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TITLE PAGE
- Food Science of Animal Resources -
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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title	Highlighting the microbial community of Kuflu cheese, an artisanal Turkish mold-ripened variety, by high-throughput sequencing
Running Title (within 10 words)	Bacteria, yeast and filamentous mold community of Kuflu cheese
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Conflicts of interest List any present or potential conflicts of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	The author is grateful to Selcuk University Scientific Research Projects Coordinatorship for their support to the study with the 21401100-project number.
Author contributions (This field may be published.)	Conceptualization: Demirci T Data curation: Demirci T Formal analysis: Demirci T Methodology: Demirci T Software: Demirci T Validation: Demirci T Investigation: Demirci T Writing - original draft: Demirci T Writing - review & editing: Demirci T
Ethics approval (IRB/IACUC) (This field may be published.)	This article does not require IRB/IACUC approval because there are no human and animal participants.

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Abstract

9
10 Kuflu cheese, a popular variety of traditional Turkish mold-ripened cheeses, is
11 characterized by its semi-hard texture and blue-green color. It is important to elucidate
12 the microbiota of Kuflu cheese produced from raw milk to standardize and sustain its
13 sensory properties. This study aimed to examine the bacteria, yeasts, and filamentous
14 mold communities in Kuflu cheese using high-throughput amplicon sequencing based
15 on 16S and ITS2 regions. *Lactococcus*, *Streptococcus*, and *Staphylococcus* were the
16 most dominant bacterial genera while *Bifidobacterium* genus was found to be
17 remarkably high in some Kuflu cheese samples. *Penicillium* genus dominated the
18 filamentous mold biota while the yeasts with the highest relative abundances were
19 detected as *Debaryomyces*, *Pichia*, and *Candida*. The genera *Virgibacillus* and
20 *Paraliobacillus*, which were not previously reported for mold-ripened cheeses, were
21 detected at high relative abundances in some Kuflu cheese samples. None of the genera
22 that include important food pathogens like *Salmonella*, *Campylobacter*, *Listeria* were
23 detected in the samples. This is the first experiment in which the microbiota of Kuflu
24 cheeses were evaluated with a metagenomic approach. This study provided an
25 opportunity to evaluate Kuflu cheese, which was previously examined for fungal
26 composition, in terms of both pathogenic and beneficial bacteria.

27 Keywords: Blue-green cheese; mold-ripened; bacteriome; mycobiota; metagenomic

28 Introduction

29 Artisanal cheeses are passed on from one generation to another and produced in
30 a particular way that is unique to a certain local area, region, or country with little or no
31 processing. They are generally known for their diverse microbiota, potent sensory
32 properties, small risks, and some benefits. There are various question marks about
33 artisanal cheeses considering their exposure to incomplete heat-treatment and their
34 being made without a starter culture and their ambiguous microbial composition, all of
35 which might result in a potential for high pathogen risks. Aside from all the pros and
36 cons, demand for artisanal cheese increases progressively and traditional manufacturers
37 try to adapt their production conditions and methods to vat milk with each passing day.
38 In this aspect, determining the microbial community of artisanal cheeses is the key to
39 enabling a safer and more beneficial cheese production by preserving the manufacturing
40 practices, characteristic flavor, aroma, and texture of the product for many years. Hence,
41 numerous studies have been executed on microbial diversity and dynamics of artisanal
42 cheeses and possible microbial interactions during ripening over the last few years
43 (Demirci et al., 2021; Dimov et al., 2021; Dugat-Bony et al., 2016; Onmaz et al., 2021).
44 As a result of this interest, novel molecular techniques, such as 16S/ITS-based high-
45 throughput sequencing, are frequently used to detect the microbiota of regional cheeses
46 and their ripening roles worldwide (Dimov et al., 2021; Murugesan et al., 2018).

47 In Turkey, there are over 150 cheese varieties displaying an amazing diversity
48 of organoleptic and textural properties. While most of them are produced in small
49 quantities to meet local cheese requirements, some of them are commercialized
50 throughout the country such as Ezine cheese, Tulum cheese, and Kuflu cheese. Kuflu
51 cheese is a mold-ripened variety which is consumed in many rural and urban areas of
52 Turkey. Despite the lack of clear historical records, Kuflu cheese manufacturing has a

53 long story in Turkey and is the most typical artisanal cheese in the Central Anatolia
54 Region (mainly in Konya, Karaman provinces). Such cheeses are made from skimmed
55 or semi-skimmed unpasteurized ewe's milk or its mixture with goat's and cow's milk in
56 small quantities without any starter microorganisms, and these cheeses are usually
57 characterized by blue-green molds that spontaneously grow on their surface.

58 With regards to the bacterial or fungal composition of artisanal Kuflu cheese or
59 other mold-ripened Turkish varieties, some researchers have executed important studies
60 using culture-dependent methods (Seri and Metin, 2021) and culturomics (Onmaz et al.,
61 2021), however, to the best of our knowledge there is no metagenomic study of the
62 microbiota of such cheese. As a matter of fact, metagenomic next generation sequencing
63 has a superior capability to characterize the conserved and variable regions of the
64 bacterial 16S or eukaryotic rRNA genes for the purpose of taxonomic classification.
65 Normally, both bacteria and mold starter cultures are used to produce mold-ripened
66 cheeses, otherwise, limited contamination of the microbiota is expected. However, the
67 Kuflu cheeses examined in this study were traditionally produced under completely
68 uncontrolled conditions. It is thought that the detection of pathogenic bacteria, intestinal
69 commensals, and various contaminants is important for the food safety of Kuflu cheese,
70 as well as an important literature information for artisanal mold-ripened cheeses. The
71 determination of beneficial bacteria and yeasts is also valuable in terms of the health
72 benefits that can be attributed to this type of cheese. For this reason, the cheeses were
73 not produced by us and random Kuflu cheese samples that have a controversial place in
74 terms of consumers were obtained from the bazaars. In this manner, it is essential to
75 take metagenomic approaches to characterize the bacterial, yeast, and filamentous mold
76 diversities and discuss their presence in Kuflu cheeses. In the present study, both
77 bacterial and fungal biota in ten artisanal mold-ripened Kuflu cheese samples produced

78 in Konya (Turkey) were characterized by high-throughput 16S DNA and ITS
79 sequencing. The richness and distribution uniformity of both bacterial and fungal
80 communities identified in Kuflu cheeses were determined by alpha diversity indices
81 (Shannon, Simpson) and similarity/dissimilarity of microbial communities of cheeses
82 was also detected by beta diversity indices (PCoA).

83

84 Materials and Methods

85 Kuflu cheese samples

86 A total of ten homemade artisanal Kuflu cheeses were supplied from ten
87 different local producers of Konya province. From these samples, the first Kuflu cheese
88 (MC1) was made from only raw cow's milk, the second (MC2) from only raw goat's
89 milk and all the other cheeses were produced from the mixture of raw ewe's (about
90 20%) and raw cow's milk (about 80%). The samples were collected in October 2021
91 and cheese producers declared that they put these cheeses for sale after they have
92 matured for 3-4 months. Cheese samples were quickly transferred to the laboratory and
93 kept at -20 °C until DNA extraction. The images of Kuflu cheeses analyzed in this work
94 are presented in Fig. 1.

95 Proximate analyses of Kuflu cheeses

96 The pH values of cheese samples were analyzed by a calibrated pH-meter
97 (Starter 3100 model, Ohaus). The titratable acidity of samples was determined
98 according to the titration method using 0.1 N NaOH (AOAC, 2016) and expressed as g
99 lactic acid/100 g cheese. As for fat content, it was analyzed by Gerber method (Kleyn et
100 al., 2019). Salt contents of samples were detected using the AgNO₃ titration method
101 suggested by Hayaloglu et al. (2005) and the results were provided as the salt content

102 (%) in the dry matter. In addition to these, moisture content was detected by the
103 gravimetric method (AOAC, 2016).

104 DNA extractions from cheese samples

105 For extraction of total DNA directly from Kuflu cheese samples, the
106 GeneMATRIX Bacterial DNA Purification Kit (EURX, Molecular Biology Products)
107 was utilized based on the kit manufacturer's instructions. Samples were taken from
108 different points of the cheeses to represent the whole cheese.

109 Bacterial 16S rRNA gene amplification and sequencing

110 The V3-V4 hyper-variable regions of the 16S rRNA gene were used for the
111 examination of bacterial composition in Kuflu cheese samples (Demirci et al., 2022).
112 (5'): TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN
113 GGC WGC AG and (5'): GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA
114 GGA CTA CHV GGG TAT CTA ATC C were used as forward and reverse primers,
115 respectively. After amplification with specific primers, purification was performed. At
116 the index PCR step, the Nextera XT index kit (FC-131-1001/FC-131-1002) specific to
117 Illumina technology was used. Library preparation and sequencing were performed
118 using the NovaSeq Reagent kit according to the Illumina 16S metagenomic sequencing
119 library protocol based on the yielding 2×250-bp (paired-end reads).

