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1 **Specific metagenomic asset drives the spontaneous fermentation of Italian sausages**

2

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13

14

15 **Abstract**

16 Metagenomics is a powerful tool to study and understand the microbial dynamics that occur during
17 food fermentation and allows to close the link between microbial diversity and final sensory
18 characteristics. Each food matrix can be colonized by different microbes, but also by different
19 strains of the same species. In this study, using an innovative integrated approach combining
20 culture-dependent method with a shotgun sequencing, we were able to show how strain-level
21 biodiversity could influence the quality characteristics of the final product. The attention was placed
22 on a model food fermentation process: Salame Piemonte, a Protected Geographical Indication (PGI)
23 Italian fermented sausage. Three independent batches produced in February, March and May 2018
24 were analysed. The sausages were manufactured, following the production specification, in a local
25 meat factory in the area of Turin (Italy) without the use of starter cultures. A pangenomic approach
26 was applied in order to identify and evaluate the lactic acid bacteria (LAB) population driving the
27 fermentation process. It was observed that all batches were characterized by the presence of few
28 LAB species, namely *Pediococcus pentosaceus*, *Latilactobacillus curvatus* and *Latilactobacillus*
29 *sakei*. Sausages from the different batches were different when the volatilome was taken into
30 consideration, and a strong association between quality attributes and strains present was
31 determined. In particular, different strains of *L. sakei*, showing heterogeneity at genomic level,
32 colonized the meat at the beginning of each production and deeply influenced the fermentation
33 process by distinctive metabolic pathways that affected the fermentation process and the final
34 sensory aspects.

35

36 **Keyword:** volatile compounds, shotgun metagenomics, metabolic pathways, spontaneous
37 fermented sausages

38

39 1. Introduction

40 In the last ten years, in order to investigate and understand the microbial community structure of
41 different fermented foods, shotgun DNA sequencing has been applied (De Filippis F., La Stora A.,
42 Villani F., 2019; Ferrocino et al., 2018; Hellmann et al., 2020; Kobus et al., 2020). Nevertheless,
43 only few studies aimed at characterizing the microbiome of fermented foods and its link to the
44 quality properties of the final product through a metagenomics/pangenomics approach. A better
45 knowledge of the microbial resource is essential to characterize, track and monitor the genetic
46 microbial diversity and the spread of genetic traits (Lee, Whon, Roh, & Jeon, 2020; Moreno Zolfo,
47 Francesco Asnicar, Paolo Manghi, Edoardo Pasolli, 2018; Suárez, Weckx, Minahk, Hebert, &
48 Saavedra, 2020; Suo et al., 2020). Differences at strain level could influence the metabolic and
49 ecological adaptation capabilities of microbes and thereby how they could successfully occupy a
50 wide range of habitats (Eisenbach, Geissler, Ehrmann, & Vogel, 2019) and have a different impact
51 on the colonised environment. A pangenomic analysis could facilitate the comprehension of the
52 microbial ecology in food fermentation and the explanation of peculiarities that characterize
53 specific products by highlighting strain abundance and diversity (De Filippis et al., 2019;
54 Mataragas, 2020; Peng et al., 2018).

55 In this study, metagenomic DNA-seq, integrated with GC-MS analysis, was used to better
56 understand strains distribution through *in situ* monitoring of metabolic pathways and microbial
57 genes in spontaneously fermented Italian sausage. It is already known that during spontaneous
58 fermentation the presence of *Latilactobacillus sakei* is usually associated with the presence of
59 *Latilactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Pediococcus*, *Staphylococcus xylosus* and
60 *Staphylococcus equorum* (Greppi et al., 2015). These bacteria have a different role during the
61 maturation process. Coagulase-negative Staphylococcaceae (CNS) are responsible for proteolytic
62 and lipolytic metabolic activity on meat components, while lactic acid bacteria (LAB) for the
63 acidification process, due to lactic acid production, while they also deliver important volatile

64 organic compounds (acetic acid, ethanol, acetoin and pyruvic acid) (Ferrocino et al., 2018; Polka,
65 Rebecchi, Pisacane, Morelli, & Puglisi, 2014) through carbohydrate catabolism.

