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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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(Article begins on next page)

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

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4 Federica Cardinali<sup>1</sup>, Giorgia Rampanti<sup>1</sup>, Giuseppe Paderni<sup>2</sup>, Vesna Milanović<sup>1</sup>, Ilario Ferrocino<sup>3</sup>,  
5 Anna Reale<sup>4</sup>, Floriana Boscaino<sup>4</sup>, Nadja Raicevic<sup>2</sup>, Maša Ilincic<sup>2</sup>, Andrea Osimani<sup>1,\*</sup>, Lucia  
6 Aquilanti<sup>1</sup>, Aleksandra Martinovic<sup>2</sup>, Cristiana Garofalo<sup>1</sup>

7

8 <sup>1</sup>Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche,  
9 Breccie Bianche, 60131 Ancona, Italy

10 <sup>2</sup>Centre of Excellence for Digitalisation of Microbial Food Safety Risk Assessment and Quality  
11 Parameters for Accurate Food Authenticity Certification, University of Donja Gorica, Podgorica,  
12 Montenegro

13 <sup>3</sup>Department of Agricultural, Forest, and Food Science, University of Turin, Largo Paolo Braccini 2,  
14 Grugliasco, Torino, Italy

15 <sup>4</sup>Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Via Roma 64, 83100  
16 Avellino, Italy

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26 \*corresponding author: Andrea Osimani, a.osimani@univpm.it

27 **Abstract**

28

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

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

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## 79 1. Introduction

80

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

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

98 Mirecki et al. (2015) were the first to describe the production technology of *Njeguški* cheese, noting  
99 that it was originally a hard, full-fat cheese made from raw sheep's milk. However, due to changes in  
100 consumer eating habits, *Njeguški* cheese is now mainly manufactured using a mixture of sheep's,  
101 cow's, or goat's milk and is marketed as a semi-hard, full-fat cheese. Traditionally, the cheesemaking  
102 process is performed manually. The cooled raw milk is filtered through cheesecloth and then heated  
103 to about 35°C. Natural rennet from a lamb's stomach is added to the milk. After 30–60 minutes, the  
104 cheese curd forms and is cut into pieces about 5 mm in size. The curd is then broken by hand in the

105 whey, which is gradually heated to about 40-45°C. The curd is transferred into wooden or metal  
106 molds (20 cm in diameter) and pressed for a total of 24 hours, with the cheese being turned after 12  
107 hours of pressing. A wooden circular board is used for pressing, and a stone is placed on top for  
108 additional weight. The cheese is then removed from the mold, placed in a wooden chest, and dry  
109 salted for 2 days, with salt being added 2-3 times per day. The optimum maturation period for  
110 *Njeguški* cheese is 1 month, and its ripening occurs on wooden shelves in stone cellars. To remove  
111 molds from the cheese surface, it is washed with cold, salty water and then dried with a clean cloth.  
112 The ripened cheese can also be exposed to smoke or soaked in maize, wheat, or olive oil for further  
113 maturation. Ready-to-eat *Njeguški* cheese has the shape of a low cylinder with a height of 3-5 cm, a  
114 diameter of 15-25 cm, and a weight of about 1-2.5 kg. The crust is crack-free and has a golden-yellow  
115 colour. The cheese texture is homogeneous with a few "cheese eyes" up to 0.5 cm in diameter; it is  
116 not brittle and is easy to cut (Martinovic & Mirecki, 2021; Martinovic et al., 2018; Mirecki et al.,  
117 2015).

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

129 Despite the great popularity and ancient origin of *Njeguški* cheese, only a few scientific studies have  
130 been conducted on this spontaneously fermented cheese. These studies have mainly focused on

131 defining its production technology and basic composition (Mirecki et al., 2015), studying its  
132 production using lactic acid bacteria starter cultures (Bojanic Rasovic et al., 2017; Martinovic et al.,  
133 2018), and investigating its  $\alpha$ -tocopherol content during ripening (Jokanovic et al., 2022). However,  
134 deciphering the microbial profile of spontaneously fermented foods significantly contributes to  
135 protecting their origin, quality, and technology, as it is well known that the autochthonous microbial  
136 population is the most important factor influencing the quality and traditional characteristics of  
137 fermented foods, especially dairy products. Indeed, cheese microbiota contribute to the aroma, taste,  
138 texture, safety, and nutritional characteristics of the products (Cardinali et al., 2022a,b; Mirecki et al.,  
139 2015; O'Sullivan et al., 2013). Therefore, the present study aims to fill the gap of knowledge  
140 regarding the microbiota of *Njeguški* cheese through culture-dependent techniques and metagenomic  
141 analysis. Furthermore, for the first time, a complete characterization of the cheese in terms of gross  
142 composition, physico-chemical and morpho-textural features, biogenic amine content, and volatilome  
143 has been conducted. This study will serve as a baseline for further defining biomarkers of quality and  
144 authenticity, contributing to the process of protecting the origin of *Njeguški* cheese.

145

## 146 **2. Materials and methods**

147

### 148 *2.1. Sampling*

149

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

157 and a height of 3.5 cm. The scheme depicting the general manufacturing procedure of *Njeguški* cheese  
158 is shown in Supplementary Figure 1.

159

## 160 2.2 Microbiological analyses

161

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

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



183

### 184 *2.3 Detection of staphylococcal enterotoxins*

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

190

### 191 *2.4 Microbial DNA extraction, sequencing, and bioinformatics*

192

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

197 A total of 18 DNA samples (6 for each producer) were quantified using the QUBIT dsDNA Assay  
198 kit (Life Technologies, Milan, Italy) and standardized to  $5 \text{ ng } \mu\text{L}^{-1}$ . Two  $\mu\text{l}$  of each DNA sample was  
199 amplified for microbiota analysis by using the primers and conditions for the amplification of the V3-  
200 V4 region of the 16S rRNA gene as described by Klindworth et al. (2013). The mycobiota was studied  
201 by the amplification of the D1-D2 domain of the 26S rRNA gene according to Mota-Gutierrez,  
202 Ferrocino, Rantsiou, & Cocolin (2019). Pair-end sequencing (2X250bp) was performed with a MiSeq  
203 Illumina instrument (Illumina, San Diego, CA, USA) using V2 chemistry according to the  
204 manufacturer's instructions. Raw reads were analyzed by using the Quantitative Insights Into  
205 Microbial Ecology (QIIME2) (Bolyen et al., 2019). Primers and adapters were first trimmed by using  
206 Cutadapter and then quality filtered using the DADA2 algorithm (Callahan et al., 2016). Low-quality  
207 bases, chimeric sequences, and sequences shorter than 300 bp were filtered out by using the dada2  
208 denoise-paired plug in of QIIME2. Amplicon Sequence Variants (ASVs) generated by DADA2 were