120 Fungal ITS gene amplification and sequencing

121 Fungi community was characterized by sequencing the ITS2 regions using
122 specific ITS2 forward primer (5'): TCG TCG GCA GCG TCA GAT GTG TAT AAG
123 AGA CAG GCA TCG ATG AAG AAC GCA GC and reverse primer (5'): GTC TCG
124 TGG GCT CGG AGA TGT GTA TAA GAG ACA GTC CTC CGC TTA TTG ATA
125 TGC. For this purpose, 15 ng of genomic DNA was employed. The ITS2 gene was

126 amplified with specific primers. Indexing was constructed in the same way as for the
127 16S rRNA gene. AMPure beads (Beckman Coulter Genomics) were used for the
128 purification of the resulting libraries. For a more sensitive determination of library
129 quantification, Qubit™ 4 fluorometer was used. Library preparation and sequencing
130 were performed using the NovaSeq Reagent kit according to the Illumina ITS2
131 metagenomic sequencing library protocol (yielding 2×250-bp) on Illumina NovaSeq
132 6000.

133 Bioinformatic data analysis

134 After sequencing, the obtained data were converted to FASTA format for
135 bioinformatic analysis. At first base, reads, primer, and barcodes with Phred score lower
136 than 20 were trimmed. QIIME 1.9.1 (Caporaso et al., 2010), with a pipeline reported by
137 Berni Canani et al. (2017). After the evaluation of the read quality, similar sequences
138 were clustered to detect the taxonomy of each sample. The Operational Taxonomic
139 Units (OTU) were identified. OTUs representing less than 0.001% were excluded from
140 the evaluation. To understand the microbial composition in the samples, the identified
141 microorganisms were converted to percentages in each sample. The major OTUs in
142 phylum- and genus-level were filtered at 1% and the stacked column charts were
143 constructed using MS Excel. Shannon's diversity and Simpson's diversity indexes were
144 used to calculate rarefaction curves (Willis, 2019). The principal coordinate analysis
145 (PCoA) was performed with the QIIME2 Emperor using the Bray-Curtis distance
146 matrix to demonstrate the variation in microbial communities among the Kuflu cheese
147 samples. Additionally, a Venn diagram was created to observe the differences of the
148 identified bacterial and fungal genera in Kuflu cheese samples depending on the type of
149 milk used in production. To establish the correlation between some parameters

150 Pearson's correlation coefficients were calculated using R software with the corrplot
151 package (<https://www.r-project.org>).

152 BioProjects for metagenomic data

153 The raw metagenome sequence data of bacterial and fungal profiles of Konya
154 Kuflu cheese samples was added to the Sequence Read Archive (SRA) division of the
155 National Centre for Biotechnology Information (NCBI) with the accession number of
156 BioProject PRJNA993662 and BioProject PRJNA993704, respectively.

157

158 Results

159 pH, TA, and chemical composition of Kuflu cheeses

160 The TA, pH, moisture, salt in dry matter, and fat-in-dry matter of Kuflu cheese
161 samples are shown in Table 1. The pH values ranged from 5.17 (MC10) to 6.69 (MC6)
162 with an average value of 5.79. The TA values were found between 1.37 (MC6) and 2.81
163 g lactic acid/100 g cheese (MC10). The moisture content of the experimental Kuflu
164 cheeses showed a high variability (in the range of 39.9-56.7%). Turkish Standards
165 Institute (TSI) reported that the salt content of mold-ripened cheeses could be at most
166 5% in dry matter, but it was determined that one of the experimental cheeses (MC8,
167 5.21%) had a salt content outside of this standard. In the MC5, MC2 and MC8 samples
168 more fat in DM was detected than all other Kuflu cheeses, respectively 16.5, 25, and
169 27.5%.

170 Sequences

171 In this study, we used two hyper-variable regions (V3 and V4) of the bacterial
172 16S rRNA and the number of clean reads obtained per sample ranged from 69,639 to

173 83,243 with an average length of 250 bases for Kuflu cheese samples. Also, ITS2 region
174 was used for fungal microbiota and the number of clean reads obtained per samples
175 ranged from 76,706 to 98,674 with an average length of 250 bases.

176 The bacterial phyla and genus abundance of Kuflu cheese by HTS

177 Fig. 2A indicates the relative abundances of bacteria in ten Kuflu cheeses at the
178 phylum level. The predominant taxa at the phylum level were *Firmicutes* (63.89-
179 83.32%), *Bacteroidetes* (2.24-21.44%), *Proteobacteria* (2.28-17.80%), and
180 *Actinobacteria* (which together accounted for more than 99.5% of the total relative
181 bacterial abundance) in studied Kuflu cheese samples. Minor phylum which had low
182 abundances with sequence frequencies of <0.5% included *Verrucomicrobia*,
183 *Fusobacteria*, *Deinococcus-thermus*, respectively. Although all cheese samples showed
184 a similar bacterial diversity at the phylum level, the lowest *Firmicutes* abundances
185 (63.89, 64.18, 68.78%) were observed in the MC7, MC9, and MC2 samples,
186 respectively, whilst the highest *Bacteroidetes* abundances were in MC2 and MC10
187 samples (21.44 and 12.22%, respectively) and the highest *Proteobacteria* abundances
188 were in MC7 and MC1 cheeses with 17.80 and 17.61%, respectively. Furthermore, the
189 MC9 (15.22%), MC3 (14.06%), and MC7 (11.57%) cheeses contained greater
190 proportions of *Actinobacteria* compared to the other samples while MC1 and MC10
191 samples had lower *Actinobacteria* phylum with 1.07 and 1.00% relative abundances,
192 respectively.

193 At the genus level, a total of 28 different genera were determined for 10 Kuflu
194 cheese samples (Fig. 2B). Among these bacteria, *Lactococcus* genus dominated the
195 microbiota of 5 cheese samples with relative abundances ranging from 29.18% (MC7)
196 to 52.4 % (MC10). *Streptococcus* genus showed dominance in 2 cheeses containing

197 MC2 (51.03%) and MC5 (36.22%) samples, also *Staphylococcus* spp. were the highest
198 genus in MC6 (26.03%) and MC8 (29.42%) cheeses. MC8 cheese showed a dissimilar
199 bacterial proportion at the genus level, particularly with the highest abundance of
200 *Paraliobacillus* (24.56%), *Brevibacterium* (8.23%), *Halomonas* (5.68%), and
201 *Virgibacillus* (4.71%). As for the genus *Virgibacillus*, it was also present at a high
202 relative abundance of 10.96% in MC6 samples. On the other hand, *Chryseobacterium*
203 was relatively abundant in MC2 (18.75%) and MC10 (11.05%) cheeses among all
204 studied samples. A large number of bacterial OTUs such as those affiliated with
205 *Bifidobacterium*, *Psychrobacter*, *Escherichia*, *Prevotella*, and *Acinetobacter* genera was
206 commonly detected in the Kuflu cheese samples.

207 The yeast and filamentous mold phyla and genera abundances of Kuflu cheese samples
208 by HTS

209 The taxonomic distribution of fungal biota at phylum level is shown in Fig. 3A.
210 No important variation was reported for fungi diversity of ten Kuflu cheese samples and
211 only two phyla were observed affiliated with *Ascomycota* and *Basidiomycota*. It was
212 found that *Ascomycota* potently dominated the mycobiota of all cheese samples ranging
213 from 97.11% (MC3) to 99.85% (MC7). *Basidiomycota* was detected relatively high in
214 only two cheese samples (2.55% in MC3 and 1.09% in MC6), and for most cases, their
215 prevalence was never >0.2%. Regarding fungi communities at the genus level,
216 *Penicillium* dominated nine cheese samples with relative abundances ranging from
217 65.38 % (MC1) to 91.46 % (MC10) and in only one cheese sample (MC2) it was the
218 second prevalent genus (42.42%) after the *Debaryomyces* genus (Fig. 3B). *Pichia* genus
219 was more abundantly present in MC7 (30.28%) and MC1 (17.37%) cheeses than in all
220 the other cheeses while its relative abundance showed differences ranging from 0.16%

221 (MC8) to 7.17% (MC10) except for MC2 (not detected). As for *Debaryomyces* genus,
222 MC2 cheese sample displayed higher abundances (51.03%). The genus *Candida* was
223 the third prevalent genus in MC1 cheese with 14.08% relative abundance while it was
224 the second in MC4 (10.99%) and MC9 (10.32%). *Kluyveromyces* (between 0.04% and
225 1.78% in all cheeses), *Saccharomyces* (between 0.03% and 0.57% in 7 cheeses),
226 *Fusarium* (<0.1% in 6 cheeses) constituted the other fungi groups of Kuflu cheese
227 samples, and *Aspergillus* genus, one of the important filamentous mold groups, was
228 observed in only MC8 cheese (0.03%).

