoin, and acetone were the most represented ketones. Samples from producer C were also characterized by high amounts of 2-heptanone and 2-pentanone. Ketones are crucial in defining the aroma of dairy products, as they can be derived from the raw materials, and are also formed during ripening by the activities of the dominant microflora. Furthermore, as highlighted by different authors (Ruiz et al., 2023), ketones are associated with fruity and floral notes, making their presence positive for cheese flavor.

2-Butanone and acetoin, which have a buttermilk, fruity, and ethereal odor, were identified as the main odorant in the traditional *Beaten* (*Bieno sirenje*) ewe milk cheese (Sulejmani et al. 2014), *Cheddar* cheese (Arora et al., 1995) and other raw milk cheeses such as Spanish soft PDO *Torta del Casar* (Delgado et al., 2010), highlighting that they have an important role in the flavour profile of these raw milk cheeses. In contrast, 2-heptanone, which has an herbaceous, sweet, and spicy odor, has been found to be an influential volatile compound in *Emmental* and *Gorgonzola* cheeses (Curioni et al., 2002).

A considerable occurrence of different acids was detected in all the samples. Butanoic, hexanoic and acetic acids were detected mainly in the samples from producer C, while 3-methylbutanoic and isobutanoic acids were predominant in the cheese samples from producers A and B.

In general, acetic acid is associated with sour, pungent, and vinegary notes and is synthesized from the catabolism of lactose, citrate, and free fatty acids, whereas butanoic and hexanoic acids are associated to cheesy, buttery, and sometimes rancid odors, and usually they increase during ripening in hard cheeses (Ianni et al., 2020).

Alcohols were another chemical family abundantly found in the *Njeguški* cheeses, where isoamyl alcohol, ethanol, and phenylethyl alcohol were the most common alcohols identified as key odorants. In particular, isoamyl alcohol was predominant in the samples from producers A and B and was also the major alcohol detected in *Van herby* cheese, a Turkish cheese made from raw and pasteurized ewe's, ewe's and cow's, and mixture of ewe, cow, and goat milk (Ocak et al., 2014), and in *Bryndza*, a traditional Slovak ewe's spreadable cheese (Štefániková et al., 2020). Ethanol had high relative abundance in all the samples, as also found in other cheeses such as Turkish white cheese, *Gokceada*, and *Cheddar* (Hayaloglu et al., 2013; Hou et al., 2014; Oluk, 2023).

The *Njeguški* cheeses were also characterized by high amounts of esters and acetates, including four main compounds such as ethyl acetate, ethyl butanoate, isoamyl acetate, and ethyl hexanoate. Esters are volatile organic compounds usually found in fermented dairy products that are responsible for fruity odors (such as apple, banana, and pineapple notes) and can contribute strongly to the fruity aroma of the cheese. Typically, esters in dairy products are formed through two enzymatic mechanisms: esterification and alcoholysis. The former involves the formation of esters from alcohols and carboxylic acids, whereas alcoholysis is the production of esters from alcohols and acylglycerols or from alcohols and fatty acyl-CoAs derived from the metabolism of fatty acids, amino acids and/or carbohydrates. In cheese, this reaction may be spontaneous or mediated by microbial esterases from lactic acid bacteria and yeasts (Ocak et al., 2014).

Sulphur compounds and terpenes were minor compounds in all the samples. Furthermore, the samples were characterized by traces of aldehydes, which, as also pointed out by Coda et al. (2006), are transient in nature and do not accumulate in cheese as they tend to be reduced in the corresponding alcohols or, alternatively, oxidize in the respective acids. The only aldehyde detected was the 3-methyl-butanal. This compound is considered a key aroma compound that imparts a nutty flavor to cheese, and its presence has often been associated with *Lc. lactis*, which is often used as a starter to improve cheese flavor and quality (Chen et al., 2022). Additionally, in the samples analyzed, *Lc.*

lactis, together with *Str. thermophilus*, *D. hansenii*, and *K. marxianus*, was one of the predominant species in the *Njeguški* cheese microbiome.