209 rarefied at the lowest sequences per sample and used for taxonomic assignment using the QIIME  
210 feature-classifier plugin against the Greengenes 16S rRNA gene database ([version 13\\_5](#)) for the  
211 microbiota, and the manually built database for the mycobiota (Mota-Gutierrez et al., 2019).  
212 Taxonomy assignment at the highest taxonomic resolution reached for 16S rRNA gene and 26S rRNA  
213 gene was confirmed by double checking on BLAST suite tools. The raw read data generated by  
214 sequencing were deposited in the NCBI Sequence Read Archive (SRA) under the Bioproject  
215 Accession Number PRJNA1133970.

216

### 217 *2.5 Physico-chemical analysis*

218

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

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

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

224 The salt (sodium chloride) content was determined through ion chromatography analysis. Briefly, 2  
225  $\pm$  0.1 g of sample was weighted, added with 20 mL of water, mixed for 20 min (orbital mixer KS  
226 501Digital, IKA® Werke, Staufen, Germany), centrifuged at 800 rpm for 5 min (Rotanta 460 R,  
227 Hettich GmbH & Co. KG, Tuttlingen, Germany), filtered through a 0.45  $\mu$ m syringe filter and  
228 analyzed by ion chromatography (ICS 5000 Dionex, ThermoFisher Scientific, Milan, Italy). The  
229 chromatography conditions were: Dionex IonPack CS12A 4x250 mm column and Dionex IonPack  
230 CG12A 4x50 mm precolumn (Thermo Fisher Scientific, Milan, Italy), 50  $\mu$ L injection, 1 mL min<sup>-1</sup>  
231 flow, 100 mM SRS and 20 mM methanesulphonic acid as mobile phase.

232 The proximate composition of the samples was determined as follows: protein (%), assessed by  
233 Kjeldahl method (AOAC, 981.10); fat (%), assessed by Soxhlet extraction (AOAC, 991.36); ash (%),

234 determined in a convection oven (AOAC, 920.153); finally, carbohydrate (CHO) content was  
235 calculated by difference (Cardinali et al., 2022a).

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

238 The analyses were carried out in triplicate for each biological replicate, and the results were reported  
239 as mean values  $\pm$  standard deviation.

240

#### 241 *2.6 Biogenic amines content*

242

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

246

#### 247 *2.7 Morpho-textural analyses*

248

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

254 For each cheese, cylindrical specimens (height: 15 mm, diameter: 20 mm) were obtained, and then  
255 subjected to uniaxial compression with a CT3-4500 texture analyzer (Brookfield Engineering  
256 Laboratories Inc., Middleboro MA, USA) equipped with a 36 mm diameter cylindrical probe (mod.  
257 TA-AACC36) at  $1.5 \text{ mm s}^{-1}$  applying a non-destructive deformation (40 %) (Osimani et al., 2023).  
258 Specimens were positioned between the load cell and the fixture base table of the instrument, and a  
259 4500 g load cell was used.

260

261 *2.8 HS-SPME-GC/MS analysis of volatile components*

262

263 Headspace volatiles from each sample were analyzed by headspace solid-phase microextraction (HS-  
264 SPME) coupled to gas chromatography–mass spectrometry (GC-MS) (HS-SPME-GC/MS), using a  
265 7890 Agilent GC system coupled to an Agilent 5975 (Agilent Technologies, Santa Clara, California,  
266 USA) inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler (Gerstel,  
267 Mülheim, Germany), as described by Reale et al (2016) with some modifications. Briefly, 2 g of  
268 samples were placed into a 20 mL headspace vial, and 5  $\mu$ L of 3-octanol (internal standard, 100 mg/L  
269 standard solution) was added. The vial was placed in a thermostatic block (40 °C) on a stirrer, the  
270 fiber was inserted and maintained in the sample headspace for 30 min, then removed and immediately  
271 inserted into the GC/MS injector for the desorption of compounds. The extraction was performed  
272 automatically by the multipurpose sampler of the GC/MS system. A silica fiber, coated with 75  $\mu$ m  
273 of Carboxen/Polydimethylsiloxane (CAR/PDMS (Supelco, Bellefonte, PA, USA) was used for  
274 analysis. the operating conditions were as follows: HP-Innowax capillary column (Agilent  
275 Technologies, 30 m  $\times$  0.25 mm ID, film thickness 0.32  $\mu$ m), gas carrier was helium (flow 1.5  
276 mL/min), and SPME injections were splitless (straight glass line, 0.75 mm ID) at 240 °C for 20 min,  
277 during which time thermal desorption of the analytes from the fiber occurred. The oven parameters  
278 were as follows: initial temperature of 40 °C held for 3 min, followed by an increase to 240 °C at a  
279 rate of 5 °C/min, and then held for 0 min. The injector, the quadrupole, the source and the transfer  
280 line temperature were maintained at 240 °C, 150 °C, 230 °C and 200 °C, respectively. Electron  
281 ionization mass spectra in full-scan mode were recorded at 70 eV electron energy in the range 31–  
282 500 amu. VOCs identification was achieved by comparing mass spectra with the Nist library (NIST  
283 20) and by matching the retention indices (RI) calculated according to the equation of Van Den Dool  
284 and Kratz (1963) and based on a series of alkanes. The data are expressed like relative peak area