229 Dissimilarity and co-occurrence analyses of bacterial and fungal microbiota

230 In the alpha diversity analysis, the Shannon and Simpson indices were
231 calculated to estimate diversity and richness of the Kuflu cheese samples (Fig. 4A, B, C,
232 D). Alpha diversity indices reveal the structure of a bacterial community in terms of
233 richness (number of OTU), evenness (distribution of abundances) or both. At this point,
234 higher values of Shannon index indicate a greater genus diversity while lower Simpson
235 indices show that a specific genera is dominant (Demirci et al., 2022). The greatest
236 bacteria richness by Shannon index was detected in MC1 cheese sample (with a total
237 102 OTU-genera) while MC4 cheese presented the lowest Shannon index (with total 49
238 OTU-genera). As for Simpson index, MC3 cheese had the closest value to 0 (with
239 46.82% relative abundance for the most prevalent genus, which was *Lactococcus*)
240 whereas MC1 cheese was located at the farthest point (with 11.11% relative abundance
241 for the most predominant genera). Considering the fungal biota richness taken from
242 Shannon index, MC6 cheese sample harbored a significantly greater richness and
243 diversity with total of 35 different fungi OTUs. MC10 cheese had a very high
244 dominance of one genus with a high proportion of sequences affiliated with *Penicillium*
245 (91.46% of total OTU-genera) with the lowest Simpson index. To compare the

246 similarities in microbiome compositions of each cheese sample, the beta diversity
247 analysis was calculated and presented in the form of principal coordinate analysis
248 (PCoA) using Bray-Curtis dissimilarity indexes (Fig. 5). While the MC2 cheese sample
249 differs from other cheeses in terms of bacterial community, MC3 cheese differs from
250 the others in terms of fungal composition.

251 Venn diagrams were created to show the differences between the bacterial and
252 fungal genera (>1% relative abundance) identified in the Kuflu cheeses produced from
253 different types of milk (Fig. 6). A greater diversity in bacterial (Fig. 6A) and fungal
254 (Fig. 6B) genera was observed in Kuflu cheeses produced using both sheep and cows
255 according to the Venn chart. It was noted that the contribution of cow milk was the
256 lowest in the diversity of microbial genera. Regarding bacterial genera, *Lactobacillus*,
257 *Lactococcus*, *Streptococcus*, *Enterococcus*, *Chryseobacterium*, and *Prevotella* were
258 common genera in all cheese varieties (Fig. 6A). As for fungal genera, *Penicillium*,
259 *Candida*, and *Debaryomyces* genera were determined as joint groups (Fig. 6B). Kuflu
260 cheeses, produced with a mixture of sheep-cow milk, contained 11 more bacterial
261 genera and 6 more fungal genera. These differences reflect differences in the microbiota
262 of the milk types used in the production (Butts et al., 2021).

263 Correlation between the dominant microorganisms and pH, TA, and chemical
264 composition

265 Correlation analysis between the relative abundance of dominant bacteria and
266 fungal flora and pH, TA, and some chemical properties of Kuflu cheese samples was
267 performed and significant correlations ($p<0.05$) were displayed in Fig 7. The relative
268 abundance of *Lactobacillus* genera was significantly positively correlated ($p<0.05$) with
269 *Escherichia* and *Candida* spp. in Kuflu cheeses whilst *Streptococcus* genus was
270 positively correlated with *Debaryomyces*. On the other hand, the abundance of

271 *Staphylococcus* genus was significantly positively correlated ($p<0.05$) with
272 *Virgibacillus* and *Paraliobacillus* abundances, whereas it was negatively correlated with
273 *Chryseobacterium*. The relative abundance of *Penicillium* was also positively correlated
274 ($p<0.05$) with *Debaryomyces* genera. Again, fat in dry matter was significantly
275 positively correlated ($p<0.05$) with *Paraliobacillus* and *Debaryomyces* while moisture
276 was negatively correlated with *Chryseobacterium* and *Debaryomyces*.

277 Discussion

278 Wide ranges in pH, TA, and chemical composition were determined in Kuflu
279 cheeses. The pH values ranged from 5.17 (MC10) to 6.69 (MC6) with an average value
280 of 5.79. The average pH value was found to be lower than that previously determined
281 by Hayaloglu and Kirbag (2007) who studied on Kuflu cheese. Also, Hayaloglu et al.
282 (2008) stated that the measured pH values were between 5.52 and 7.22, while the
283 average value was 6.29. The differences in the regions where the cheeses are produced
284 and in the raw materials and the differences arising from the manufacturer and
285 production method may be the reason for this discrepancy. The average TA of Kuflu
286 cheese samples (2.32 lactic acid/100 g) were higher than that reported by Hayaloglu et
287 al. (2008) in line with pH results. The moisture content of the Kuflu cheeses showed a
288 high variability (in the range of 39.9-56.7%). Similar results were reported by
289 Hayaloglu and Kirbag (2007) in Kuflu cheeses (37.65-53.65%). TSI has been reported
290 that the moisture content cannot be more than 45% in mold-ripened cheeses. From this
291 point of view, it was determined that 9 out of 10 cheeses analyzed contained more
292 moisture content than the value specified in the standard. Besides, TSI reported that the
293 salt content of mold-ripened cheeses could be at most 5% in dry matter, but it was
294 determined that MC8 cheese had a high salt content (5.21%) outside of this standard. In
295 the MC5, MC2, and MC8 samples more fat in DM was detected than all other Kuflu

296 cheeses, respectively 16.5, 25, and 27.5%, however, average fat in DM value (11.8%)
297 was similar to the study conducted by Hayaloglu et al. (2008) who reported that the
298 average fat in DM value was 12.18%. In fact, there can be serious discrepancies in the
299 chemical compositions of such cheeses, which are traditionally produced under
300 uncontrolled conditions, even in those manufactured in the same location. Indeed,
301 Sengul et al. (2006) found very different results in the chemical composition of mold-
302 ripened Civil cheeses and attributed this to the differences that may occur in the
303 manufacturing procedures and ripening conditions of traditional raw milk cheeses.

304 In the present study, at phylum level, *Firmicutes* phylum showed dominance in
305 all cheese samples with relative abundances ranging from 63.89% (MC7) to 83.32%
306 (MC4) followed by *Bacteroidetes* with 2.24-21.44% (MC8 and MC2, respectively) and
307 *Proteobacteria* with 2.28-17.61% (MC3 and MC1, respectively). Apart from these, the
308 *Actinobacteria* constituted one of the important phyla in Kuflu cheese samples with
309 1.00-14.06% (MC10 and MC3, respectively). In fact, several previous studies revealed
310 that *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* were predominating
311 phyla in cheeses and also in ripened cheeses (Dugat-Bony et al., 2016; Irlinger et al.,
312 2015), however, Unno et al. (2021) stated that bacterial biota of three types of surface
313 mold-ripened cheeses (Brie de Meaux, Brie de Melun, and Coulommiers) were
314 dominated by *Firmicutes*, followed by *Proteobacteria*, *Actinobacteria*, but not
315 *Bacteroidetes*.

316 At the genus level, *Lactococcus*, *Streptococcus*, *Lactobacillus*, and
317 *Staphylococcus* genera in general constituted the top four genera relative abundance
318 with more than 50% -except for MC8- in Kuflu cheese samples, however, in some
319 cheeses, *Chryseobacterium* (in MC10), *Bifidobacterium* (in MC3, MC7, and MC9),
320 *Paraliobacillus* (in MC8), *Virgibacillus* (in MC6), *Leuconostoc* (MC3), and

321 *Brevibacterium* (MC8) genera replaced one of them. Besides that, MC6 and MC8
322 cheese samples had a distinctive bacterial genera composition unlike the other cheeses
323 because of the high prevalence of *Virgibacillus* (10.96%), *Psycrobacter* (8.65%),
324 *Paraliobacillus* (3.17%) and *Paraliobacillus* (24.16%), *Brevibacterium* (8.23%),
325 *Halomonas* (5.68%), *Virgibacillus* (4.71%), respectively. Regarding the taxonomic
326 distribution of bacteria at genus level, *Streptococcus* spp. and *Lactococcus* spp., which
327 are known as starter LAB, were the most dominant genera except for MC6 and MC8
328 cheeses which were dominated by staphylococci. This finding was not unexpected since
329 *Streptococcus* spp. (mainly *S. thermophilus*) and *Lactococcus* spp. (mainly *L. lactis*)
330 were previously identified among the most widespread and dominant genera in the
331 untreated and ripened cheese microbiota with *Leuconostoc* spp., *Lactiplantibacillus*
332 spp., and *Enterococcus* spp. by Montel et al. (2014). On the other hand, the genus
333 *Lactobacillus* was found to be one of the most prevalent three genera in 9 out of the 10
334 Kuflu cheese samples with relative abundances changing between 9.07-28.51% and it
335 took the first and second places in MC1 (28.51%) and MC4 (25.59%) samples,
336 respectively. Lactobacilli species -especially *L. delbrueckii* subsp. *bulgaricus*- was used
337 as a starter in blue-veined cheeses whilst facultatively heterofermentative species of the
338 lactobacilli can be found in several mold-ripened and blue cheeses during ripening
339 (Desmaures, 2014).