66 In order to discover the strain level variation an assembly-based approach is often applied, in this
67 way a comparison between the different assembled metagenomes could be perform. This approach
68 uses sequence reads assembly (contigs) that can be grouped and assigned to discrete population bins
69 (Dilthey, Jain, Koren, & Phillippy, 2019; Lunt et al., 2020; Quiñones et al., 2020) to generate
70 multiple whole genomes. A metagenome-assembled genomes (MAGs) phylogeny is a way through
71 which we can show the genetic variability after the assembly, mapping and binning process
72 (Karnachuk et al., 2020; C. Luo et al., 2015). Genetic variation is then described through single
73 nucleotide polymorphisms (SSNP) or using genes as units of comparison (Méric et al., 2014; Prior,
74 Harmsen, Mellmann, & Mellmann, 2020; Sheppard, Jolley, & Maiden, 2012). Different SSNP
75 profiles represent individual strains. The major limitation is that this approach is applicable only for
76 organisms with enough coverage to be assembled and binned, so only part of the genomes in a
77 complex community can be explored (C. Luo et al., 2015; Quince, Walker, Simpson, Loman, &
78 Segata, 2017).

79 To obtain a strain-level phylogeny reconstruction an assembly-free metagenomic profiling can be
80 done mapping the raw reads to reference genes or directly to contig bins. The principal advantage of
81 this method is the potential to perform large scale strain-level analyses, but uncharacterized bacteria
82 are difficult to profile (C. Luo et al., 2015; Quince et al., 2016, 2017). However, the genomes of the
83 strains reconstructed through this approach are characterized by lower quality than those obtained
84 directly from isolates (Segata, 2018). For this reason, it is always necessary to compare
85 metagenomics data with culture-dependent methods in order to confirm the level of diversity. It can
86 be possible to obtain a complete overview of the specific microbiome that characterizes a food
87 matrix only by a combination approach, to avoid the possibility to overestimate or underestimate
88 part of the obtained data.

89 With the final goal of enriching the current knowledge of strain biodiversity and its influence on the
90 final product quality, the present study takes as a model the ecosystem developing during
91 spontaneous fermentation of Salame Piemonte PGI, a fermented sausage from the North West of
92 Italy. A blended approach, employing culture-dependent methods and metagenomics, was chosen in
93 order to better link diversity of strains with volatile profiles of the investigated fermented sausages
94 (Greub, 2012).

95 The metagenomics approach adopted in this study has the capability to create a profile based on the
96 presence or absence of the genes in order to characterize the microbial organisms at single strains'
97 resolution (Manghi et al., 2018). This method was applied only seldom to a food matrix in the
98 understanding of strain dynamics during a fermentation process. However, this approach can shed
99 light on how the various microorganisms can influence food bioprocesses (Bora, Keot, Das, Sarma,
100 & Barooah, 2016). Better knowledge is necessary to investigate how we can control and obtain the
101 final sensory characteristics of the products by selecting the right consortia as starter cultures.

102

103 **2. Materials and methods**

104 **2.1. Sausages manufacturing**

105 Salame Piemonte PGI were manufactured in a local meat factory in the area of Turin according to
106 the production specification. The formulation used in the manufacturing included pork meat (lean
107 from the shoulder and fat from the belly), salt (maximum 3%); pepper (maximum 0.4%); spices and
108 aromatic plants (garlic, cloves, whole, crushed or infused with wine, nutmeg). Meat batter was then
109 stuffed into casings, resulting in sausages of about 35 cm long and 3 kg in weight. Fermentation and
110 ripening for 50 days were carried out in a climatic chamber (Ferrocino et al., 2018). Temperature
111 and relative humidity decreased from 21°C to 15°C and from 88% to 70% respectively, in the first
112 week of fermentation, after which the temperature was set up between 12-14°C and the relative
113 humidity as 74-88% until the end of the maturation process. The fermentation and ripening
114 program, as well as the recipe followed during manufacture, was the same for all the three batches.

115 Three independent batches of sausages produced in February, March and May without using the
116 starter culture were analyzed. At each sampling point (T4, T8, T15, T30 and T50 days of
117 fermentation) 3 sausages were collected placed in a sterile stomacher bag (Sto-circul-bag; PBI,
118 Milan, Italy) cooled at 4 degree and immediately transported to the laboratory for the following
119 analysis. A total of 15 samples for each batch were collected.

120

121 **2.2. DNA extraction**

122 At each sampling point, 3 aliquots of about 10 g from each sausage were collected from the core
123 and individually homogenized with 90 ml of buffered peptone water (Oxoid, Milan, Italy) for 2 min
124 in a stomacher (LAB blender 400; PBI, Italy).

125 One ml was collected and centrifugated in order to extract the total DNA from each sample. The
126 total DNA from fermented sausage was extracted using the RNeasy Power Microbiome kit
127 (QIAGEN Group) according to the manufacturer's instructions. One microliter of RNase (Illumina
128 Inc. San Diego. CA) was added to digest RNA in the DNA samples with an incubation of 1 h at
129 37°C.

130 Whole metagenomics (150 bp paired-end reads) was performed on a NextSeq 550 Illumina machine
131 by the Genewiz company (Leipzig, Germany). The analysis was done on the pool of DNA of the
132 three sausages from each sampling point.

