Molecular investigation of bacterial communities during the manufacturing and ripening of semi-hard Iranian Liqvan cheese

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19 April 2024

1 **Molecular Investigation of Bacterial Communities during the Manufacturing and Ripening of**

2 **Semi-hard Iranian Liqvan Cheese**

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18   **Abstract**

19   Liqvan (or Lighvan) is a traditional Iranian cheese from the East Azerbaijan province of Iran, which is

20   made of raw ewe´s milk without the addition of a starter. The grazing pastures, environmental conditions

21   and the ancient regional production methods allocate a distinctive microbial ecology to this type of

22   cheese, and these factors are consequently associated with the quality of the product. In this study, the

23   microbiota of the milk, curd and cheese has been investigated using culture independent approaches.

24   Denaturing gradient gel electrophoresis (DGGE) of the bacteria, 16S rRNA based high-throughput

25   sequencing and enumeration of the live bacterial community by means of quantitative PCR (qPCR) have

26   been used for this purpose. The results showed that the main bacterial population in the milk belonged to

27   both microbial contaminants and lactic acid bacteria (LAB). However, both of these populations were

28   totally replaced by LAB during ripening. The present survey contributes by describing the microbiota of

29   this ancient cheese in more detail during fermentation and ripening.

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31   **Keywords:** Liqvan cheese, Illumina analysis, RNA-based methods

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33   **Introduction**

34   Liqvan (or Lighvan) cheese is the most famous traditional, semi-hard, feta like, starter-free, Iranian

35   cheese. It is manufactured from raw ewe's milk in the Liqvan village in the stratovolcano mountainous

36   area of Sahand in the East Azerbaijan province of Iran. The production process is conducted within 2h

37   from milking. Ewe's milk is cooled to 25°C and coagulated with homemade or industrial lamb rennet.

38   After 2 hours of drainage, the coagulum is cut into 15x15 cm cubes and placed in 15-20% saline water,

39   where it is left for 9 to 10 hours. The curd is consequently covered with dry salt and kept in a basin for 2-

40   3 days, during which whey drainage continues. During this period, the cubes are turned (upside down) at

41   least five times. Finally, the resulting curd is placed in 10–12% saline water in metal containers. Ripening

42   takes place in deep natural or manufactured caves over a period of 6 months. This traditional

43   manufacturing process, which has remained unchanged for centuries in this specific area, has attracted

44   particular interest in the last few years (Edalatian et al., 2012; Kafili et al., 2009; Navidghasemizad et al.,

45   2009; Barouei et al., 2008). Typical sensory properties of such traditional cheeses mostly affected by

46   dairy animals' type, breeds and nutrition as well as their indigenous microbiota which drive biochemical

47   process during cheese making. However, this traditional way of production has made also some problems

48   during these days. First of all Standard Organization of Iran recommends strictly the pasteurization of

49   milk to improve the safety of dairies and this process might eliminate the key functional microorganisms

50   involved at cheese ripening and acidification. In spite of food safety, product standardization is necessary

51 for entrance at international marketing systems so identification and characterization of active starter and

52 non-starter microorganisms during each step of production seems to be important.

53 Food microbiota is currently monitored by means of a variety of culture dependent and independent

54 techniques. DNA and/or RNA based approaches are considered indispensable tools for the detection,

55 identification and characterization of microorganisms in food, and they have led to a profound change in

56 the microbial ecology study of fermented foods in recent decades. These techniques are faster and more

57 reliable than conventional culturing methods, which fail to reproduce ecological niches and symbiotic

58 relationships (Carraro et al., 2011, Cocolin et al., 2013; Ndoye et al., 2011). Moreover, Next Generation

59 Sequencing (NGS) approaches have been used successfully to monitor microbial communities in foods,

60 especially in milk-based foods lately (Aldrete-Tapia et al., 2014; Bassi et al., 2015; Dugat-Bony et al.,

61 2015; Garofalo et al., 2015; Minervini et al., 2015; O'Sullivan et al., 2015; Alessandria et al., 2016;

62 Dalmasso et al., 2016; De Pasquale et al., 2016; Escobar-Zepeda et al., 2016).

63 Previous studies on Liqvan diversity, based on culture-dependent and on DNA based DGGE method, led

64 to the identification of some dominant bacteria (Edalatian et al., 2012; Kafili et al., 2009;

65 Navidghasemizad et al., 2009; Barouei et al., 2008). However, to the best of the authors' knowledge, no

66 RNA based studies on this cheese have been carried out so far. Moreover, in order to have a more

67 complete picture of viable microbiota thriving in this cheese during ripening process, we used illumina-

68 sequencing method as a new powerful tool for analyzing of milk-based foods. This thorough analysis of

69 RNA-based microbiota, will help us to follow the trace of possible pathogenic microorganisms and their

70 survival during manufacturing.