340 One of the most prevalent non-LAB detected in Kuflu cheeses was
341 *Staphylococcus* genus which was found in MC6 (26.03%) and MC8 (29.42%) cheese
342 samples with the highest presence amongst bacterial OTUs and again was present in
343 MC5 and MC1 samples with 14.48% and 9.24% relative abundances, respectively.
344 Many studies on fermented-food microbiota have reported that many coagulase-
345 negative staphylococci (CNS) are part of the indigenous microbiota which are

346 originated from raw materials and food environment, and they are known to be food-
347 related beneficial microbes. The presence of this genus at such high levels in mold
348 ripened Kuflu cheeses may be due to the competitive characteristics attributed to many
349 staphylococci owing to their capability to easily grow anaerobically at low water
350 activity, in high concentration of NaCl, and at low temperatures. On the other hand, the
351 genus *Bifidobacterium* was detected with increasing abundances in three cheese samples
352 including MC7, MC3, and MC9 with 11.24%, 13.80%, and 14.65%, respectively.
353 Additionally, this genus presented small but noticeable amounts in MC4 and MC5
354 cheese samples with 2.23% and 2.48%. Previously, this genus with probiotic potential
355 was detected in cheeses made with similar production methods, such as Camembert,
356 Brie, and Reblochon cheeses (Bondue et al., 2020). To the best of our knowledge, there
357 is no report on the presence of *Bifidobacterium* in Turkish mold-ripened cheeses.

358 Surprisingly, the presence of the *Paraliobacillus* (24.56%) in MC8 cheese,
359 which was marginally detected in other cheese samples except for MC6 (3.17%), was
360 remarkably high. *Paraliobacillus* species have not been detected in any cheese before.
361 In fact, the most striking feature of this group bacteria, which was detected at a high
362 prevalence in only one Kuflu cheese sample, is that it needs to have high pH (pH 8) and
363 NaCl (5%) concentrations for optimum growth (Chen et al., 2009). Indeed, the Kuflu
364 cheese sample in which *Paraliobacillus* genus was detected very high (MC8), was the
365 cheese sample with the highest NaCl in dry content among all samples (Table 1).
366 *Chryseobacterium* genus was also detected in Kuflu cheese samples. This genera was
367 reported in dairy products by previous researchers, for instance homemade yogurts
368 (Demirci et al., 2022), Halloumi cheese (Kamilari et al., 2020), but it was not detected
369 in Tulum or Kuflu cheeses in any study. Lastly, *Chryseobacterium* genus with a high

370 relative abundance caused MC2 and MC10 cheeses to be distinguished from the other
371 Kuflu cheese samples in terms of sensorial properties (Fig. 1).

372 With regard to fungi, at the phylum level, members of the *Ascomycota* heavily
373 dominated all of the Kuflu cheese samples with relative abundances ranging from
374 97.11% to 99.85% corroborating the results already given by Dimov et al. (2021) who
375 demonstrated that almost all fungi (99.9%) detected in Bulgarian green cheeses
376 belonged to *Ascomycota* phylum. Nonetheless, in this present study, MC3 and MC6
377 samples differed from the other cheeses with a relatively high occurrence of
378 *Basidiomycota* (2.55% in MC3 and 1.09% in MC6) which were mainly represented by
379 the genera *Panaeolus* (1.3 % in MC3) and *Trichosporon* (1.23% in MC3 and 0.95% in
380 MC6). Although *Panaeolus* genus is an unexpected contaminant in cheese,
381 *Trichosporon* has been detected in cheese before (Gelen and Ceylan, 2021).

382 Regarding the taxonomic distribution of fungi at genus level, members of the
383 *Penicillium* genus were determined with increasing relative abundances in 9 out of the
384 10 Kuflu cheese samples ranging from 65.38% to 91.46% (in MC1 and MC10,
385 respectively). These findings regarding the high abundances of *Penicillium* in mold-
386 ripened Kuflu cheeses were confirmed by a recent study of Onmaz et al. (2021) in
387 which the microbiota of mold-ripened Turkish cheeses. Indeed, this is an expected
388 result since *Penicillium* spp. are known emblematic of blue-veined cheese varieties and
389 they can be inoculated as a starter culture during cheese making process such as in
390 Roquefort, Stilton, and Gorgonzola as well as appearing as the dominant filamentous
391 mold of the environment in spontaneously molded cheeses (Desmasures, 2014; Montel
392 et al., 2014). Differently, fungal community of MC2 cheese sample was dominated by
393 *Debaryomyces* genus with 51.03% relative abundance followed by *Penicillium* genus
394 (42.4%). Also, *Debaryomyces* genus was the second most prevalent fungi at genus level

395 in MC8 and MC6 cheeses with 22.38% and 12.94% relative abundance, respectively.
396 This may be attributed to the high fat content of MC2 and MC8 in dry matter, since
397 *Debaryomyces* genus is known to be lipophilic (Song et al., 2022). It was previously
398 reported that they were among the most important yeast genera for several cheese
399 varieties, for example, Yildiz et al. (2021) showed that *Debaryomyces* genus –mostly *D.*
400 *hansenii*- was the most dominant in moldy Civil cheese followed by *Pichia* and
401 *Candida*. On the other hand, many functional/probiotic properties are attributed to
402 relevant yeast species such as antimicrobial killer toxin production, binding mycotoxin
403 etc. (Esen and Çetin, 2021), therefore, the high rates of this genus in Kuflu cheeses are
404 important in terms of the health benefits provided to the consumers of this cheese.
405 Furthermore, two Kuflu cheese samples (MC7 and MC1) were characterized by an
406 elevated representation of the *Pichia* genus with 30.28% and 17.37% relative
407 abundances, respectively. Previous studies have reported that this yeast was one of the
408 most prevalent genus in Bulgarian green cheeses (Dimov et al., 2021), traditional
409 Turkish moldy Civil cheeses (Yildiz et al., 2021) and blue cheese varieties such as
410 Roquefort, Cabrales, and Gorgonzola (Desmaures, 2014; Dimov et al., 2021; Yildiz et
411 al., 2021).

412 As for *Candida* genus, it was found in all Kuflu cheese samples except for MC8
413 and MC10 and was amongst the most prevalent three yeast OTUs in MC1 (14.08%),
414 MC4 (10.99%), and MC9 (10.32%). These are not surprising results, because this genus
415 was reported to be one of the most common yeasts in several cheese varieties such as
416 Tilsit, Reblochon, Surk, moldy Civil, Roquefort, Gorgonzola, Danablu, Bleu
417 d'avuergne, Bleu de Bresse, and Valdeon cheeses (De Boer and Kuik, 1987).
418 Surprisingly, one of the most remarkable findings of this study is the fact that
419 *Aspergillus* genus, commonly associated with allergic bronchopulmonary aspergillosis

420 producing potential, were not detected in any of the Kuflu cheese samples contrary to a
421 study conducted by Onmaz et al. (2021) on mold-ripened Turkish cheese varieties.
422 Besides, another finding of this study showed that *Dipodascus* genus was amongst the
423 five most prevalent genera in half of the Kuflu cheese samples with relative abundances
424 ranging from 0.29% to 2.58% and also *Kluyveromyces* genus did not constitute an
425 important part of the yeast community of Kuflu cheese (0.1-1.78%), which is different
426 from the previous reports on mold-ripened cheeses (Desmaures, 2014; Yildiz et al.,
427 2021). In fact, *Kluyveromyces* genus including especially *Kluyveromyces marxianus* and
428 *Kluyveromyces lactis*, which were frequently detected in previous blue/mold-ripened
429 cheese studies, were found in negligible amounts in the Kuflu cheese samples produced
430 in Konya while *Yarrowia* genus was not detected at all (Dimov et al., 2021; Gkatzionis
431 et al., 2014; Onmaz et al., 2021). The absence or low levels of these yeasts is a
432 disadvantage for Kuflu cheeses since there are some strong data showing that these
433 yeast species have probiotic properties and improve the aroma profile of cheeses as they
434 are capable of producing high amounts of volatile compounds (Esen and Çetin, 2021).
435 This may be due to the fact that these fungi are less salt-tolerant compared to *D.*
436 *hansenii* and they cannot metabolize compounds, such as lactate, towards the end of
437 ripening. It should also be noted that different bacterial and fungal genera that were not
438 found in previous studies on Kuflu cheese, such as *Virgibacillus*, *Paraliobacillus*, and
439 *Bifidobacterium*, were detected in the present study. Also, *Dipodascus* genus formerly
440 *Geotrichum*, was detected in Kuflu cheeses with a percentage between 0.28 and 4.28%
441 except for MC7 and MC8 samples. The lower abundance of this genus in Kuflu cheese
442 as compared to other mold-ripened cheeses can be attributed to their salt sensitivity.