133

134 **2.3. Metagenomics analysis**

135 Raw sequences were first mapped against the draft genome of *Sus scrofa domesticus* L., to remove
136 porcine sequences, using Bowtie2 (Langmead & Salzberg, 2012) in end-to-end sensitive mode.
137 Reads were quality filtered with Solexa v3.1.7.1 QA++ software (Cox, Peterson, & Biggs, 2010)
138 (Q<20) and by Prinseq v0.20.4 (reads <60bp and dereplicated) (Schmieder, 2011). The
139 phylogenetic characterization of the shotgun sequences was achieved at species level of taxonomy
140 by using MetaPhlAn2 (Segata et al., 2012) with default parameters.

141 Assembly was performed with MetaSPAdes v3.14.1 (Nurk, Meleshko, Korobeynikov, & Pevzner,
142 2017) while QUAST v5.0.2 (Gurevich, Saveliev, Vyahhi, & Tesler, 2013) software was used for
143 the quality check of the contigs. Genes prediction from each contig was performed by
144 MetaGeneMark v3.25 (Zhu, Lomsadze, & Borodovsky, 2010). The sausages gene catalog was
145 obtained using the pipeline describe by Ferrocino *et al.* (2018). Genes were concatenated and
146 clustered, using USEARCH v3.25 (Edgar, 2010), and aligned against the NCBI-NR database by
147 BLASTn tools. Clean reads were then mapped against the annotated catalog with Bowtie2 v2.4
148 (Langmead & Salzberg, 2012). The functional analysis against the KEGG database was conducted
149 using MEGAN (Mitra, Stärk, & Huson, 2011) software. The KEGG gene count table was internally
150 normalized in MEGAN with the function “normalized count”. The rarefaction analysis was
151 performed on selected genes with coverage and identity > 98% in MEGAN.

152

153 **2.4. *De novo* extraction of strain genomes from metagenomes**

154 MetaBat2 (Kang et al., 2019) software was used on contigs to reconstruct draft genomes (bins).
155 CheckM (Parks, Imelfort, Skennerton, Hugenholtz, & Tyson, 2015) software was then used to
156 evaluate completeness and contamination. Bins where then imported in Focus (Silva, Cuevas,
157 Dutilh, & Edwards, 2014) for taxonomic assessment. High quality bins where then imported in
158 PROKKA (Seemann, 2014) for gene prediction and annotation. Bins were classified following
159 Bowers et al (2017) standards as: high-quality draft (>90% completeness, <5% contamination),
160 medium-quality draft (>50% completeness, <10% contamination) or low-quality draft (<50%
161 completeness, <10% contamination). The pangenome calculation and phylogenetic analysis of bins
162 were obtained by Roary (Page et al., 2015).

163

164

165 **2.5. Microbial analysis**

166 Sausage homogenates were used for lactic acid bacteria (LAB) counts, a_w and pH determination.
167 Decimal dilutions in quarter-strength Ringer's solution were prepared and spread in triplicate on de
168 Man-Rogosa-Sharpe (MRS; Oxoid) agar incubated at 30°C for 48 h in anaerobic condition. Fifteen
169 colonies from MRS at each sampling point were randomly isolated and purified. The pH was
170 measured by pH probe of a digital pH meter (micropH2001; Crison, Barcelona, Spain) according to
171 the manufacturer's instructions.

172

173 **2.6. Molecular typing by rep-PCR LAB population**

174 LAB isolates were subjected to DNA extraction and genetic fingerprints were obtained by using
175 repetitive extragenic palindromic PCR (rep-PCR) with the (GTG)₅ primer according to Iacumin *et*
176 *al.* (2006). The rep-PCR profiles were normalized, and cluster analysis was performed using the
177 BioNumerics software (version 6.1; Applied Maths, Sint-Martens-Latem, Belgium). The
178 dendrograms were calculated on the basis of the Dice coefficient of similarity, with the unweighted
179 pair group method using average linkages (UPGMA) clustering algorithm. After cluster analysis, 2
180 isolates from each cluster at >80% of similarity were selected and subjected to identification. The
181 identification of LAB was performed by amplifying the 16S rRNA gene (Ercolini *et al.*, 2010).
182 Amplicons were sent for sequencing to GATC-Biotech (Cologne, Germany). To determine the
183 closest known relatives of the 16S rRNA gene sequences obtained, searches were performed in
184 public data libraries (GenBank) with the BLAST search program.