71 Therefore, in this study, the microbial population of the bacterial communities of Liqvan cheese have

72 been studied during its manufacturing through reverse transcriptase PCR (RT-PCR)-DGGE. Furthermore,

73 the total counts and diversity of the viable bacterial populations in the raw milk, curd and ripened cheese

74 have been investigated using RT quantitative PCR (RT-qPCR) and 16S rRNA gene amplicon sequencing,

75 respectively.

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78 **Material and methods**

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80 **Sample collection**

81 Samples of raw milk, curds and cheeses were collected aseptically on-site at five different local authentic

82 production units (A to E) in the Liqvan village, which covers an area of 19.76 square kilometers in the

83 south-western part of Tabriz province. All the milk samples collected at the same day after transferring to

84 the units and cooled at 25 °C. While three days curds gathered after coagulation and drainage. The milk

85 and curds were transferred directly to the lab, albeit the cheeses were collected after 180 days of ripening

86 from the same source of milk. Three trials as replicates were taken from each dairy for every sampling.

87 The samples were transported at 4-8 °C and kept at -20 °C.

88 This cheese mainly characterized by a pH value and total fat and protein percentage around 5.02±0.5,

89 20.3±2.0 and 15.7±1.0, respectively.

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91 **Reverse transcription and PCR amplification of the microbial community**

92 Aliquots of 2 milliliters of milk were centrifuged for 10 min at 14,000 × g under refrigerated conditions (4

93 °C). The samples were placed at -80 °C for 30 min to allow separation of the fat layer, which was then

94 removed by means of a sterile spatula. After thawing, the supernatant was discarded and cell pellets were

95 re-suspended in 1 ml ¼ strength Ringer solution. The mixture was centrifuged at 14,000 × g for 10 min at

96 4 °C, and the supernatant was again discarded. The pellets were re-suspended in a 50 µl lysozyme

97 solution (50 mg/ml, Sigma, Milan, Italy) and incubated at 37 °C for one hour. The lysate was then

98 subjected to nucleic acid extraction, using the Master-Pure complete DNA and RNA Purification Kit

99 (Epicentre, Madison, WI, USA), according to the manufacturer´s instructions.

100 In the case of the curds and cheeses, five grams of samples was homogenized with 20 ml of ¼ strength

101 Ringer solution. One ml of this solution was transferred to a 1.5 ml micro-tube, and the same procedure as

102 the one described for the milk was followed. The resulting RNA was then treated with 3 µl of TURBO

103 DNase (Ambion, Milan, Italy) and incubated for 3 h at 37 °C in order to eliminate the DNA. Complete

104 DNA digestion was confirmed using 1 µl of extracted RNA in PCR reactions with primers 338f and 518r

105 (Muyzer et al., 1993). If a PCR product was obtained, the DNase treatment was repeated. The resulting

106 RNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Milan, Italy) and

107 standardized at 500 ng/µL.

108 RT reaction was carried out using M-MLV reverse transcriptase (Promega, Milan, Italy). An aliquot of

109 500 ng of RNA was mixed with 1 µl of random primer (Promega Milan, Italy) for each sample. A volume

110 of 10 µl was reached by adding DNase- and RNase-free sterile water (Sigma), and the solution was

111 incubated at 75 °C for 10 min. The mixture was then immediately placed on ice for 5 min, and then added

112 to a microtube vial containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 2 mM

113 of each dNTP, 1 µl of 200 U/µl M-MLV and 0.96 U of RNasin ribonuclease inhibitor (Promega). Reverse

114 transcription was carried out at 42 °C for 1 h (Alessandria et al., 2010). The PCR reaction was performed

115 in triplicate in a MyCycler (BioRad, Hercules, CA, USA) on each sample.

116 The V3 region of the 16S rRNA gene in the bacteria was amplified with primers 338f-GC/518r (Muyzer

117 et al., 1993). PCR amplification was carried out adopting the same procedure used in previous studies on

118 the ripening of hard cheese (Bautista-Gallego et al., 2014).

DGGE analysis and band sequencing

The RT-PCR products were analyzed by means of DGGE, using the DCode system apparatus (BioRad, Hercules, CA, USA). Polyacrylamide gels (8% W/V, Acrylamide-Bisacrylamide 40% solution 37.5:1, 0.8 mm thickness) were prepared using 30-50% urea-formamide denaturing gradients (100% corresponded to 8 M urea and 40% (wt/vol) formamide). The gels were run for 240 min at 130 V, stained with SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Milano), visualized under UVtransillumination and photographed by using UVIpro Platinum 1.1 Gel Software (Eppendorf).

Identification of the microbial populations was carried out by cutting selected bands from the DGGE gels. DNA was eluted in 50 µl water overnight at 4 °C, and again run in DGGE after re-amplification in order to check the electrophoretic mobility with respect to the excised bands. After the check, DNA was amplified again with non GC-clamp primers and sent for sequencing to GATC-Biotech (Cologne, Germany). A fingerprint database was created using Bionumerics software, version 4.6 (Applied Maths, Sint Marten Latem, Belgium).