443 The greatest bacterial richness by Shannon index was detected in MC1 cheese
444 sample with total 102 OTU-genera while MC4 cheese showed the lowest one with total

445 49 OTU-genera. Kuflu cheese samples containing an average of 62 bacterial OTU-
446 genera had a better bacterial richness than Bulgarian green cheese according to the
447 findings of Dimov et al. (2021). Regarding fungal richness by Shannon index, MC6 and
448 MC3 cheeses had the highest OTUs at genus level (35 and 27 OTU-genera,
449 respectively). MC10 cheese had a very high dominance of one genus with a high
450 proportion of sequences belonging to *Penicillium* (91.46% of total OTU-genera) with
451 the lowest Simpson index. Average fungal OTUs determined in this study are lower in
452 comparison to the findings of Dimov et al. (2021) who studied on the Bulgarian green
453 cheese batches.

454 As shown Fig. 7, the relative abundance of *Lactobacillus* genera was
455 significantly positively correlated ($p<0.05$) with *Candida* in Kuflu cheeses. This is
456 actually an unexpected result because there are studies showing that lactobacilli species
457 have anti-candidal activities (Vasquez-Munoz and Dongari-Bagtzoglou, 2021).
458 Although these studies are mostly related to the human opportunistic pathogen *Candida*
459 *albicans*, in a study conducted on traditional white pickled and soft cheeses, very low
460 abundance of lactobacilli were found in cheeses with a high abundance of *Candida*
461 *zeylanoides* (Golic et al., 2013). Meanwhile, there are also studies that found a positive
462 correlation between *Candida* and lactobacilli in parallel with this present study
463 (Fujinami et al., 2021). *Streptococcus* genus was positively correlated with the relative
464 abundance of *Debaryomyces* genus. Similarly, Stellato et al. (2015) observed that *D.*
465 *hansenii* is also high in cheeses where *Streptococcus thermophilus* is high. On the other
466 hand, *Staphylococcus* genus was significantly positively correlated ($p<0.05$) with
467 *Virgibacillus* abundance whereas it was negatively correlated with *Chryseobacterium*.
468 Positive interaction between staphylococci and *Virgibacillus* genus was previously
469 observed in fish sauce (budu) produced using autochthonous *Virgibacillus*

470 *halodenitrificans* PS21 and *Staphylococcus simulans* PMRS35 and in shrimp paste
471 produced using *Virgibacillus* sp. SK37 and *Staphylococcus nepalensis* JS11 together
472 (Kanjian et al., 2021; Yu et al., 2022). Again, a previous study revealed a positive
473 correlation between *Staphylococcus* and *Chryseobacterium* in boiled milk samples in
474 contrary to this current study (Joishy et al., 2019). This situation can be attributed to the
475 possibility of dominance of different species in the genera mentioned in both studies.
476 Also, the relative abundance of *Penicillium* was significantly positively correlated
477 ($p<0.05$) with *Debaryomyces* genera in parallel with the findings of Stellato et al. (2015)
478 who determined the positive correlation between *Penicillium* and *Debaryomyces* genera
479 in cheese samples. In another study, the researchers have shown that *D. hansenii*, which
480 is frequently found in blue cheeses, stimulates the growth of *P. roqueforti*, that is, there
481 is a positive interaction between them (Gkatzionis et al., 2014). The relative abundance
482 of *Paraliobacillus* and *Debaryomyces* was significantly positively correlated ($p<0.05$)
483 with fat in dry matter. This is no surprising result because especially *Debaryomyces*
484 genera is known for having high lipolytic activity which may contribute to the flavor
485 and texture development of fermented products (Song et al., 2022). Moisture was
486 negatively correlated with the relative abundance of *Debaryomyces* and
487 *Chryseobacterium*. *Debaryomyces* species, especially *D. hansenii*, is particularly
488 tolerant of high salt or high sugar environments, therefore, its negative correlation with
489 moisture content is not surprising.

490 Conclusion

491 As a result of the study, *Firmicutes* was found as the most dominant bacterial
492 phyla, while *Lactococcus*, *Streptococcus*, and *Staphylooccus* were detected as the most
493 dominant bacterial genera, respectively. The genus *Virgibacillus*, which had not been
494 detected in mold-ripened cheeses before, was detected with high relative abundances in

495 some Kuflu cheeses. As for the fungal composition, *Ascomycota* was very dominant at
496 the phylum level as expected, and the genus *Penicillium* was found to be the most
497 prevalent in most of the cheeses at genus level. No OTU belonging to *Aspergillus* was
498 detected in Kuflu cheeses. At the same time, none of the important food pathogens were
499 detected in these cheeses. This study enlightens the researchers and producers interested
500 in mold-ripened cheeses and Kuflu cheese, and it is also important to improve the
501 quality of this uncontrolled moldy cheese variety. Overall, this work may be an initial
502 study to exhibit the benefits and risks of Kuflu cheeses in the context of microbial
503 composition using metagenomic data, and it has also shown that Kuflu cheeses can
504 assist in the isolation and identification of the first detected strains in Kuflu cheeses
505 such as *Bifidobacterium* and *Lactobacillus* species using culture-dependent methods.
506

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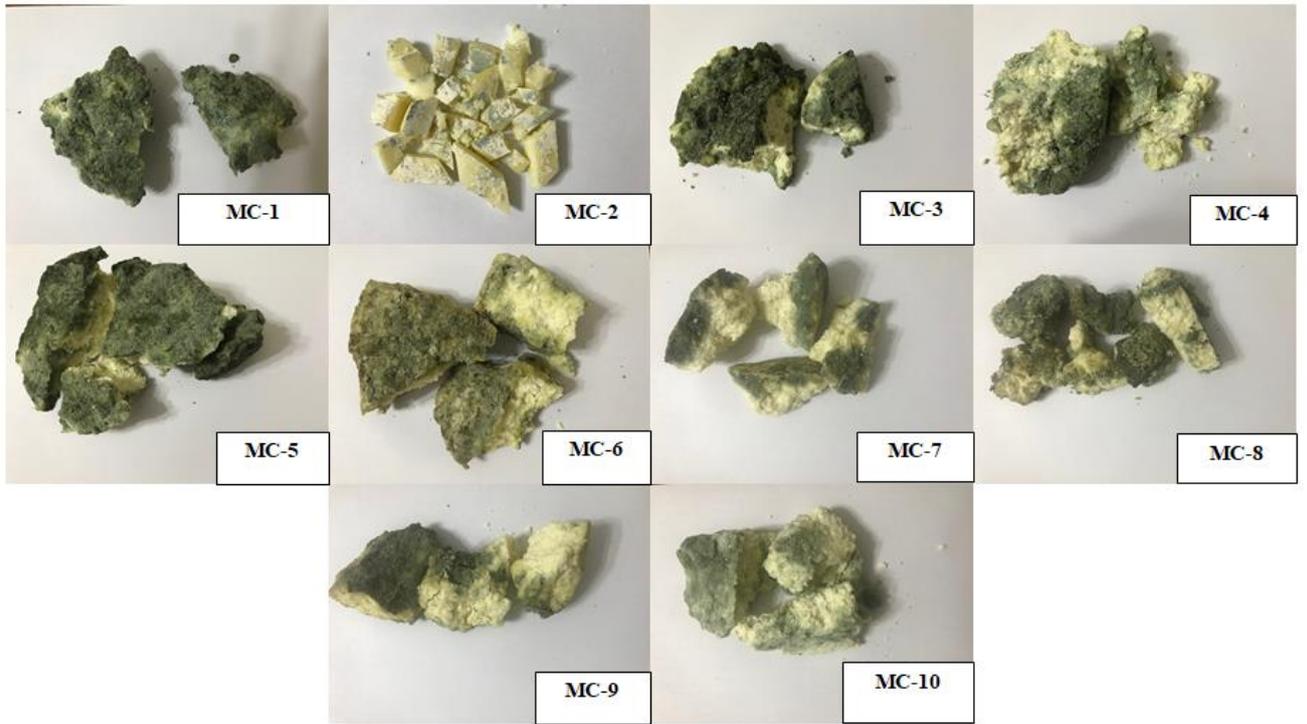
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623 **Table 1.** Chemical composition, pH, and TA of Kuflu cheeses.