185

186 **2.7. Analysis of volatile organic compounds**

187 The volatile organic compounds (VOCs) in sausage samples were extracted using headspace (HS)
188 solid-phase microextraction (SPME) and analysed by gas chromatography-mass spectrometry
189 (GC/MS). All samples were analysed in triplicates. The analysis was conducted using a 20 ml vial
190 filled with 3 g of mixed sample to which 10 μ l of 2-octanol in ultrapure water (100 ppm) was added
191 as internal standard. A HS-SPME followed by GC/MS was carried out according to the SPME

192 extraction previously described (Ferrocino et al., 2018) by using a GC-2010 gas chromatograph
193 equipped with a QP-2010 Plus quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan)
194 and a DB-WAXETR capillary column with 30 m length, 0.25 mm internal diameter, 0.25 μ m film
195 thickness (J&W Scientific Inc., Folsom, CA). Semiquantitative data (μ g/kg) were obtained by
196 measuring the m/z peak area of the quantifier ion of each identified compound in relation to that of
197 the m/z ion of the added internal standard.

198

199 **2.8. Statistical analyses**

200 For taxonomic composition alpha diversity indices were calculated using the diversity function of
201 the vegan package (Dixon, 2003). Indices were analysed using the pairwise comparisons using
202 Wilcoxon rank sum test to assess differences between the batches. The OTU table obtained by
203 metaphlan2 was used to produce the Principal component analysis (PCA) in R environment
204 (www.r-project.org) by using the made4 package of R. ADONIS and ANOSIM statistical test was
205 used to detect significant differences in the overall microbial community by using the OTU table.
206 Not-normally distributed data were evaluated by Wilcoxon matched pairs test or the Kruskal-
207 Wallis test as appropriate.

208 The normalized KEGG gene table was used to obtain the Co-Abundance Gene Groups (CAGs)
209 by using the canopy-based algorithm. KEGG genes were clustered into CAGs and their abundance
210 was calculated as the sample-wise median gene abundance (Nielsen et al., 2014; Zhang et al.,
211 2015).

212 Determination of differentially abundant KEGG genes was conducted using the DESeq2 package
213 (Love, Huber, & Anders, 2014) in R as well as the GAGE Bioconductor package (Luo, Friedman,
214 Shedden, Hankenson, & Woolf, 2009) in order to identify genes and biological pathways
215 overrepresented or underrepresented between samples. P values were adjusted for multiple testing
216 using the Benjamini-Hochberg procedure, which assesses the false-discovery rate (FDR).

217 Pairwise Spearman's correlations between taxa, CAGs, and volatile organic compounds were
218 assessed by the R package *psych*, and the significant correlations ($R > 0.5$ or >-0.5 and $FDR < 0.05$)
219 were plotted in a correlative network by using Cytoscape v. 2.8.143 (Shannon et al, 2021). All the
220 results are reported as mean values of 3 replicates for sampling point.

221 The genes presence/absence table obtained from the reconstructed bins was used to calculate the
222 distance matrix on Bray Curtis's distance by the *vegdist* function in package *vegan* of R. The matrix
223 was used to build the principal coordinate analysis (PCoA) through the *dudi.pco* function in *made4*
224 package. The website iTOL v5 (Letunic & Bork, 2007) was used to visualize the phylogenetic trees
225 of the pangenome obtained through Roary (Page et al., 2015).

226 One-way ANOVA was used to analyze the effect of ripening time on the dependent variables (pH,
227 microbial count and volatilome data) separately for each batch. This statistical analysis was carried
228 out by the software IBM SPSS® Statistics 25 using the Duncan's Multiple Range Test (MRT) post
229 hoc test and 0.05 as level of significance.

230

231

232 **3. Results**

233 **3.1. Microbiota composition of sausages**

234 The microbiota composition through fermentation obtained by metaphlan2 showed the dominance
235 of *L. sakei* (around 40% of the relative abundance in all samples, Fig 1), followed by *L. curvatus*
236 (15%) and *P. pentosaceus* (15%). By comparing the three batches we can clearly observe a cluster
237 separation of the batches (Supplementary Fig 1). In detail samples manufactured in February were
238 characterized by the presence of *P. pentosaceus*, while *L. curvatus* were associated with samples
239 manufactured in March and May production was characterized by *L. sakei* (Fig 1).

240 Moreover, the alpha diversity indexes didn't show differences in terms of observed species and
241 *chao1* index, but only the Shannon index showed differences ($FDR < 0.05$) between samples
242 belonging to the February batch across time and between the three batches (Supplementary Fig 2).