Construction of a/the LAB standard curve for viable bacterial enumeration

The viable bacterial populations in the milk, curd and cheese were determined according to Alessandria et al. (2016). Milk, curd and cheese were sterilized. At the same time, overnight pure cultures of *Lactococcus lactis* subsp. *lactis, Lactobacillus plantarum, Lactobacillus pentosus, Enterococcus faecium* and *Lactobacillus helveticus* was prepared. The count of each culture is reached to approximately 10^9 (CFU/g or ml, which checked by culturing methods) and mixed. Serial dilutions of this overnight cultures were prepared using ¼ strength Ringer and 10 ml of each dilution (which contains the final concentrations of 10^8 to 10 CFU/g or ml), inoculated to 10 g or ml of food samples.

 The samples were then homogenized in a Stomacher (Interscience Rockland, MA, USA) for 1 min, and 1 ml of each mixture was subjected to RNA extraction and reverse transcription, as described above. QPCR amplifications, targeting the V3 region of the 16S rRNA gene, were performed in a final volume of 25 µl, using SSo Advanced Sybr Green Supermix (Bio-Rad, Italy). One µl of cDNA was amplified with 338f and 518r primers at a final concentration of 400 nM in a Chromo 4 real-time PCR Detection System (Biorad, Milan, Italy) (Alessandria et al., 2010). In all cases and at each step the bacterial counts was checked by triple plate counting routine methods and Standard curves were constructed by plotting the threshold cycle (C_T) values against log CFU/g or CFU/ml on MRS agar. MRS agar plates were incubated at 37 °C for 48 h in microaerophilic conditions. The correlation coefficients ($R2$) and efficiency of amplification were calculated as described in Higuchi et al. (1993).

Quantification of the viable bacteria in the milk, curd and cheese was carried out by amplifying 1 µl of cDNA, and using the standard curves for the calculation of the counts.

Bacterial RNA analysis by means of Illumina high-throughput sequencing

A high-throughput sequencing approach was applied to a total of 15 samples (5 samples from milk, curd and cheese). cDNA, obtained as explained above, was used to study the microbial diversity of the active populations. The V3–V4 region of the 16S rRNA gene was amplified using the following primers: the 16S Amplicon Forward Primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and the 16S Amplicon Reverse Primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'), as reported in Klindworth et al. (2013). Twenty-five µl PCR reactions were prepared using 12.5 mL of the 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA), 1 µM of each primer, 2.5 µL of cDNA template and PCR grade water. Twenty five cycles of denaturation (95 °C/ 30 sec), primer annealing (55 °C/30 sec) and primer elongation (72 °C/30 sec), followed by a final elongation step (72 °C/ 5 min), were carried out.

The PCR products were purified by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc. San Diego, CA), according to the manufacturer's instructions. The sequencing reaction was performed by Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, S.C. Controllo Alimenti e Igiene delle Produzioni (Turin, Italy) with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instruction. The software used for the base-calling and Illumina barcode demultiplexing processes, were the MiSeq Control Soft. V2.3.0.3, the RTA v1.18.42.0 and the CASAVA v1.8.2.

Bioinformatics analysis

Paired-end reads were first merged using FLASH software (Magoc and Salzberg, 2011) with default parameters. Join reads were further quality filtered (at Phred > Q20) using QIIME 1.9.0 software (Caporaso et al. 2010) through multiple_extract_barcode.py and multiple_split_libraries_fastq.py script. Usearch v8.1 software (Edgar et al., 2011) was adopted for chimera filtering, using the 16S reference databases v9 (RDP classifier training database). OTUs were picked at 99% of similarity by means of UCLUST clustering methods (Edgar, 2010), and representative sequences of each cluster were used to assign taxonomy using the Greengenes 16S rRNA gene database, version 2013, by means of the RDP classifier (Wang et al. 2007). Representative sequences belonging to clusters identified as *Lactobacillus* spp. were double-checked using the BLAST (BLASTN) search program (http://www.ncbi.nlm.nih.gov/blast/) to get the species level as best hit. Statistics and plotting were carried out in the R environment (www.r-project.org). Alpha diversity indices were calculated using the diversity