Samples	pH	TA ^a	Fat in DM ^b (%)	Salt in DM (%)	Moisture (%)
MC1	5.78±0.02	2.47±0.03	6.5±0.3	3.8±0.2	50.8±1.2
MC2	5.40±0.04	2.25±0.05	25.0±2.3	3.8±0.2	39.9±0.5
MC3	6.29±0.04	2.58±0.01	6.0±0.3	3.6±0.1	48.7±1.3
MC4	5.83±0.01	2.53±0.00	7.5±0.9	3.6±0.2	56.7±2.1
MC5	5.65±0.00	2.64±0.03	16.5±0.9	2.1±0.1	48.8±1.0
MC6	6.69±0.09	1.37±0.01	6.0±0.6	3.7±0.3	51.7±1.4
MC7	5.66±0.01	2.53±0.02	6.0±0.2	4.3±0.3	45.9±1.1
MC8	5.32±0.02	2.47±0.03	27.5±1.3	5.2±0.2	48.2±0.8
MC9	6.15±0.00	1.59±0.02	9.5±0.3	4.0±0.0	48.0±2.2
MC10	5.17±0.03	2.81±0.01	6.5±0.5	4.4±0.2	49.8±0.7

624 ^aTA expressed as g lactic acid/100 g cheese.

625 ^bDM: Dry matter.

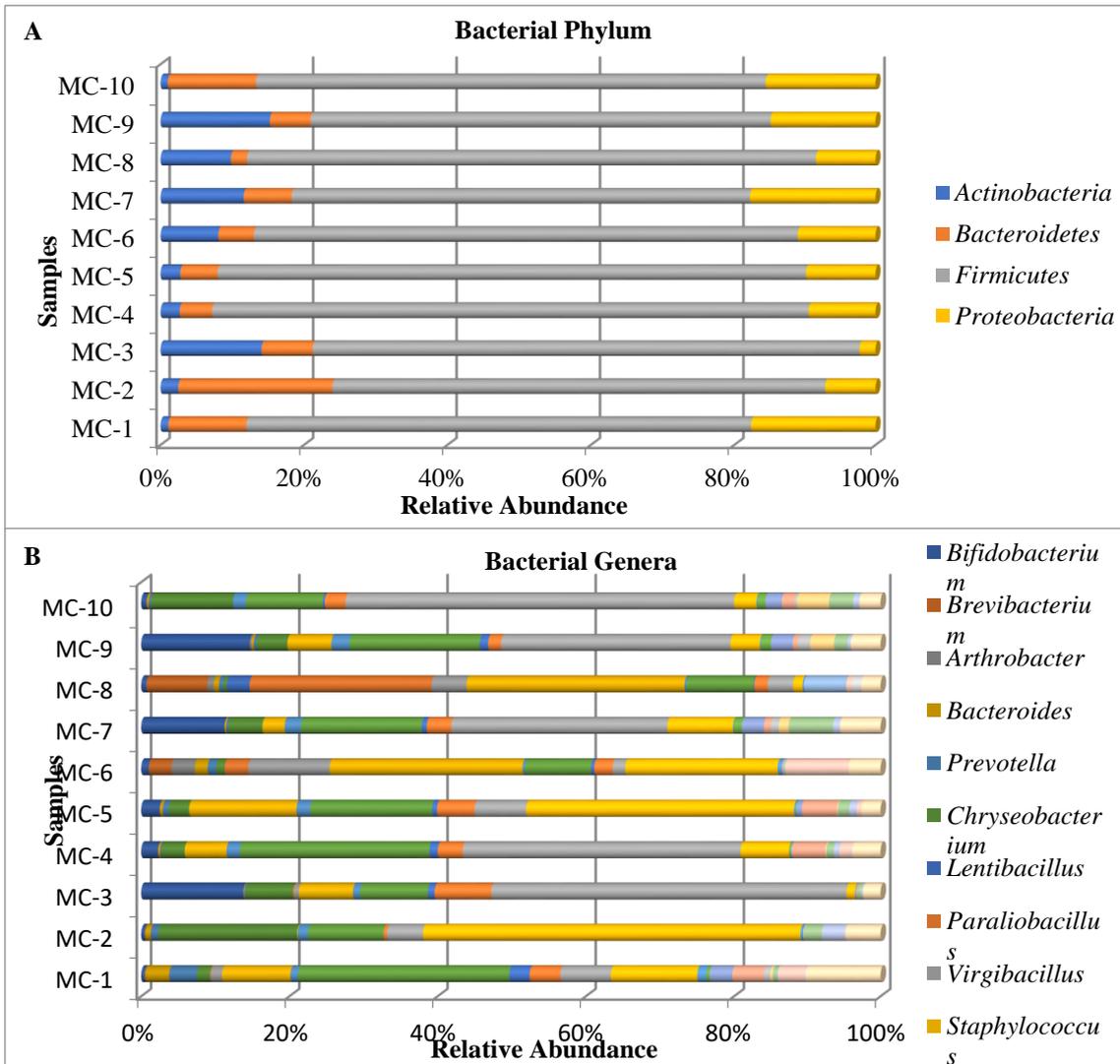


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Fig 1. The images of Kufli cheese samples analyzed in this study.

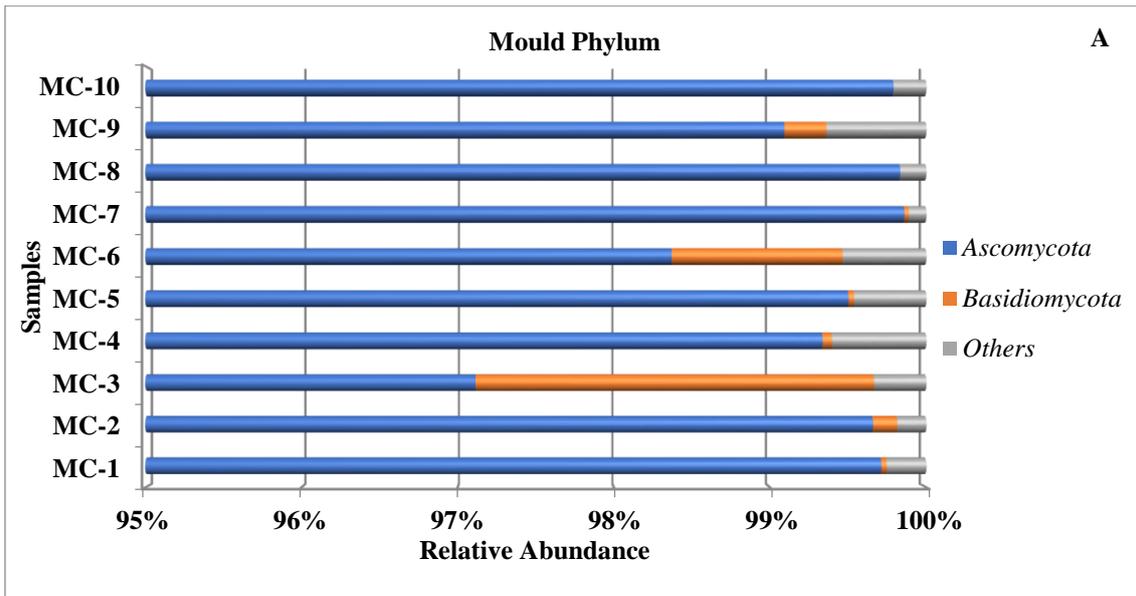
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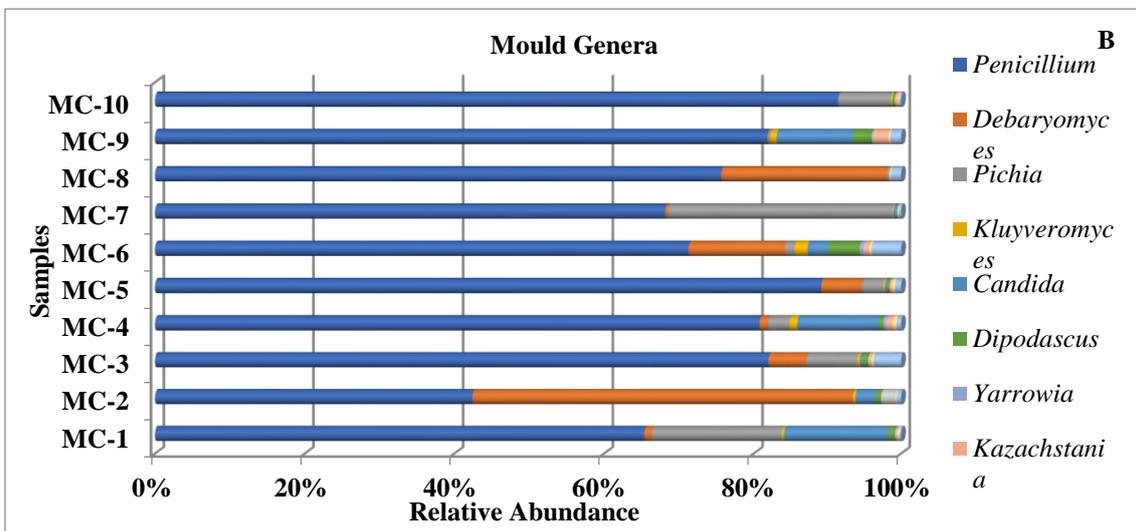
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630 **Fig. 2** Bar chart showing the relative abundance of the major taxonomic groups of bacteria
 631 at phyla (A) and genera (B) level all Kufli cheese samples. OTUs with an incidence above
 632 1% in A and B are offered.