243

244

245 **3.2. Functional diversity of sausages**

246 A total of 126.54 Gbp of raw reads were generated and after host (*Sus scrofa domesticus* L)
247 sequence removal and quality filtering 35.34 Gbp of clean reads were used for further analysis. For
248 each sample approximately 2.35 Gbp of clean reads were obtained (Supplementary Table 1). A *de*
249 *novo*-performed assembly generated a total of 1029546 contigs of more than 1000 bp in length,
250 with an average N50 of 1090.4 bp (Supplementary Table 1). The KEGG analysis assigned 2067
251 genes to 21 pathways. The pathway enrichment analysis performed through GAGE showed that
252 March samples had lower KEGG pathways related to the biosynthesis of amino acids, valine,
253 leucine and isoleucine biosynthesis, cysteine and methionine, amino sugar and nucleotide sugar,
254 alanine, aspartate and glutamate metabolism, one carbon pool by folate and quorum sensing related
255 genes, if compared against February and May samples. Moreover, by comparing May samples
256 versus February, May samples displayed higher KEGG genes related to butanoate, methane, sulfur
257 and pyruvate metabolism (data not shown).

258

259 **3.3. Different distribution of genes repertoire according to season**

260 Due to the observed different association between microbes and batches, a diverse distribution of
261 genes repertoire according to season was observed. This is probably due to the different natural
262 microbial composition of the meat. February sample (especially at the end of fermentation) showed
263 higher KEGG gene count (FDR <0.05) of alcohol dehydrogenase (EC:1.1.1.1) responsible for
264 ethanol production from acetaldehyde, butanediol dehydrogenase/diacetyl reductase (EC:1.1.1.4;
265 EC:1.1.1.303) involved in the production of acetoin, and D-lactate/ L-lactate dehydrogenase for the
266 production of lactate from pyruvate (EC:1.1.1.28; EC:1.1.1.27) (Supplementary Tables 2B and 2C).
267 The February batch also showed highest (FDR<0.05) counts of shikimate dehydrogenase
268 (EC:1.1.1.25) involved in the biosynthesis of folates and aromatic amino acids (phenylalanine,

269 tyrosine, tryptophan and indole) and tryptophan synthase (EC:4.2.1.20) involved in the
270 interconversion between serine and tryptophan and between tryptophan and indole (Supplementary
271 Tables 2A and 2B) derived from the amino acids metabolism. Branched-chain amino acid
272 aminotransferase (EC:2.6.1.42), as well as ketol-acid reductoisomerase (EC:1.1.1.86) coming from
273 amino acids metabolism and catalyzing the conversion from valine to methyl-oxo butanoate were
274 found most abundant in February samples.

275 The March batch displayed the highest counts of KEGG genes for carbohydrate metabolism like
276 acetate kinase (EC:2.7.2.1), aspartate-semialdehyde dehydrogenase (EC:1.2.1.11) and acetyl-CoA
277 C-acetyltransferase (EC:2.3.1.9) playing an important role in the production of propanoate and
278 butanoate (FDR <0.05, Supplementary Tables 2A and 2C). In addition, malate dehydrogenase
279 (EC:1.1.1.38) that boosted the conversion of pyruvate to malate and several KEGG genes involved
280 in the hydrolyzation of oligosaccharides as alpha-glucosidase (EC:3.2.1.20),
281 alpha-N-arabinofuranosidase (EC 3.2.1.55) were associated with March samples (Supplementary
282 Tables 2A and 2C).

283 March samples were also rich in KEGG genes of the amino acids metabolism related to biogenic
284 amine biosynthesis like agmatine deiminase (EC:3.5.3.12), thus taking part in the putrescine
285 synthase pathway, converting agmatine into N-carbamoyl-putrescine, a precursor of putrescine. In
286 addition, KEGG genes responsible for arginine interconversion, such as arginine deiminase
287 (EC:3.5.3.6) (Supplementary Tables 2A and 2C), were associated with March samples. Genes
288 belonging to glycerophospholipid metabolism, such as glycerol-3-phosphate dehydrogenase
289 (NAD(P)+) (EC:1.1.1.94) and glycerol-3-phosphate cytidyltransferase (EC:2.7.7.39)
290 (Supplementary Tables 2A and 2C), were more abundant in March samples.

291 May samples displayed at the end of fermentation the highest abundance of methylglyoxal synthase
292 (EC:4.2.3.3) and glutamate decarboxylase (EC:4.1.1.15) that from alanine route convert L-
293 glutamate in 4-aminobutanoato and ribokinase (EC:2.7.1.15) in the pentose phosphate pathway
294 (Supplementary Tables 2A and 2B).

295 May samples were also rich in threonine synthase (EC:4.2.3.1) and in genes of histidine metabolism
296 as histidinol dehydrogenase (EC:1.1.1.23), linked to the pentose phosphate pathway
297 (Supplementary Tables 2A and 2B). In addition, we observed the highest presence of
298 1,3-propanediol dehydrogenase (EC:1.1.1.202), a key KEGG gene involved in the lipid metabolism
299 that allows the interconversion of propanal to propanol (Supplementary Tables 2A and 2B).