5. Conclusion

Njeguški cheese is one of the most popular autochthonous Montenegrin fermented foods that so far has not been deeply investigated in terms of microbiota, composition, and volatilome. The results of the present study will serve as a basis for a comprehensive knowledge of such food product for implementation of a production disciplinary, and standardization of the product, as well as for drawing attention to safety and health issues related to production.

Lc. lactis, *Str. thermophilus*, *D. hansenii*, and *K. marxianus* made up the core microbiome of *Njeguški* cheese. The presence of Enterobacteriaceae and Pseudomonadaceae or opportunistic pathogenic yeasts as *M. capitatus* and *W. pararugosa*, as well as the variable content of biogenic amines, suggests the necessity for further attention in terms of hygienic conditions to be applied during *Njeguški* cheese production. HS-SPME-GC/MS analysis showed a well-defined volatilome profile of the *Njeguški* cheese, where alcohols, esters and acetates, ketones, and acids were the main chemical groups involved in aroma formation.

Further studies could be also performed on *Njeguški* cheese produced either in winter season or by using selected autochthonous starter cultures to compare overall data and to overcome limitations on safety aspects.

CRediT authorship contribution statement

Federica Cardinali: Formal analysis, Investigation, Writing – review & editing. **Giorgia Rampanti:** Formal analysis, Investigation, Writing – original draft. **Giuseppe Paderni:** Investigation. **Vesna Milanović:** Investigation. **Ilario Ferrocino:** Formal analysis, Investigation. **Anna Reale:** Formal analysis, Investigation. **Floriana Boscaino:** Formal analysis, Investigation**. Nadja Raicevic:** Investigation. **Maša Ilincic:** Investigation, Writing – original draft. **Andrea Osimani:** Writing – original draft, Writing – review & editing. **Lucia Aquilanti:** Writing – review & editing. **Aleksandra Martinovic:** Funding acquisition, Resources, Writing – review & editing. **Cristiana Garofalo:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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FIGURE CAPTIONS

Figure 1. Principal coordinate analysis (PCoA) based on weighted unifrac distance matrix for microbiota (a) and mycobiota (b). Samples are color-coded according to the producer.

Figure 2. Panel a) Circular ideogram showing the bacterial biota distribution (>1 % of the relative frequency in at least 2 samples) among *Njeguški* cheese samples. ASVs and samples are connected with a ribbon, and its thickness is proportional to the abundance of an ASV in the connected sample. The outer circle displays the proportion of each ASV in a given sample and vice versa. Panel b) Boxplots showing the differences in relative abundance of ASVs based on ANOVA test ($p < 0.05$) in cheese samples among the three producers for bacterial biota. Statistically significant differences are indicated by different letters on top of each graph.

Figure 3. Panel a) Circular ideogram showing the fungal biota distribution (>1 % of the relative frequency in at least 2 samples) among *Njeguški* cheese samples. ASVs and samples are connected with a ribbon, and its thickness is proportional to the abundance of an ASV in the connected sample. The outer circle displays the proportion of each ASV in a given sample and vice versa. Panel b) Boxplots showing the differences in relative abundance of ASVs based on ANOVA test ($p < 0.05$) in cheese samples among the three producers for fungal biota. Statistically significant differences are indicated by different letters on top of each graph.

Figure 4. Principal Component Analysis (PCA) of biogenic amines in *Njeguški* cheese from three different batches (I, II, III) and producers (A, B, C).

CAD, cadaverine; PHE,2-phenylethylamine; HIS, histamine; PUT, putrescine; SPD, spermidine; TYR, tyramine; TRP, tryptamine.

Figure 5. Principal Component Analysis (PCA) of volatile compounds in *Njeguški* cheese from three different batches (I, II, III) and producers (A, B, C).