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

289

## 290 *2.9 Statistical analysis*

291

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

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

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

305

## 306 **3. Results**

307

### 308 *3.1 Microbial counts*

309

310 The results of the viable counts are reported in Table 1. Average counts of presumptive lactobacilli  
311 ranged from  $8.29 \pm 0.25$  (producer C) to  $8.57 \pm 0.31$  log cfu g<sup>-1</sup> (producer B), with no statistically  
312 significant differences among producers. For presumptive lactococci, average viable counts ranged  
313 from  $8.37 \pm 0.27$  (producer C) to  $9.04 \pm 0.17$  log cfu g<sup>-1</sup> (producer A), with samples from producer C  
314 showing the lowest values. For Enterobacteriaceae average counts ranged from  $4.99 \pm 0.58$  (producer  
315 A) to  $5.25 \pm 0.44$  log cfu g<sup>-1</sup> (producer C), with no statistically significant differences among  
316 producers. Similarly, no statistically significant differences were seen among producers for *E. coli*,  
317 with average viable counts ranging from  $3.61 \pm 0.86$  (producer A) to  $3.89 \pm 0.61$  log cfu g<sup>-1</sup> (producer  
318 C). Regarding Pseudomonadaceae, average counts ranged from  $4.80 \pm 0.16$  (producer C) to  $5.03 \pm$   
319  $0.27$  log cfu g<sup>-1</sup> (producer A), with no statistically significant differences among producers. Average  
320 counts of yeasts ranged from  $6.39 \pm 0.22$  (producer C) to  $6.53 \pm 0.29$  log cfu g<sup>-1</sup> (producer A), with  
321 no statistically significant differences among producers. No statistically significant differences were  
322 observed among producers for mold presence with average viable counts ranging from  $4.54 \pm 0.15$   
323 (producer C) to  $4.63 \pm 0.26$  log cfu g<sup>-1</sup> (producer A).

324 Finally, coagulase positive staphylococci viable counts were below the detection limit of the analysis  
325 (< 1 log cfu g<sup>-1</sup>). *L. monocytogenes*, *Salmonella* spp., and staphylococcal enterotoxins were never  
326 detected.

327

### 328 *3.2 Microbiota and mycobiota composition*

329

330 A total of 1,142,654 denoised reads (63,481 reads on average per sample) for bacteria and 2,441,952  
331 (135,664 reads on average per sample) for fungi were analyzed, with a coverage greater than 99 %.  
332 No statistically significant differences ( $p > 0.05$ ) were observed in alpha diversity (Shannon index)  
333 for both microbiota and mycobiota among *Njeguški* cheese samples from different producers (A, B,  
334 and C) (Supplementary Figure 2). By contrast, significant differences ( $p < 0.05$ ) emerged in beta-  
335 diversity (Figure 1). The composition of the bacterial biota of *Njeguški* cheese samples is shown in

336 Figure 2 (panel a) and Supplementary Table 1. Moreover, the ASVs of bacterial taxa showing  
337 statistically significant differences among the cheese samples are reported in Figure 2 (panel b).  
338 Overall, the bacterial biota of *Njeguški* cheese samples was dominated by lactic acid bacteria, namely  
339 *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus* spp., *Lactococcus* spp., and  
340 *Streptococcus* spp. *Lactococcus lactis* was significantly more abundant in cheeses from producer B  
341 (approximately 50 % of the relative frequency) and was found at relative frequencies between 30–40  
342 % in cheeses from producers A and C. Instead, cheeses from producer A showed higher relative  
343 frequencies of *Lactobacillus* spp. (approx. 30 %) compared to producers B and C (3 % and 9 %,  
344 respectively). All cheese samples from producer C showed a higher incidence of Bifidobacteriaceae  
345 (approx. 4.50 % of the relative frequency) compared to the other samples (approx. 1 % and 0.12 %  
346 in producer A and B, respectively). *Lactococcus garvieae*, *Lacticaseibacillus zaeae*, and *Enterococcus*  
347 spp. were also detected in cheese samples from the three different producers with relative frequencies  
348 ranging from 0.23 % to 2.72 %. Notably, cheese samples from producer B showed a higher presence  
349 of Enterobacteriaceae (approx. 3.70 % of the relative frequency) compared to the cheeses from the  
350 other producers. A minor fraction of ASVs belonged to other lactic acid bacteria taxa as well as to  
351 spoilage microorganisms.

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

362 *Wickerhamiella pararugosa* (approx. 21.50 % of the relative frequency) compared to the other  
363 samples. Conversely, *Torulaspota delbrueckii* was predominant in samples from producer A (approx.  
364 16 % of the relative frequency). *Saccharomyces cerevisiae* and *Geotrichum* spp. were equally  
365 distributed in all cheese samples. Beyond the microorganisms listed above, minority taxa were  
366 sporadically detected at very low relative frequencies, including *Candida sake*, *Geotrichum fragans*,  
367 *Kurtzmaniella zeylanoides*, *Pichia* spp., *Starmerella apicola*, *Torulaspota quercuum*, *Trichosporon*  
368 spp., and *Wickerhamomyces anomalus*.

369

### 370 3.3 Physico-chemical characterization

371

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

374 In more detail, pH average values ranged between  $4.38 \pm 0.32$  (producer C) and  $4.80 \pm 0.31$  (producer  
375 B), whereas water activity ( $a_w$ ) average values ranged from  $0.97 \pm 0.01$  (producer A and C) to  $0.98 \pm$   
376  $0.01$  (producer B). No differences were observed for pH and water activity average values,  
377 irrespective of the producer.

378 Concerning humidity, average values ranged between  $38.37 \pm 1.66$  % (producer C) and  $45.58 \pm 3.03$   
379 % (producer A), with average counts of samples from producer C showing the lowest values.

380 The proximate composition analysis revealed no statistically significant differences among the  
381 carbohydrate, lipids, and total saturated fatty acids content of the samples from the three producers.