300

301 **3.4. Co-Abundance Gene Groups (CAGs)**

302 We established co-abundance associations of KEGG genes and then clustered into sixteen co-
303 abundance groups (CAGs) in order to identify signature patterns in the different batches
304 (Supplementary Fig 3). CAG2 and CAG3 were associated with the February batch and CAG6 with
305 the March batch (FDR < 0.05). In details CAG6 was composed by KEGG genes related to pyruvate
306 metabolism and arginine and proline metabolism, in particular, agmatine deiminase (EC:3.5.3.12),
307 involved in the biogenic amines production. CAG2 and CAG3 were related to sulphur metabolism,
308 phenylalanine, tyrosine and tryptophan biosynthesis, methane metabolism, glycolysis, butanoate
309 metabolism, pentose phosphate pathway and fatty acid biosynthesis.

310

311 **3.5. Strain-Level Differences association - Draft genomes by metagenome binning**

312 Several bins were identified in the sausage metagenomes. We found the presence of 6, 7 and 9 *L.*
313 *sakei* draft genomes in the batches of February, March and May respectively; 5 and 2 *L. curvatus* in
314 the batches of March and February, respectively; 6, 2 and 5 *P. pentosaceus* in the batches of
315 February, March and May, respectively (Supplementary Table 3A). The genomes of *P. pentosaceus*
316 were reconstructed mainly from the February batch, while genomes of *L. curvatus* and *L. sakei*
317 from the batches in March and May, respectively (Supplementary Table 3A). Following Bowers et
318 al (2017) only 2 strains of *L. sakei* were classified as high quality, 4 medium quality and the other
319 one low quality; for *L. curvatus* 6 were medium quality and one low quality; *P. pentosaceus* were
320 classified as 2 high quality, 5 medium quality and 6 low quality. The three groups of reconstructed

321 genomes *L. sakei*, *L. curvatus* and *P. pentosaceus* (Fig 2) showed specific genes associated with
322 each group of strains. We found that D-lactate dehydrogenase genes were present only in *P.*
323 *pentosaceus* genomes and enolase and mevalonate kinase genes showed the highest prevalence for
324 this species. *L. sakei* genomes displayed the highest presence of 3-dehydroquinate dehydratase
325 (belonging to the biosynthesis of secondary metabolites pathways), arginine deiminase and
326 carbamate kinase 1 (belonging to the arginine biosynthesis pathways), lactose permease and L-
327 lactate dehydrogenase genes. Interesting phosphate propanoyl transferase and propanediol
328 dehydratase genes (belong to propanoate metabolism pathways) were found in *L. curvatus* and *P.*
329 *pentosaceus* genomes, but were absent in *L. sakei* genomes. Acetolactate synthase and adenine
330 deaminase genes showed the lowest presence in *L. sakei* genomes.

331 *L. sakei* and *P. pentosaceus* genomes were associated with key genes of glycolysis and pyruvate
332 metabolism (6-phosphofructokinase, pyruvate kinase, pyruvate oxidase) and pentose phosphate
333 pathway (glucose-6-phosphate 1-dehydrogenase, ribokinase). The presence of these genes was low
334 in *L. curvatus* genomes.

335

336 **3.6. *L. sakei* strain-level differences**

337 It is well known that fermented sausages are an ecological niche for *L. sakei* and in order to
338 discover its potentials during fermentation we further analysed in depth its genome content. For this
339 purpose, to confirm the results obtained by the binning methods after metagenome assembly,
340 contigs belonging to *L. sakei* 23K (1.99 Mbp in size), used as reference strain, were extracted.
341 Reconstructed genomes from contigs of each sample displayed the presence of a part of the full
342 genome (due to assembly and coverage limitations) of about 1.33 Mbp in median value
343 (Supplementary Table 3B). Principal coordinates analysis (PCoA) (Supplementary Fig 4) based on
344 shell and cloud genes of the 15 reconstructed *L. sakei* clearly highlights a separation of the strains
345 based on the production batch due to a different genes patterns.

346 Based on occurrence of *L. sakei* gene repertoire we observed that genomes reconstructed from
347 March samples had several genes with higher prevalence, if compared with February and May
348 samples (Fig 3). In detail, genes involved in glutamate metabolism and pentose phosphate pathway
349 like pyruvate transaminase and ribokinase were dominant in this batch. Moreover, the lowest
350 presence of agmatine deiminase and putrescine carbamoyl-transferase gene (genes involved in the
351 agmatine deiminase pathway) was determined. Other genes not observed in May-associated *L. sakei*
352 genome ($P < 0.05$) were: carbamate kinase gene (involved in purine metabolism, glutamate
353 metabolism, arginine and proline metabolism and nitrogen metabolism), ribokinase and lactose
354 permease.