187 function of the vegan package (Dixon, 2003). The Shannon-Wiener diversity index H' was further
188 analyzed using the t-test to assess any differences between the milk, curd and cheese samples. Weighted
189 UniFrac distance matrices and OTU tables were used to perform Adonis and Anosim statistical tests in R
190 environment. A filtered OTU table was generated at 0.5% abundance in at least two samples through
191 QIIME. PICRUSt (Langille et al., 2013) was used to predict the abundance of the gene families, on the
192 basis of 16S rRNA sequence data, as recently described (Ferrocino et al., 2016, Bokulich et al., 2015).
193 Nearest Sequenced Taxon Indexes (NSTI) were calculated in order to evaluate the accuracy of the
194 metagenome predictions (Langille et al., 2013). KEGG orthologs were then collapsed at a hierarchy level
195 of 3, and the table was imported into R. The made4 package was adopted, and hierarchical Ward-linkage
196 clustering, based on the Spearman correlation coefficients of the proportion of the activities belonging to
197 lipid carbohydrates and amino acid metabolism pathways, was used to produce a heatplot.
198 All the sequencing data were deposited at the Sequence Read Archive of the National Center for
199 Biotechnology Information (SRP074051).
200
201 **Results**
202 **Identification and total bacterial counts during ripening**
203 Eleven different bands, corresponding to the bacterial V3 region, were observed in the milk, curd and
204 cheese samples (Tab. 1). *Lactococcus lactis* and *Leuconostoc mesenteroides* were the predominant
205 species that recovered from all of the different types of samples, while *Streptococcus thermophilus* was
206 only found in the curd. On the other hand, bands related to *Enterobacter aerogenes* and *Pseudomonas*
207 *fluorescens* were mostly abundant in the milk and curd, but there was no trace of them in the cheese
208 samples. Other LAB were randomly distributed between curd and cheese except *Lactococcus*
209 *raffinolactis* that was not found in cheese.
210 The results of the quantification RT-PCR showed 8.5± 0.2 log CFU/ml for milk and 5.8± 0.2 and 3.5± 0.1
211 log CFU/g for curd and cheese, respectively. These data, which all approved by plate counting methods
212 (data was not shown) underline that, the total bacterial populations decreased during the ripening process.
213
214 **16S rRNA gene sequencing**
215 A total of 1,216,136 raw reads (2x250bp) were obtained after sequencing. After joint, a total of 431,012
216 reads passed the filters applied through QIIME, with an average value of 28,734 reads/sample, and a
217 sequence length of 457 bp. The estimated sample coverage for milk, curd and cheese are 94.69± 1.24,
218 95.21± 0.77 and 95.43± 1.38, respectively which indicates that there was a satisfactory coverage for all of
219 the samples (ESC > 94%). Moreover, The average OUT numbers between different producers are
220 2333.8± 515.63 in milk, 1545.6± 489.01 in curd and 1594.4± 298.14 in cheese while Chaol values that

221 reflect the operational taxonomic unit richness are ranged from 3933.67± 440.16, 2758.24± 904.50 and

222 2419.38± 306.59 between milk, curd and cheese . In addition, overall sample diversity index (Shannon)

223 are calculated as 6.47± 0.38, 5.17± 0.65 and 5.63±0.42 in milk, curd and cheese, respectively. Besides,

224 the alpha-diversity showed that there was a higher level of complexity (P < 0.05) in the milk samples than

225 in the curds and cheeses and between the cheeses and the curds (P < 0.05). It was possible to show,

226 through a principal coordinate analysis (PCoA), with a Weighted UniFrac distance matrix as well as from

227 Adonis and Anosim statistical tests, that the samples were grouped together according to their type (P <

228 0.001). In addition, no differences were found between the milk, cheese and curd samples for the different

229 repeats, according to the Adonis and Anosim statistical tests (P > 0.001). Adonis and Anosim statistical

230 tests of Weighted UniFrac distance matrix showed. significant difference among sample type (P < 0.001).

231 Only OTUs with a relative abundance of 0.5% in at least two samples are shown in Figure 1. The

232 abundance of the OTUs from the milks, curds and cheeses was averaged. The raw milk showed a varied

233 microbiota, characterized by a predominance of *Moraxellaceae* (20% of the relative abundance),

234 *Leuconostoc mesenteroides* (16%), *Staphylococcaceae* (13%), *Lactococcus raffinolactis* (7%),

235 *Pseudomonas* sp. (5%), *Enterobacteriaceae* (6%), and *Lactococcus lactis* (6%).

236 The curd samples showed a predominance of *Leuconostoc mesenteroides* (43%), *Lactococcus lactis*

237 (11%), *Enterobacteriaceae* (18%) and *Streptococcus* sp. (8%). The cheese samples were characterized by

238 a predominance of *Lactococcus lactis* (30%), *Leuconostoc mesenteroides* (20%), *Lactobacillus*

239 *fuchuensis* (10%), *Lactobacillus* sp. (10%) and *Pediococcus* sp. (10%).

240 The main differences between the three types of analyzed samples were due to the presence of several

241 contaminant OTUs, such as *Pseudomonas* and *Moraxellaceae,* which were found to be significantly more

242 abundant (g-test, P < 0.001) in the milk samples, while *Enterobacteriaceae* discriminated the curd

243 samples. On the other hand, the cheese samples were characterized by the presence of several lactic acid

244 bacteria (LAB), such as *Lactobacillus curvatus, Lactobacillus zeae*, *Lactobacillus fuchunensis*,

245 *Lactococcus lactis* and *Lactobacillus pentosus* (g-test, P < 0.001).