382 In detail, carbohydrate average content ranged from  $1.93 \pm 0.91$  (producer B) to  $2.37 \pm 0.49$  %  
383 (producer C), lipids average content ranged from  $27.94 \pm 1.09$  (producer A) to  $30.11 \pm 1.07$  %  
384 (producer C), whereas total saturated fatty acids average content ranged from  $18.70 \pm 1.32$  (producer  
385 A) to  $19.59 \pm 0.27$  % (producer B). However, for protein, salt, and ash significant differences were  
386 observed among producers, with samples of producer C showing the highest content and samples of  
387 producer A showing the lowest content. In detail, proteins average values were comprised between



388 21.42 ± 1.70 (producer A) and 25.08 ± 1.36 % (producer C), salt average content was comprised  
389 between 0.70 ± 0.12 (producer A) and 1.78 ± 0.36 % (producer C), and ash average content was  
390 comprised between 2.97 ± 0.28 (producer A) and 4.05 ± 0.87 % (producer C).

391

### 392 3.4 Biogenic amines content

393

394 The results of biogenic amines analyses carried out on the *Njeguški* cheese samples are reported in  
395 Table 3. The analyses revealed a significant variation in biogenic amines content of the cheese among  
396 samples except for spermine that was below the detection limit of the analysis for all of the samples  
397 (< 1 mg kg<sup>-1</sup>). Only samples from batches II and III of producer A showed tryptamine levels > 1 mg  
398 kg<sup>-1</sup>, whereas batch I from producer A was characterized by the presence of only spermidine and  
399 tyramine. 2-phenylethylamine was detected only in batch I from producer B. Overall, significantly  
400 higher levels of biogenic amines as cadaverine, histamine, putrescine, and spermidine was found in  
401 samples from producer C.

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

409

### 410 3.5 Morpho-textural characterization

411

412 The results of colour analyses carried out on the *Njeguški* cheese samples are reported in Table 4.  
413 Cheese colour evaluation revealed no statistically significant differences in *L\** parameter (lightness)

414 among producers ranging from 76.54 (producer C) to 80.65 (producer A), whereas for the  $a^*$   
415 parameter (redness/greenness), the average value (-1.19) of samples from producer C was the lowest.  
416 Finally, concerning  $b^*$  parameter (blueness/yellowness), the average value (17.27) of samples from  
417 producer A was the lowest.

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

424

### 425 *3.6 Volatile organic composition*

426

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

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

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

439 Among ketones, the most representative were acetoin, 2-butanone and acetone, found in all the

440 samples. Minor amounts were found of 2-pentanone, 2-heptanone, 2-nonanone mainly in the samples  
441 from producer B and C.

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

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

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

457

## 458 **4. Discussion**

459

### 460 *4.1 Microbial populations*

461

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

464 Concerning viable counts, a general lack of significant differences among lactic acid bacteria (in  
465 terms of presumptive lactobacilli and lactococci), Enterobacteriaceae, *Escherichia coli*,

466 Pseudomonadaceae, yeasts and molds loads recorded in the analyzed *Njeguški* cheese samples. These  
467 results suggest that, although manufactured from different producers in Montenegro, the production  
468 technology allows similar products to be obtained.

469 High counts of presumptive lactobacilli and lactococci were found in all samples, and the data are in  
470 accordance with the study by Martinovic et al. (2018) aimed at monitoring the microbial population  
471 in experimental *Njeguški* cheese during ripening, showing maximum number of presumptive  
472 lactococci in 14-day-old cheeses up to 9 log cfu g<sup>-1</sup>, and the maximum number of presumptive  
473 lactobacilli in 7-day-old cheese up to about 8 log cfu g<sup>-1</sup>. Other studies on semi-hard raw sheep's  
474 cheeses from Mediterranean countries reported similar values. In detail, Schirone et al. (2013)  
475 studying twelve different *Pecorino* cheeses from the Abruzzo region (central Italy) and Cardinali et  
476 al. (2022a) studying *Queijo da Baixa* PDO cheese (Portugal) reported viable counts of lactobacilli up  
477 to 9 log cfu g<sup>-1</sup>. In the latter study, counts of lactococci ranged from 6.32 to 9.07 log cfu g<sup>-1</sup>, which  
478 were similar to those detected in the *Njeguški* cheese samples analyzed in the present study.

479 Moreover, presumptive lactobacilli and lactococci counts were in line with those reported by  
480 Cardinali et al. (2022b) in the Portuguese *Queijo de Nisa* PDO cheese with values ranging from 7.60  
481 to 8.84 log cfu g<sup>-1</sup> and 7.66 to 9.01 log cfu g<sup>-1</sup>, respectively. These data were quite expected, as the  
482 main role of lactic acid bacteria during cheese manufacturing and ripening is well-known. Lactic acid  
483 bacteria naturally occur in milk and become predominant during cheese manufacturing thanks to their  
484 ability to ferment carbohydrates forming organic acids (mainly lactic and acetic acids), ethanol and  
485 CO<sub>2</sub>, through homo- or heterofermentative metabolism. Besides organic acids, lactic acid bacteria  
486 produce other metabolites (e.g., hydrogen peroxide, antifungal peptides, and bacteriocins) with  
487 inhibitory effect against the growth of spoilage and pathogenic bacteria, thus increasing food safety  
488 and prolonging the shelf-life of the cheese. The biosynthetic activity of lactic acid bacteria also plays  
489 a key role that ensures the following effects: the development of a wide range of volatile compounds  
490 (e.g., organic acids, heterocyclic compounds, aldehydes, ketones, etc.), textural changes (e.g.,  
491 exopolysaccharides production that increase the product viscosity), and production of valuable

492 nutritional compounds (such as vitamins, polyphenols, polysaccharides, and polyunsaturated fatty  
493 acids) that enhance cheese nutritional profile. Lactic acid bacteria metabolism also consists of the  
494 hydrolyzing activity of polymers that improve digestibility and bioavailability of the products and  
495 influence the aromatic profile of products (e.g. amino acids) (Garofalo et al., 2022; Terzić-Vidojević  
496 et al., 2020). Moreover, some lactic acid bacteria strains are recognized for their probiotic functions  
497 that promote human health (Garofalo et al., 2022; Terzić-Vidojević et al., 2020).