355

356 **3.7. Volatilome profile of fermented sausages**

357 The data showed a specific signature of the volatilome that characterized each batch
358 (Supplementary Table 4). In detail the March batch showed the highest values of 2-butanone,
359 methyl propionate, ethyl propanoate, 2-butanol, 2,3-pentanedione, hexanal, methyl hexanoate, 2,3-
360 octanedione, propanoic acid, 2-octen-1-ol and hexanoic acid ($P < 0.05$, Supplementary Fig 5). In
361 addition, the three batches at the end of ripening were different ($P < 0.05$) for the concentration of
362 the following compounds: isobutyric acid showed the lowest values in February samples, ethyl
363 isovalerate had the lowest values in samples from March, and octanal, ethyl lactate and butanoic
364 acid had the lowest values in May samples (Supplementary Fig 5).

365

366 **3.8. Correlations between microbiota, VOCs and CAGs**

367 Our understanding of sausage microbiome is a strict connection between microbes' function and
368 metabolomic development. In our study we observed that February samples at end of ripening
369 showed high level of ethanol and ethyl lactate and the presence of these VOCs were linked to the
370 high abundance of the KEGG genes of alcohol dehydrogenase (EC:1.1.1.1) and D-lactate

371 dehydrogenase (EC:1.1.1.27) (Fig 4A) belonging to CAG2 and CAG3 groups respectively. The
372 genome analysis of *P. pentosaceus* confirmed the specific presence of the D-lactate genes (Fig 2).
373 In addition, we observed that the March batch showed high levels of butanoic acid, methyl
374 propionate, propanoic acid and ethyl propanoate (Supplementary Fig 5). The presence of these
375 VOCs was confirmed by the high abundance of the KEGG genes belonging to butanoate and
376 propanoate pathways (CAG6 group) (Fig 4B). Genes belonging to propanoate pathways were found
377 also in *L. curvatus* genomes.

378 In order to have a better comprehension of this fermented food model, a correlation network (Fig 5)
379 based on microbiota, VOCs and CAGs was visualized. Through the network it was determined that
380 the presence of specific species was positively or negatively correlated to specific VOCs and CAGs.
381 In fact, focusing only on the three species that characterized each batch, we could observe that *P.*
382 *pentosaceus* was positively correlated with hexanal, heptanone, acetoin, ethyl alcohol, heptanal,
383 nonanal, octanal as well as with CAG2, CAG3 and CAG5 (Fig 5, FDR<0.05). *L. sakei* was
384 positively correlated to ethyl isovalerate and negatively correlated to propanoic acid, acetic acid and
385 dimethyl sulfone. *L. curvatus* showed a positive correlation with CAG6 and a negative correlation
386 to heptanal, ethyl isovalerate and CAG5 (Fig 5, FDR<0.05).

387

388 **3.9. Microbiota evolution and culture dependent strain diversity of LAB population**

389 During the first 4 days of ripening (Supplementary Table 5) the pH values were around 5 for each
390 batch. This value remains quite constant until the end of the fermentation process, with the March
391 batch maintaining a higher pH if compared with the others (Supplementary Table 5, P<0.05).
392 Regarding the LAB population, by culture dependent approach, we observed a fast load increase in
393 the first 4 days of fermentation around 9 Log CFU/g with no further increase till the end, with the
394 highest values in May samples (Supplementary Table 5, P<0.05).

395 By culture dependent approach we isolated 224 strains from MRS plates. The results of the
396 identification of the colonies isolated from the three batches showed that: February samples was

397 characterized by 77% *L. sakei* and 23% *P. pentosaceus*; March samples showed 62% *L. sakei* and
398 32% *L. curvatus*; and May samples displayed 97% of *L. sakei* and 3% of *P. pentosaceus* (data not
399 show). It should be pointed out that *L. curvatus* was never isolated from May and February samples
400 while *P. pentosaceus* was never isolated from March samples. The molecular characterization
401 performed by rep fingerprinting approach on the main population isolated from all batches
402 belonging to *L. sakei* showed the presence of 51 rep-biotypes. In detail it was possible to observe
403 that March samples showed the highest presence of unique *L. sakei*, while May and February
404 samples had several common *L. sakei* rep-biotypes (data not show). This trend was also observed
405 for the pH, in fact the batch from March showed different values at the last two sampling times
406 (Supplementary Table 5, $P > 0.05$).