246 The OTU co-occurrence/exclusion pattern is shown in Figure 2, where only significant correlations are

247 reported (False Discovery Rate - FDR < 0.05). The characteristic OTU of the cheese samples, such as

248 *Lactobacillus curvatus, Lactobacillus zeae*, *Lactobacillus fuchunensis*, *Lactococcus lactis* , *Lactobacillus*

249 *pentosus* and *Lactobacillus kefiri,* showed the highest number of negative correlations, including a strong

250 exclusion of *Lactococcus* sp., *Moraxellaceae*, *Enterobacteriaceae*, *Enterococcus* sp., *Staphylococcaceae*

251 and other contaminants. *Moraxellaceae* showed a positive correlation with *Pseudomonas* sp., *Rothia* sp.

252 and *Staphylococacceae*.

253 As far as the predicted metagenomes are concerned, the weighted nearest sequenced taxon index (NSTI)

254 of the samples, expressed as the mean ± SD, was 0.028 ± 0.003. This index is the average branch length

255   that separates each OTU from a reference bacterial genome, weighted by the abundance of that OTU in

256   the sample. Thus, an NSTI score of 0.028 indicates a satisfactory accuracy for all of the samples (98%). It

257   was possible to differentiate the three different sample types (cheese, curd and milk) on the basis of the

258   predicted gene repertoires, associated with their microbiota (Fig. 3). A sub-cluster was mainly identified

259   for samples derived from the milk samples, and another cluster was identified for most of the cheeses and

260   curd samples. The dominant spoilage-related microbiota from the milk samples could considered, as a

261   microbial proteolitic consortium, due to presence of a presumptive abundance of genes related to lysine,

262   valine and the tryptophan metabolism. Besides, metabolic pathways related to a lipid metabolism, such as

263   a steroid metabolism, and to biosynthesis of the unsaturated fatty acids are also include. The other group

264   (cheese and curd samples) showed a higher presumptive abundance of genes related to a carbohydrate

265   metabolism, such as starch and sucrose metabolisms, amino-sugar and fructose metabolisms as well as

266   amino acid catabolism related genes.

267

268   **Discussion**

269   Liqvan is a traditional Iranian cheese that has been found to represent an interesting case for the study of

270   fermented food as it originates from a particular region in Iran, and it has long been prepared the same

271   way using an ancient methodology. Moreover, as this cheese is made from raw milk without the addition

272   of starter cultures, it can be speculated that its microbial ecology mainly depends on indigenous

273   microbiota. In this context, the first step towards protecting the microbial diversity of this traditional

274   regional food is to elucidate the evolution of the active microbial populations during its manufacturing

275   and ripening, in order to guarantee both the safety and the quality of the final products (Alegría et al.,

276   2012). Previous studies on Liqvan cheese (Edalatian et al., 2012; Kafili et al., 2009; Navidghasemizad et

277   al., 2009; Barouei et al., 2008), which were mainly based on the DNA PCR-DGGE method, showed that

278   *Lactococcus lactis, Lactococcus raffinolactis, Lactobacillus plantarum, Lactococcus garvieae,*

279   *Lactobacillus sakei, Lactobacillus casei* and *paracaei, Leuconostoc mesenteroides, Streptococcus* sp. and

280   *Enterobacter* sp. are the predominant groups of bacteria in this cheese.

281   To the best of our knowledge, no data have been reported on RNA based DGGE or Illumina analysis of

282   the microbiota of this traditional cheese, yet. In our research, the microbial ecology and active bacterial

283   communities of Liqvan cheese have been investigated using different culture-independent methods, at an

284   RNA level, during the ripening process.

285   Fifteen samples from raw milk, curd and cheese were collected from five different producers in different

286   households in the Liqvan village, East Azerbaijan province of Iran, the Mydanchay district of the central

287   part of the city of Tabriz, and were examined. The microbial investigation focused on bacteria population

288   targeting the V3 region of the 16S rRNA gene.

289   RNA-seq DGGE analysis of our samples indicates mainly that lactic acid bacteria mostly includes

290   *Lactococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp. and *Streptococcus* sp. are detected in all stages of

291   production while spoilage bacteria like S*taphylococcus* sp., *Enterobacter* sp. and *Pseudomonas* sp. are

292   detected only at milk samples and not in curds and cheese.

293   Phylogenetic assignment of NGS sequence data analysis revealed that microbial α-diversity was more

294   remarkable at milk level and corresponds to *Firmicutes, Proteobacteria* and *Actinobacteria* however, this

295   diversity of population decreased during ripening dominated by *Firmicutes*.