498 The detection of Enterobacteriaceae in raw milk cheese is common since they are part of the  
499 indigenous microbiota of raw milk, generally due to faecal contamination of raw milk, and they are  
500 also associated with poor hygienic conditions applied during cheese production (Cardinali et al.,  
501 2021, 2022a,b; Rampanti et al., 2023a). The counts of Enterobacteriaceae detected in the present  
502 study are in line with those reported by Cardinali et al. (2021) for a Portuguese raw ewe's cheese  
503 called *Queijo de Azeitão* PDO, ranging from 4.50 to 5.25 log cfu g<sup>-1</sup>, whereas they were slightly  
504 higher than those reported by Tabla et al. (2016) for a Spanish semi hard raw ewe's milk cheese at  
505 30 days of ripening, which attested at about 4 log cfu g<sup>-1</sup>. Although members of the  
506 Enterobacteriaceae family are inhibited by an acidic environment such as that found in the cheese  
507 samples under study (pH 4.38-4.80), the occurrence of high loads of about 5 log cfu g<sup>-1</sup> in the final  
508 products may indicate an initial high contamination in raw milk or the need for a longer ripening time  
509 as demonstrated by Tabla et al. (2016). Indeed, these authors observed a slow decline of  
510 Enterobacteriaceae counts up to 60 days of ripening in Spanish semi hard raw ewe's milk cheese to  
511 about 2 log cfu g<sup>-1</sup>. Enterobacteriaceae are hygiene indicators and are of great concern because they  
512 may include potential pathogenic bacteria responsible for raw milk cheese-related illnesses  
513 (Rampanti et al., 2023a; Tabla et al., 2016). Moreover, Enterobacteriaceae can produce gas whose  
514 presence is considered a defect (e.g., texture with fissures or eyes, or gas within the packaging) (Tabla  
515 et al., 2016).

516 Among Enterobacteriaceae, the occurrence of viable counts of *Escherichia coli* ranging from 3.61 to  
517 3.89 log cfu g<sup>-1</sup> is of concern since this bacterial species represents a hygiene indicator that may

518 potentially include pathogenic serotypes.

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

524 Eumycetes represent an important part of the cheese's microbial population, specifically in artisanal  
525 cheeses, influencing sensory characteristics such as appearance, flavor, aroma, and texture of the  
526 products during the ripening process (Bintsis, 2021; Cardinali et al., 2021, 2022a,b; Fröhlich-Wyder  
527 et al., 2018; Rampanti et al., 2023a,b; Ropars et al., 2012). Yeasts are capable of metabolizing the  
528 lactate produced by lactic acid bacteria and producing NH<sub>3</sub> from amino acids, being responsible for  
529 the rising of the pH on cheese surface (deacidification process) (Fröhlich-Wyder et al., 2018). Yeasts  
530 and molds may also cause cheese spoilage in terms of off-flavour, early blowing, and discolouration  
531 of the cheese (Fröhlich-Wyder et al., 2018). However, no visible alterations were detected in the  
532 *Njeguški* cheeses under study. Yeasts grow well in acidic environments and are salt-tolerant (Bintsis,  
533 2021; Fröhlich-Wyder et al., 2018), thus justifying the high viable counts detected in the *Njeguški*  
534 cheese under study. The yeasts viable counts found in the present study are in line with those detected  
535 in Spanish raw milk semi-hard cheeses reaching up to 7 log cfu g<sup>-1</sup> at the third week of maturation  
536 (Bintsis, 2021). To the author's knowledge, to date, eumycetes community of *Njeguški* cheese has  
537 never been investigated; hence, no data on eumycetes counts are available in the scientific literature  
538 for further comparison of the results. Therefore, the present study represents a significant  
539 advancement in the knowledge of the microbial population occurring in this dairy product.

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

544 The dominance of the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus* was quite expected due  
545 to the high lactic acid bacteria viable counts recorded. Among these, *Lc. lactis* is considered a key  
546 lactic acid bacteria species in dairy products manufacture since it is involved in milk acidification,  
547 the hydrolysis of milk proteins, and the production of aroma compounds such as aldehydes, ketones,  
548 amino acids, and sulphur compounds. Moreover, strains of *Lc. lactis* are able to produce bacteriocins  
549 and exopolisaccharides (EPS), thus improving both the safety and the texture of cheeses (Terzić-  
550 Vidojević et al., 2020). *Lc. lactis* was already isolated from Montenegrin soft (*Bijeli* and *Masni*) and  
551 hard (*Njeguški*) cheeses and several strains have been biochemically characterized and used in  
552 *Njeguški* pilot scale productions (Martinovic et al., 2018; Rasovic et al., 2017). Other studies reported  
553 *Lc. lactis* as a predominant bacterium in other semi-hard cheeses produced with raw sheep's milk  
554 either in the Balkan area such as Croatian *Istrian*, *Krcki*, and *Paski* cheeses (Terzić-Vidojević et al.,  
555 2020) as well as in the Mediterranean area, including Italian artisanal cheeses (Biolcati et al., 2020),  
556 Portuguese cheeses (Cardinali et al., 2021, 2022a,b; Rampanti et al., 2023b), French cheeses as  
557 *Tomme d'Orchies* cheese (Ceugniz et al., 2017) and Savoyard raw milk cheeses (Lecaude et al.,  
558 2024), as well as Spanish *San Simón da Costa* cheese (Terzić-Vidojević et al., 2020).

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

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

570 Alegria et al. (2012) reported the first detection of Bifidobacteriaceae in traditional Polish cheese.  
571 Later, Marino et al. (2017) and Mohamed et al. (2022) found the presence of Bifidobacteriaceae in  
572 Italian brined cheeses and in Egyptian cheeses, respectively, despite the high salinity of such  
573 environments. Indeed, Bifidobacteriaceae cannot survive NaCl concentrations higher than 5%, thus  
574 suggesting the presence of strains adapted to high salinities (Marino et al., 2017; Mohamed et al.,  
575 2022). Of note, the highest frequency of Bifidobacteriaceae has been found in *Njeguški* cheese  
576 samples from producer C which were characterized by the highest salt content, although below 5 %.

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

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



596 % of frequency in *Grana Padano* PDO cheese (da Silva Duarte et al., 2021), and in Portuguese  
597 cheeses as *Queijo de Azeitão* PDO cheese (Cardinali et al., 2021), *Queijo de Nisa* PDO cheese  
598 (Cardinali et al., 2022a), *Queijo de Beira Baixa* PDO cheese (Cardinali et al., 2022b).