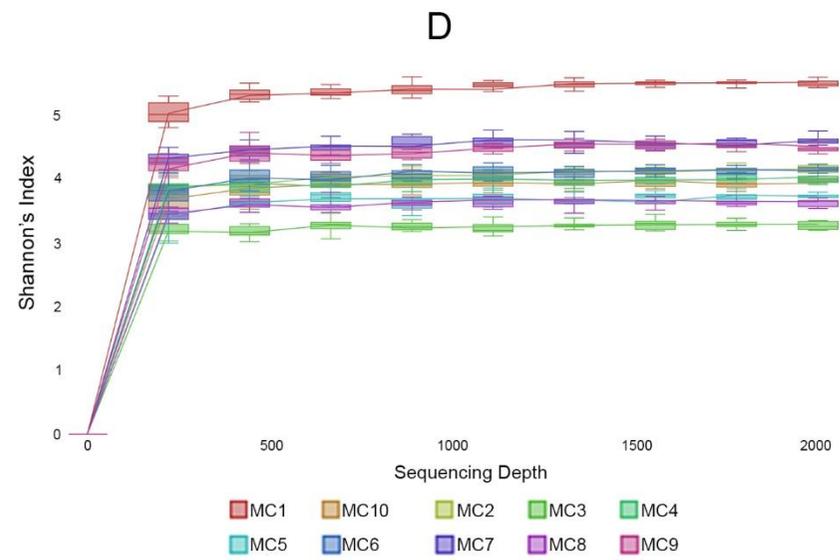
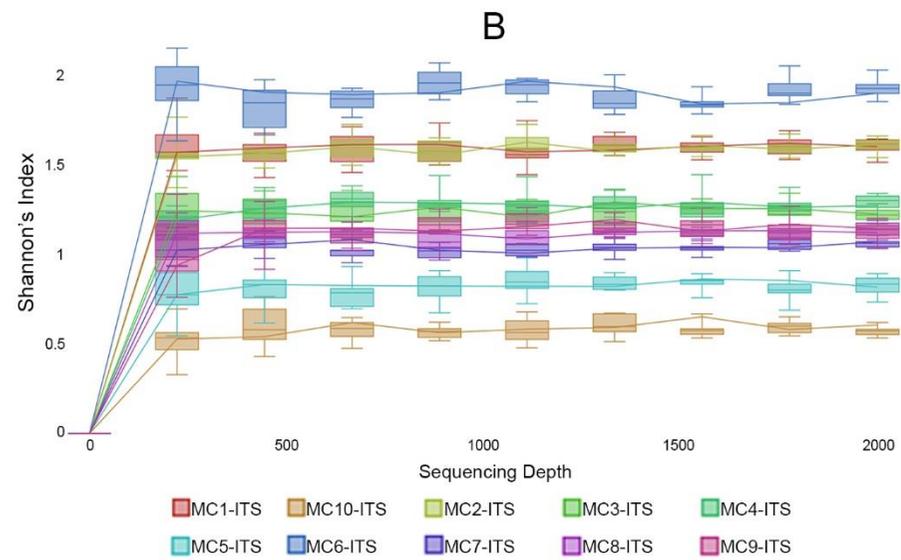
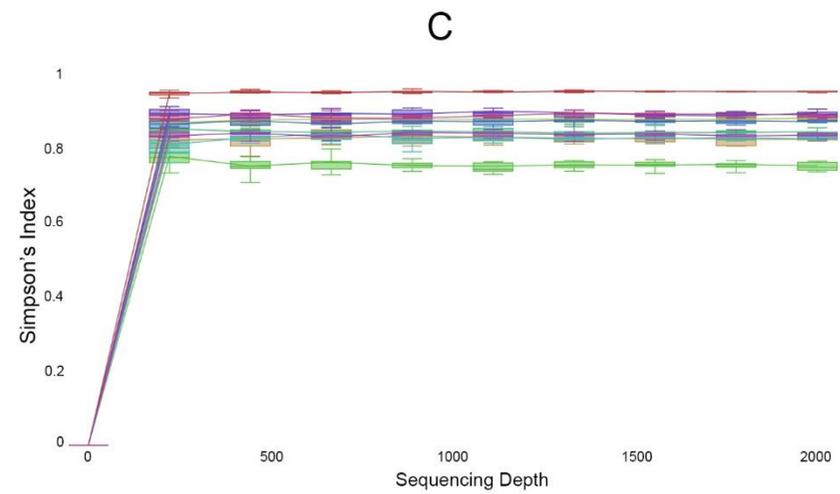
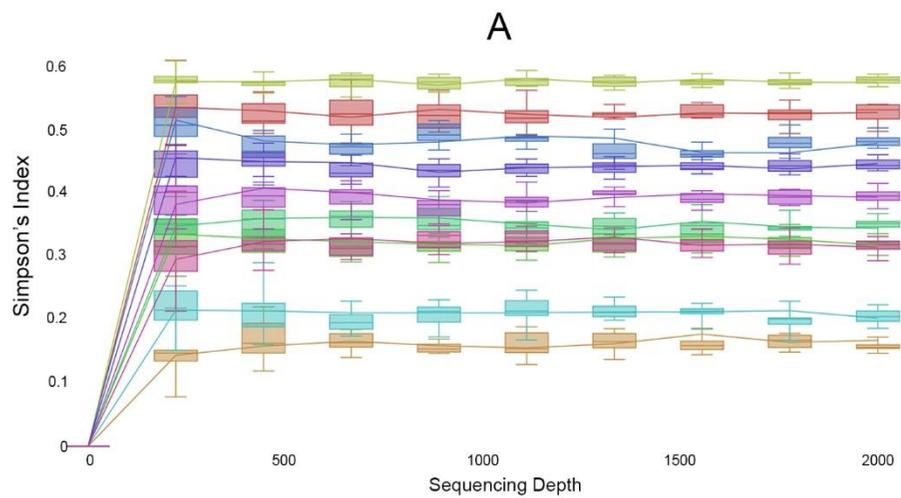


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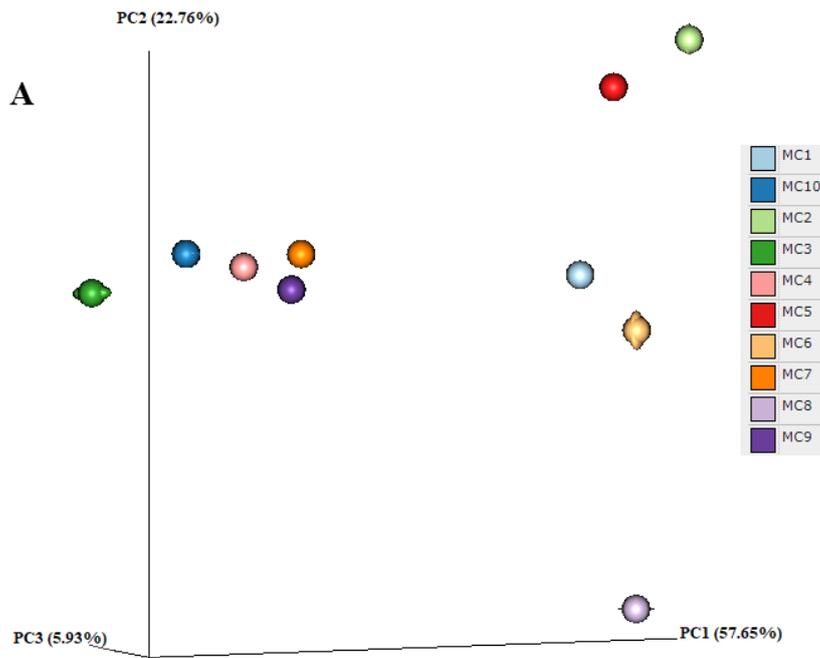
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635 **Fig. 3** Bar chart showing the relative abundance of the major taxonomic groups of fungi
 636 at phyla (A) and genera (B) level all Kufllu cheese samples. OTUs with an incidence above
 637 0.1% in A and B are offered.