296   At the family level, *Moraxcellaceae, Pseudomonadaceae, Corynebacterineae, Flavobacteriaceae,*

297   *Comamonadaceae, Staphylococaceae* and *Enterobacteriaceae* were the most abundant contaminant

298   microbiota in the milk, while *Lactobacillaceae*, *Streptococcaceae* and *Leuconostocaceae* were the

299   predominant microbial families in the curds and cheese. There are some number of notable observations

300   among the subdominant populations. Some records from sheep feces, sheep wool, soil and grass

301   contaminants like those that *Brochothrix* sp. and *Rothia* sp. are also detected at milk samples nevertheless

302   there is no trace of them in curd and cheese. Furthermore, *Streptococcus* sp. especially *Streptococcus*

303   *thermophiles* were detected exclusively in curd samples while its population decreased remarkably in

304   cheese.

305   The microbial co-occurrence/exclusion patterns also proved these results, as there was a strong co-

306   exclusion effect between *Lactobacillaceae and Enterobacteriaceae,* as well as between *Lactobacillaceae*

307   and *Staphylococcaceae*. *Lactobacillus* species co-occurred each other. These results confirmed that the

308   LAB microbial populations tended to dominate the cheese microbiota and to limit the development of

309   spoilage bacteria, as has recently been demonstrated for other types of cheeses (Alessandria et al., 2016).

310   This diversity and dynamics of prokaryotes in this study are in agreement with the results that had

311   previously been reported for Liqvan and other semi hard raw milk based cheeses (Bozoudi et al., 2016;

312   Ryssel et al., 2015; Masoud et al., 2011; Rantsiou et al., 2008) although some differences were noted

313   which may related to difference in samples origin and producers.

314   The use of predicted metagenomes has proved to be useful for the observation of the putative gene

315   repertoires in the analyzed samples. The milk samples were characterized by gene profiles that were

316   related to the aminoacid metabolism, and high proteolitic activity due to the spoilage bacteria, while

317   putative genes characterized the curd and cheese related to carbohydrate depletion, which was presumably

318   associated with the nature of the highly competitive LAB that dominated during the ripening period.

319   In conclusion, in the present work, the study of microbial population with culture independent techniques

320   has provided a better understanding of the bacterial structure of Liqvan cheese during its processing and

321   ripening.

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462 **Table 1:** Identification of the selected bands from bacterial DGGE gels and their occurence in the samples
463 considered in this study

Bacterial species	Stage of manufacturing		
	Milk	Curd	Cheese
Lactococcus lactis ssp. *lactis*	+	+	+
Lactococcus lactis ssp. *cremoris*	-	+	+
Leuconostoc mesenteroides	+	+	+
Lactococcus raffinolactis	+	+	-
Lactobacillus pentosus	-	-	+
Lactococcus garvieae	-	+	+
Enterobacter aerogenes	+	+	_
Pseudomonas fluorescens	+	-	-
Lactobacillus fuchuensis	-	-	+
Staphylococcus sp.	+	-	-
Streptococcus thermophilus	-	+	-

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478   **FIG 1:** Relative abundance of the major taxonomic groups detected by means of 16S sequencing. Only OTUs with
479   an incidence above 0.5% in at least two samples are shown. The abundance of OTUs from the milks, curds and
480   cheeses was averaged.