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

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

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

618 *K. marxianus* is frequently isolated from dairy products thanks to its ability to metabolize lactose as  
619 carbon sources producing CO<sub>2</sub> and ethanol, whereas it can use lactate after lactose is depleted (Bintsis,  
620 2021; Frölich-Wyder et al., 2018). *K. marxianus* is a respiro-fermentative, fast-growing  
621 thermotolerant species that tolerates low pH values. During maturation, *K. marxianus* strongly

622 influences the final texture and flavor of the cheese due to its proteolytic, lipolytic, and esterase  
623 activity producing esters (fruity aroma) and acetaldehyde (Bintsis, 2021; Frölich-Wyder et al., 2018).  
624 *Galactomyces* spp. is the teleomorphic genus of *Geotrichum* spp.; the filamentous yeast-like  
625 species *Geotrichum candidum* is the only species widely used as a starter culture or adjunct culture  
626 in the dairy industry for the production of felted-looking cheese or cheese ripening (Frölich-Wyder  
627 et al., 2018; Pottier et al., 2008). In the present study, *G. candidum* has not been specifically detected,  
628 whereas the contaminant species *M. capitatus* (synonym *Geotrichum capitatum*) has been found at  
629 about 10 % of the relative frequency in cheeses from producer B. Among the genus *Magnusiomyces*,  
630 *M. capitatus* is the most important clinical species since it is an emerging opportunistic yeast with  
631 thermophilic nature responsible for systemic infections such as fungemia, endocarditis, and  
632 pulmonary infections (Zhu et al., 2022). Moreover, cheeses from producer B were characterized by  
633 high relative frequency of *Wickerhamiella pararugosa* (synonym *Candida pararugosa*) (about 21.50  
634 %), an emerging and rare pathogenic yeast identified from different organs and biological fluids of  
635 humans and animals, responsible for invasive candidemia associated with high morbidity and  
636 mortality mainly in immunocompromised patients (Kumar et al., 2022).  
637 *K. unispora* is a lactose-negative yeast previously found along the ripening process of traditional  
638 Spanish and French semi-hard ewes' and goats' cheeses (Padilla et al., 2014).  
639 *T. delbrueckii* has been reported as the most frequent yeast in *Canastra* cheese, a Brazilian semi-hard  
640 cheese produced from raw cow's milk inoculated with commercial rennet and pingo, which is a  
641 natural starter derived from the cheese whey from the previous day (Bintsis, 2021). However, *T.*  
642 *delbrueckii* is well-known as the most attractive non-*Saccharomyces* yeast species typically  
643 associated with winemaking to produce higher levels of alcohols, ethyl and acetate esters during the  
644 initial steps of the process, compared to *S. cerevisiae*. Thanks to these biochemical properties and its  
645 resistance to osmotic and freezing stresses, *T. delbrueckii* is considered a promising yeast for  
646 biotechnological exploitation in a wide range of industries (Fernandes et al., 2022; Silva-Sousa,  
647 2022).

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

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

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

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

667

#### 668 4.2 Physico-chemical characterization

669

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

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

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

690 Humidity of food is a fundamental parameter that significantly impacts the quality, safety, and shelf-  
691 life of food products. The humidity content of foods strongly influences microbial growth, enzymatic  
692 activity, and chemical reactions. Overall, the humidity content of *Njeguški* cheese samples is in line  
693 with data reviewed by Teneva-Angelova (2018) for the same cheese and corresponding to moisture  
694 values ranging between 41-54 %. In detail, humidity content from producer B is in accordance with  
695 the data reported by Mirecki et al. (2015) for traditional *Njeguški* cheese ripened 40-50 days and  
696 corresponding to  $42.07 \pm 4.28$  %, whereas samples from producer A and C showed higher ( $45.58 \pm$   
697  $3.03$  %) and lower ( $38.37 \pm 1.66$  %) mean values than those reported by Mirecki et al. (2015),  
698 respectively. In particular, the latter samples show moisture content closest to hard cheeses that is  
699 generally below 40 % due to the pressure applied during the manufacturing process aimed at forcing

700 the drainage of the whey (Bintsis 2021; Terzić-Vidojević et al., 2020). Overall, moisture content of  
701 cheeses under study is in accordance with raw ewe's hard and semi-hard cheeses from Greece with  
702 moisture percentages ranging from 37.10 % (*Ladotyri Mytilinis*) to 49.40 % (*Batzos*) (Danezis et al.,  
703 2020).

704 The salt content of the cheeses under study is lower than the data reviewed by Teneva-Angelova  
705 (2018) for the same cheese and ranging from 1.90-2.30 %. In more detail, the average value of cheeses  
706 from producer C ( $1.78 \pm 0.36$  %) was slightly lower than the salt average values of *Njeguški*-type  
707 cheese produced with ewe milk ripened at 60 days ( $2.02 \pm 0.28$  %) (Jokanovic et al., 2022), and those  
708 of traditional *Njeguški* cheese of 40-50 days ripening ( $1.94 \pm 0.56$  %) (Mirecki et al., 2015). On the  
709 other hand, much lower salt content values were found for cheeses from producer B and A showing  
710 values closest to NaCl content of semi-hard raw ewe's milk cheese from Spain ripened 30 days which  
711 was attested at  $1.18 \pm 0.07$  % (Tabla et al., 2016).

712 Dairy products are generally consumed because of their nutritional and high-protein content. Indeed,  
713 beside their functional properties, proteins in cheese are among the main macronutrients necessary  
714 for the human body's metabolism (Diaz-Bustamante et al., 2023). The mean values of the total protein  
715 content of the cheeses under study were in line with the mean value reported by Teneva-Angelova  
716 (2018) and by Mirecki et al. (2015) ranging from 21 to 25 %. In detail, the cheeses from producer C  
717 showed the highest values, with an average value of protein content closest to those reported by  
718 Jokanovic et al. (2022) for *Njeguški*-type cheese produced with ewe milk at 45-60 days of ripening  
719 ( $25.45 \pm 0.79$  %- $25.98 \pm 0.49$  %). The protein content of the *Njeguški* cheeses under study was also  
720 in accordance with that reported by Danezis et al. (2020) in Greek hard raw ewe's milk  
721 *Kefalograviera* cheese that exhibited 24.10 % of protein content.