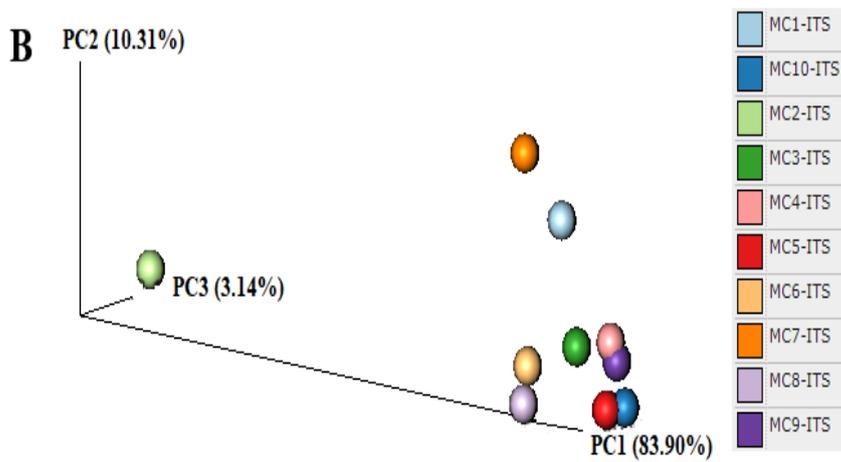


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639 **Fig. 4** A, B: Alpha diversity as Simpson and Shannon Index of fungi populations of Kufllu cheese samples, respectively (from MC1-ITS to MC10-ITS); C, D: Alpha
 640 diversity as Simpson and Shannon Index of bacteria populations of Kufllu cheese samples, respectively (from MC1 to MC10).



641



642

643 **Fig. 5** β -diversity plot showing the relatedness of bacterial (A) and fungal (B)
 644 communities with PCoA distance matrix among Kufllu cheeses. The plot is based on the
 645 Bray-Curtis dissimilarity index.

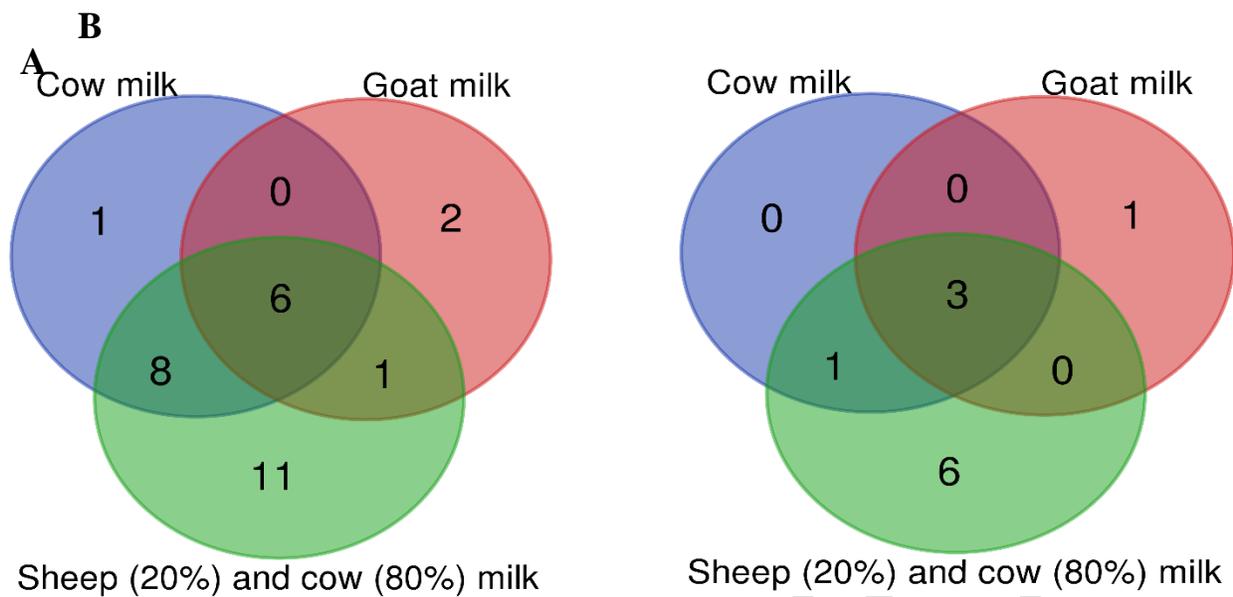
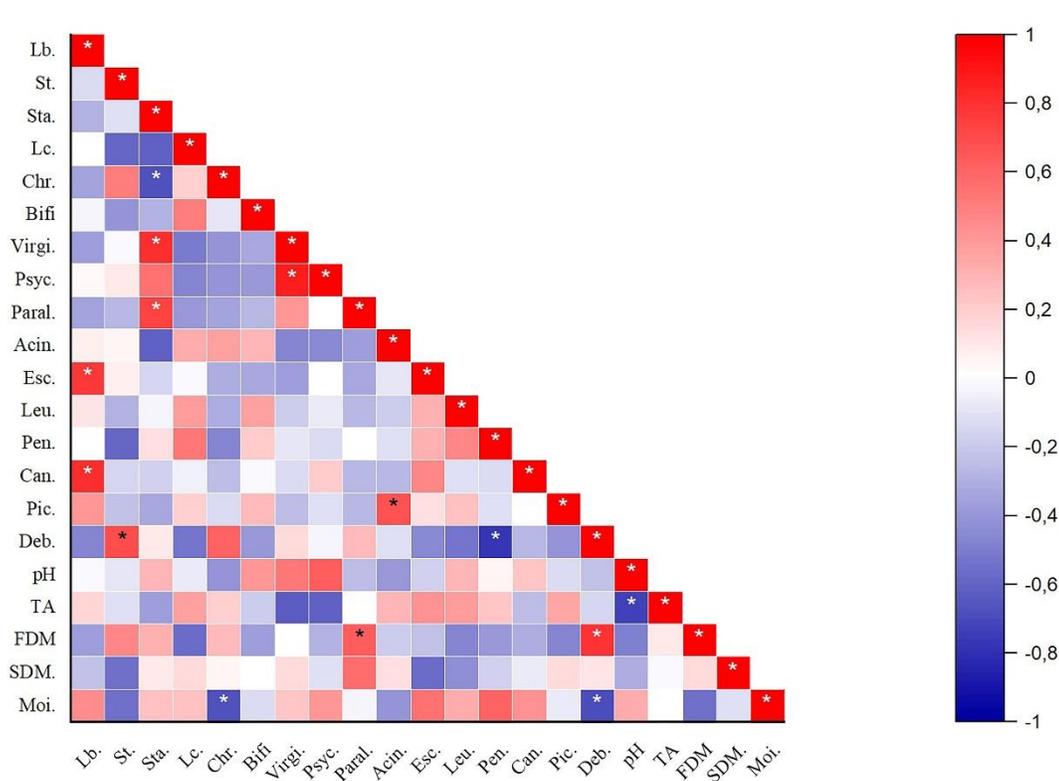


Fig. 6. Venn diagram representing different and similar genera between Kuflu cheese samples produced from different types of raw milk. Relative abundances above 1% were evaluated in the Venn diagram. A: bacterial genera, B: fungal genera.



* p<=0.05

Fig. 7. Correlation clustering heatmap of the correlation between differentially abundant microorganisms and pH, TA, and chemical composition in Kuflu cheese samples. The color scale indicated the nature of the correlation with red indicating a positive correlation and blue indicating a negative correlation. The asterisk marks represent the significance of the correlation value, * means p<0.05. Lb.: *Lactobacillus*, St.: *Streptococcus*, Sta.: *Staphylococcus*, Lc.: *Lactococcus*, Chr.: *Chryseobacterium*, Bifi.: *Bifidobacterium*, Virgi.: *Virgibacillus*, Psyc.: *Psychrobacter*, Paral.: *Paraliobacillus*, Acin.: *Acinetobacter*, Esc.: *Escherichia*, Leu.: *Leuconostoc*, Pen.: *Penicillium*, Can.: *Candida*, Pic.: *Pichia*, Deb.: *Debaryomyces*, TA: Titratable acidity, FDM: Fat in dry matter, SDM: Salt in dry matter, Moi.: Moisture.