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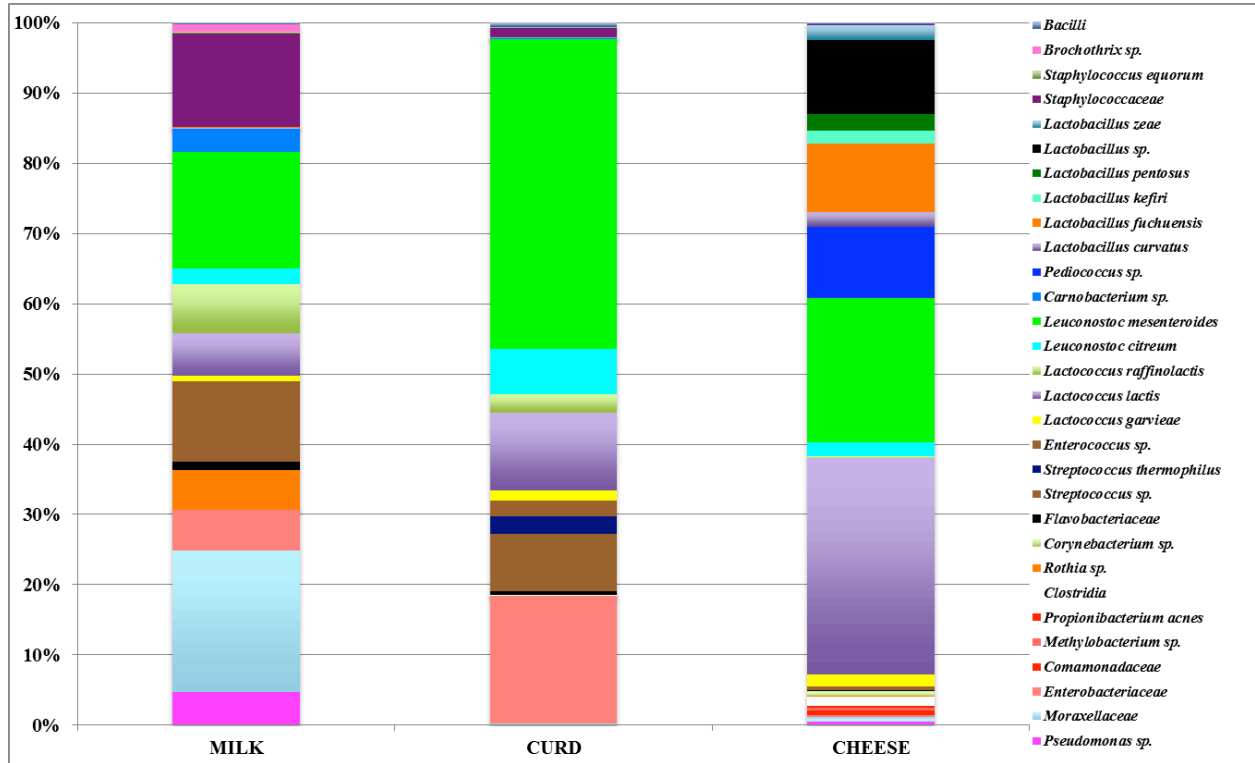
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FIG 2: Significant co-occurrence and co-exclusion relationships between bacterial OTUs. Spearman's rank
correlation matrix of OTUs with > 0.5% abundance in at least 2 samples. Strong correlations are indicated with
large squares, whereas weak correlations are indicated with small squares. The color of the scale bar denotes the
nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly
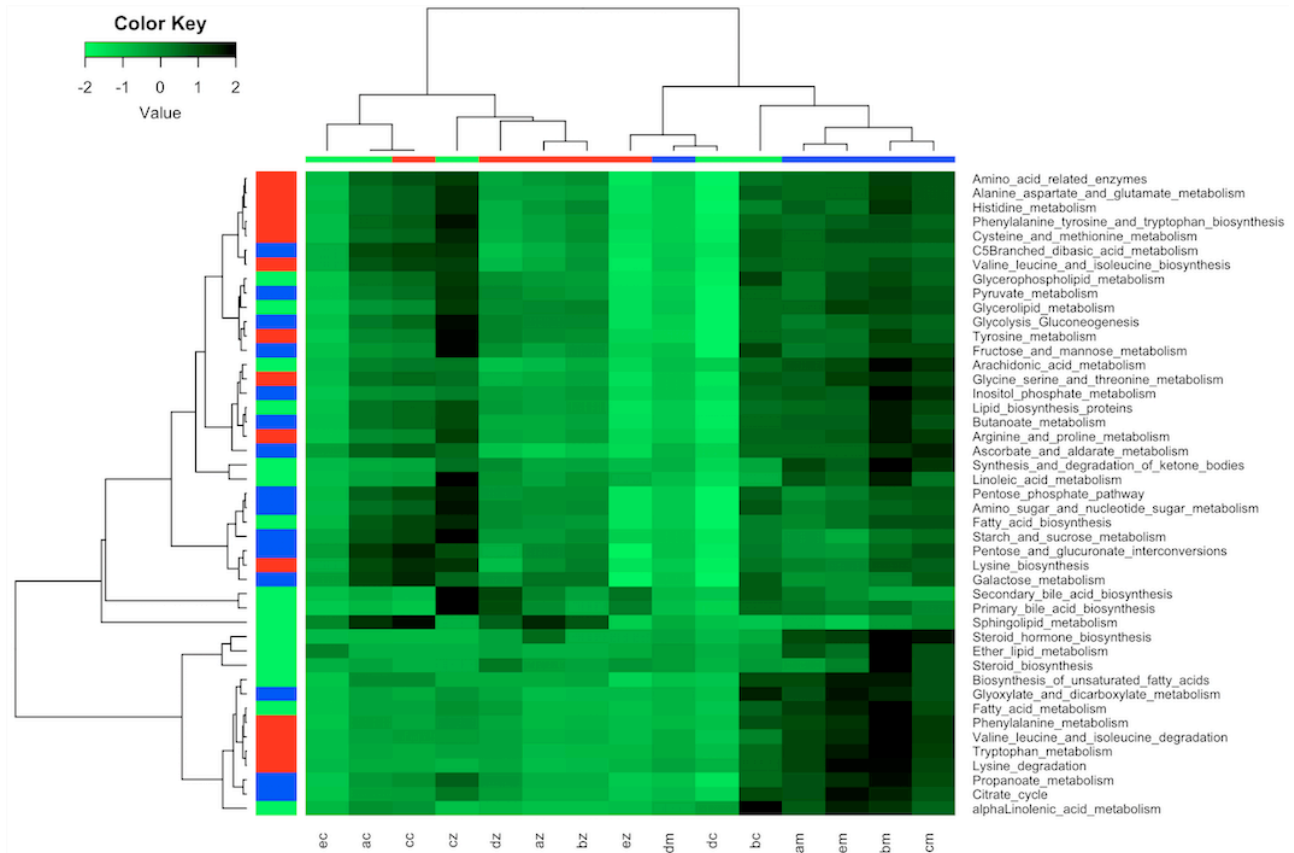negative correlation (dark red). Only significant correlations (FDR < 0.05) are shown.