722 Concerning lipids, the average values of all the cheeses under study were generally in line with the  
723 values reported by Mirecki et al. (2015) for traditional *Njeguški* cheese ripened 40-50 days ( $29.97 \pm$   
724  $3.02$  %) and by Jokanovic et al. (2022) for *Njeguški*-type cheese produced with ewe milk until 60  
725 days of ripening ( $30.24 \pm 3.17$  %). Furthermore, the lipid content of the *Njeguški* cheeses analyzed

726 was slightly higher than those reported by Tabla et al. (2016) for Spanish semi-hard raw ewe's milk  
727 cheese at 30 days of ripening and attested at  $26.80 \pm 0.60$  %, but it falls within the fat value for the  
728 semi-hard *Ladotyri Mytilinis* PDO cheese from Greece attested up to 35.30 % (Danezis et al., 2020).  
729 The lipid amount and composition are parameters that influence the most the nutritional composition  
730 as well as the color, texture and flavor of cheese (Inmaculada González-Martín et al., 2020). Cheese  
731 is particularly rich in saturated fatty acids (SFAs), which include a heterogenous group of fatty acids  
732 that contain only carbon-to-carbon single bonds. SFAs are categorized as short-chain (4-6 carbon  
733 atoms), medium-chain (8-12 carbon atoms), long-chain (14-20 carbon atoms), and very long-chain  
734 (22 or more carbon atoms). Depending on the length of the carbon chains, different effect of SFA on  
735 human health have been shown (Inmaculada González-Martín et al., 2020; Astrup et al., 2020;  
736 Paszczyk and Łuczyńska, 2020). Overall, diets with high concentrations of SFA are associated with  
737 cardiovascular disease, obesity, and cancers (Inmaculada González-Martín et al., 2020; Paszczyk,  
738 2022; Paszczyk & Łuczyńska, 2020). Moreover, for authenticity purposes the chemical composition  
739 and fatty acid profile of traditional cheeses are used as potential tracers. This issue is challenging due  
740 to the influence of multiple factors such as feeding system of the animals, variation in production  
741 methods, different breeds, and others (Danezis et al., 2020; Margalho et al., 2021; Paszczyk, 2022).  
742 No previous data on SFAs content as well as carbohydrates and ash content of *Njeguški* cheese are  
743 available for further comparison with the results.

744

#### 745 *4.3 Biogenic amines content*

746

747 Variable levels of biogenic amines were observed in the cheese samples under study, thus suggesting  
748 the presence of microorganisms with decarboxylation or amination activities. Indeed, biogenic  
749 amines are aliphatic, heterocyclic, or aromatic organic nitrogenous molecules with low molecular  
750 weight deriving from specific amino acids that after the elimination of the  $\alpha$ -carboxyl group give the  
751 corresponding amines: histamine derives from histidine, tyramine from tyrosine, tryptamine from

752 tryptophan, putrescine from ornithine, cadaverine from lysine, and 2-phenylethylamine from  
753 phenylalanine (O'Sullivan et al., 2013). Alternatively, amines as putrescine are also produced through  
754 deamination of agmatine-by-agmatine deaminase in the genera *Enterococcus* and *Lactobacillus*  
755 (O'Sullivan et al., 2013). The production of decarboxylases is primarily associated with  
756 Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae, *Clostridium* spp., and lactic acid bacteria  
757 (O'Sullivan et al., 2013; Schirone et al., 2022), and secondarily to yeasts ascribed to *Yarrowia*  
758 *lipolytica* and *D. hansenii*. The latter play a marginal role in the production of biogenic amines in  
759 semi-hard and hard cheeses. Furthermore, *Y. lipolytica* and *D. hansenii* together with *G. candidum*  
760 have been found able to degrade biogenic amines (Frölich-Wyder et al., 2018).

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

765 These hazardous substances are considered quality markers often associated with several protein-rich  
766 foods as cheese, fish, and dry sausage as well as in wine and beer manufactured under poor or  
767 uncontrolled hygiene conditions (O'Sullivan et al., 2013; Schirone et al., 2013). Specifically, cheese  
768 is correlated with histamine poisoning and tyramine toxicity (Schirone et al., 2013). The EU  
769 Regulation No 2073/2005 establishes histamine limits for fish species associated with a high amount  
770 of histidine between 100 mg kg<sup>-1</sup> and 200 mg kg<sup>-1</sup>, for fishery products subjected to enzyme  
771 maturation treatment in brine between 200 mg kg<sup>-1</sup> and 400 mg kg<sup>-1</sup>, and for fish sauce produced by  
772 fermentation of fishery products at 400 mg kg<sup>-1</sup>, while the US Food and Drug Administration  
773 indicates the limit of histamine at 50 mg kg<sup>-1</sup> as harmful to health (Belleggia et al., 2022; Schirone et  
774 al., 2022). Although there is no regulation on histamine and tyramine content in most foodstuffs,  
775 Schirone et al. (2013; 2022) reported the accepted limit of histamine as 100 mg kg<sup>-1</sup> and of tyramine  
776 as 800 mg kg<sup>-1</sup> in fermented food products. Overall, a great variability of biogenic amines levels has  
777 been found in literature among different types of cheeses, and it is attributed to several factors that

778 influence biogenic amines accumulation in dairy products as the use of starter cultures, milk  
779 pasteurization, microbial quality of raw milk, type of rennet, pH, temperature during maturation and  
780 storage, NaCl concentration, ripening and post-ripening technological process, overall manufacturing  
781 process and the sanitation procedures used (Linares et al., 2012; Schirone et al., 2013; 2022). Of note,  
782 the European Food Safety Authority (EFSA) Opinion (EFSA, 2011) reported a maximum of  
783 histamine and tyramine in hard cheese corresponding to 457 and to 1450 mg kg<sup>-1</sup>, respectively  
784 (Schirone et al., 2022). In the present study, only samples from producer C showed histamine content  
785 higher than 100 mg kg<sup>-1</sup> (Schirone et al., 2013; 2022) but below 457 mg kg<sup>-1</sup> (EFSA, 2011), while  
786 tyramine content was widely below the levels above-mentioned (EFSA, 2011; Schirone et al., 2013;  
787 2022) although a synergistic toxicity of tyramine and histamine on intestinal cell cultures has been  
788 recently reported (Schirone et al., 2022).