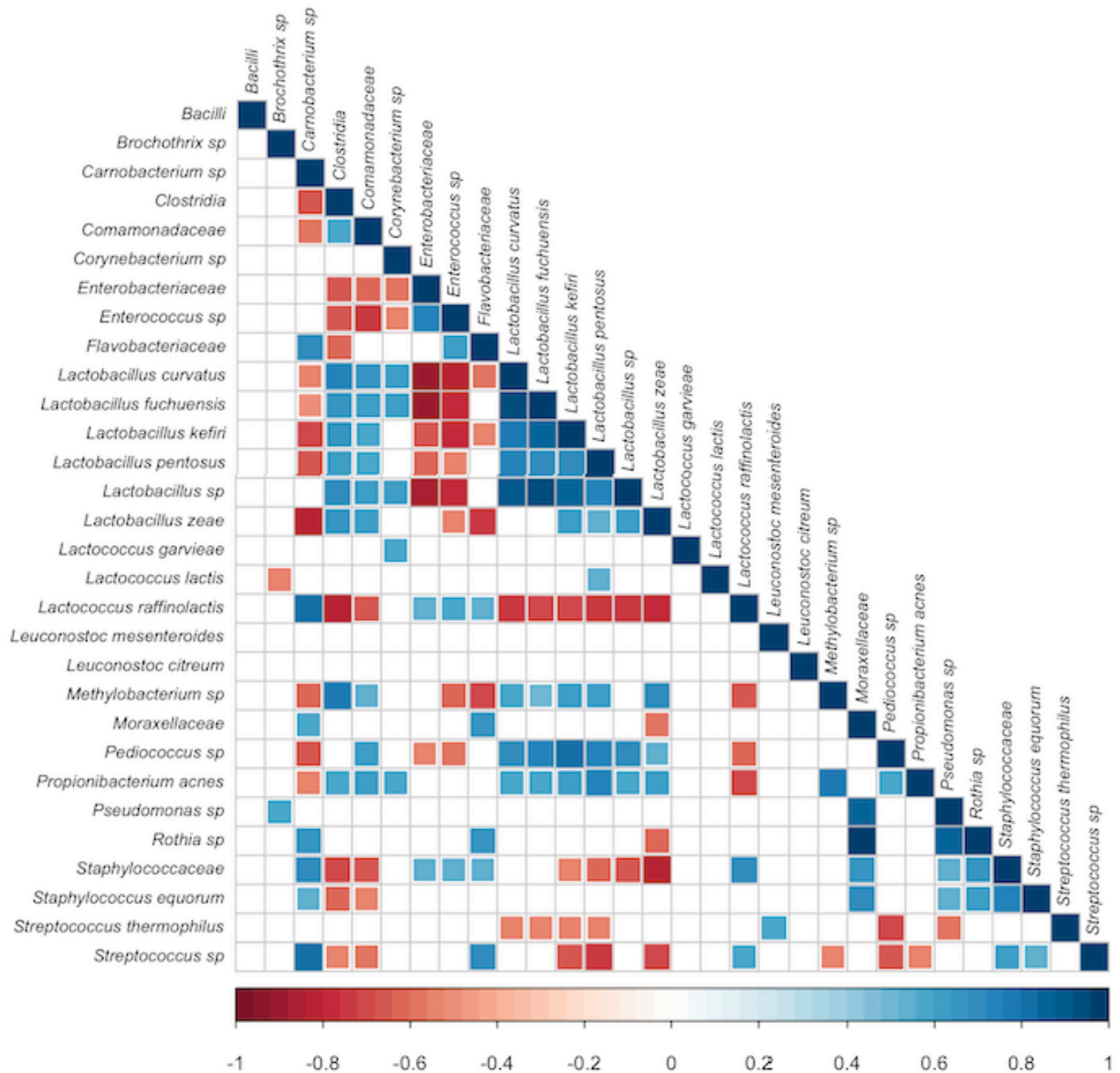
506 **FIG 3:** Heat plot of the abundances of genes presumptively belonging to carbohydrate (blue squares), amino acid
507 (red squares) and lipid (green squares) metabolism pathways in the milk (upper blue bar), curd (upper green bar) and
508 cheese (upper red bar). Rows and columns are clustered by means of Ward linkage hierarchical clustering. The
509 intensity of the colors represents the degree of correlation between the samples and KO as measured by Spearman's
510 correlations.

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