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

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

798

#### 799 *4.4 Morpho-textural characterization*

800

801 In the CIE  $L^*a^*b^*$  system, the  $a^*$  parameter is used to indicate the green–red opponent colors (<0  
802 toward green and >0 toward red), while  $b^*$  parameter denote the blue–yellow opponent colors (<0  
803 toward blue and >0 toward yellow) (Rampanti et al., 2024). All the cheese samples were characterized



804 by high lightness with yellow tonalities pronounced. Specifically, a higher yellowness and higher  
805 greenness have been found in samples from producers C that were also characterized by lower  
806 humidity, higher salt, proteins and ash content. Cheese yellowness is generally correlated to  
807 carotenoids, that are fat-soluble pigments ranging from red to yellow, available with variable levels  
808 in milk depending on kind of forages used for cows' and ewes' diets, while the greenish tonalities are  
809 due to the lack of red carotenoids (Cardinali et al., 2022a; Rampanti et al., 2023a). Overall, as reported  
810 by Rampanti et al. (2023b) different factors, such as origin of the milk, lipid content, pasture location,  
811 the amount and type of feed for sheep and cows, the grazing seasons, and ripening process, affect the  
812 color of the cheeses.

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

825

#### 826 *4.5 Volatile organic composition*

827

828 To the authors' knowledge, this is the first study on the characterization of volatile organic  
829 compounds in Montenegrin *Njeguški* cheese. In general, all the cheeses analyzed were characterized

830 by similar classes of volatile organic compounds, although their relative percentages differed. HS-  
831 SPME-GC/MS analysis identified major and minor volatile components; in particular, the volatile  
832 fraction of cheeses was dominated mainly by alcohols, esters and acetates, aldehydes and acids, while  
833 only traces of terpenes, sulfides and aldehydes were found.

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

841 2-Butanone and acetoin, which have a buttermilk, fruity, and ethereal odor, were identified as the  
842 main odorant in the traditional *Beaten (Bieno sirenje)* ewe milk cheese (Sulejmani et al. 2014),  
843 *Cheddar* cheese (Arora et al., 1995) and other raw milk cheeses such as Spanish soft PDO *Torta del*  
844 *Casar* (Delgado et al., 2010), highlighting that they have an important role in the flavour profile of  
845 these raw milk cheeses. In contrast, 2-heptanone, which has an herbaceous, sweet, and spicy odor,  
846 has been found to be an influential volatile compound in *Emmental* and *Gorgonzola* cheeses (Curioni  
847 et al., 2002).

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

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

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

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

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

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

882

## 883 **5. Conclusion**

884

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

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

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

900

## 901 **CRedit authorship contribution statement**

902

903 **Federica Cardinali:** Formal analysis, Investigation, Writing – review & editing. **Giorgia Rampanti:**  
904 Formal analysis, Investigation, Writing – original draft. **Giuseppe Paderni:** Investigation. **Vesna**  
905 **Milanović:** Investigation. **Ilario Ferrocino:** Formal analysis, Investigation. **Anna Reale:** Formal

906 analysis, Investigation. **Floriana Boscaino:** Formal analysis, Investigation. **Nadja Raicevic:**  
907 Investigation. **Maša Ilincic:** Investigation, Writing – original draft. **Andrea Osimani:** Writing –  
908 original draft, Writing – review & editing. **Lucia Aquilanti:** Writing – review & editing. **Aleksandra**  
909 **Martinovic:** Funding acquisition, Resources, Writing – review & editing. **Cristiana Garofalo:**  
910 Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing –  
911 original draft, Writing – review & editing.

912

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914

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921

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1203 **FIGURE CAPTIONS**

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1205 **Figure 1.** Principal coordinate analysis (PCoA) based on weighted unifrac distance matrix for microbiota (a)  
1206 and mycobiota (b). Samples are color-coded according to the producer.

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1208 **Figure 2.** Panel a) Circular ideogram showing the bacterial biota distribution (>1 % of the relative frequency  
1209 in at least 2 samples) among *Njeguški* cheese samples. ASVs and samples are connected with a ribbon, and its  
1210 thickness is proportional to the abundance of an ASV in the connected sample. The outer circle displays the  
1211 proportion of each ASV in a given sample and vice versa. Panel b) Boxplots showing the differences in relative  
1212 abundance of ASVs based on ANOVA test ( $p < 0.05$ ) in cheese samples among the three producers for bacterial  
1213 biota. Statistically significant differences are indicated by different letters on top of each graph.

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1215 **Figure 3.** Panel a) Circular ideogram showing the fungal biota distribution (>1 % of the relative frequency in  
1216 at least 2 samples) among *Njeguški* cheese samples. ASVs and samples are connected with a ribbon, and its  
1217 thickness is proportional to the abundance of an ASV in the connected sample. The outer circle displays the  
1218 proportion of each ASV in a given sample and vice versa. Panel b) Boxplots showing the differences in relative  
1219 abundance of ASVs based on ANOVA test ( $p < 0.05$ ) in cheese samples among the three producers for fungal  
1220 biota. Statistically significant differences are indicated by different letters on top of each graph.

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1222 **Figure 4.** Principal Component Analysis (PCA) of biogenic amines in *Njeguški* cheese from three different  
1223 batches (I, II, III) and producers (A, B, C).

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1225 CAD, cadaverine; PHE, 2-phenylethylamine; HIS, histamine; PUT, putrescine; SPD, spermidine; TYR,  
1226 tyramine; TRP, tryptamine.

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1228 **Figure 5.** Principal Component Analysis (PCA) of volatile compounds in *Njeguški* cheese from three different  
1229 batches (I, II, III) and producers (A, B, C).