407

408 **4. Discussion**

409 We characterized the metagenomes of spontaneous fermented sausages in order to provide evidence
410 that the presence of different strains can affect specific genomic repertoires and affect the sensory
411 characteristics of the final product. Since all the production parameters (ripening temperature,
412 ingredients and process procedure) were standardized between the three months, the differences
413 shown in the three batches at the end of the ripening were due to the different metagenome content
414 deriving from the natural meat microbial composition and its development.

415 Different VOCs between the batches were observed at the end of the fermentation. This is probably
416 due to the different initial meat microbial composition that are influenced by the season, but also by
417 the slaughter and transport conditions, manufacture operations and factory environments. A huge
418 impact on the VOCs formation and the development of the typical aroma can be also attributed to
419 enzymes activity (mainly peptidases and lipases) originated from endogenous (meat) or microbial
420 origins (Carrapiso, Amaro-Blanco, Manzano, Delgado-Adámez, & Ramírez, 2021; Toldrà, 1998;
421 Wang et al., 2021). As previously reported those enzymes can be involved in lipid oxidation with
422 the formation of free fatty acids, ketones and free alcohols. Myristic, linolenic and oleic acids are

423 often generated in greater amounts and strictly connect with the type of animal' feeding (Toldrà,
424 1998).

425 The metagenome reconstruction, although incomplete for some genes, showed that the highest
426 number of *L. sakei* strains were extracted from the metagenome of the batch from May, *P.*
427 *pentosaceus* from the one from February samples and *L. curvatus* from March samples. Data from
428 culture dependent methods confirmed this observation. *L. curvatus* was isolated only from March
429 samples while *P. pentosaceus* and *L. sakei* constituted the majority of the isolates from February
430 and May samples respectively.

431 The alpha diversity index (Shannon index) showed the highest value for February samples
432 compared to the other batches, this means that February samples showed the highest species
433 richness. In particular, February samples were rich in *P. pentosaceus* strains and showed its high
434 acidification capability of quickly lowering the pH, as already reported by Aro *et al.* (2010). This
435 capability could be a positive survival strategy in fermented food and a positive quality for the use
436 of this species as a starter culture in fermented meat for consumers that prefer high acidity products
437 (Kingcha *et al.*, 2012; Nur & Aslim, 2010; Porto, Kuniyoshi, Azevedo, Vitolo, & Oliveira, 2017).
438 February samples showed also the highest value for specific volatile compounds related to herbs
439 and floral note (e.g. octanal) (Olivares, Navarro, & Flores, 2009). Aldehydes are considered fresh
440 and agreeable at low levels, but unpleasant and rancid when concentrations rise; alcohols and esters
441 are essential in order to obtain the proper fermented sausage aroma by adding fruity and sweet notes
442 to the aroma (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Stahnke, 1994). We observed
443 in February samples the highest level of alcohol dehydrogenase (EC:1.1.1.1) and D-lactate
444 dehydrogenase (EC:1.1.1.27) that boosted the production of volatile compounds like ethanol and
445 ethyl lactate. *P. pentosaceus* is responsible for this behavior due to the presence of the D-lactate
446 dehydrogenase gene. The importance of D-lactate dehydrogenase gene in *Pediococcus* sp. is related
447 to the fact that is a key gene involved in the production of 3-Phenyllactic acid (PLA), a novel
448 antimicrobial molecule used to extend the shelf life of food (Mu, Yu, Jiang, & Li, 2012; Yu *et al.*,

449 2014). February samples also showed the highest counts of shikimate dehydrogenase (EC:1.1.1.25)
450 belonging to the shikimate pathway. The shikimate is an intermediate in the chorismate pathway,
451 which serves as a branching point toward the biosynthesis of aromatic amino acids and pABA
452 (Gupta, Reizman, Reisch, & Prather, 2017). Since *P. pentosaceus* strains were more present in the
453 samples from the February batch, we could assume that the high presence of these volatile
454 compounds in February samples was linked to the metabolic activity of this species. Some studies
455 report that the inoculation of *P. pentosaceus* could prevent an excessive lipid oxidation that can be
456 the cause of quality deterioration in meat products (off-flavor). In addition *P. pentosaceus* strains
457 could also promote the formation of alcohols originating from the amino acid catabolism, for
458 example the formation of ethanol from the reduction of acetaldehyde in the presence of alcohol
459 dehydrogenase (Chen, Kong, Sun, Dong, & Liu, 2015; Chen, Liu, Sun, Kong, & Xiong, 2015). In
460 fact, Figure 5A shows acetaldehyde production and consequently ethanol production from fatty acid
461 metabolism.

462 *L. curvatus* was observed as a key species in March samples, where genes related to agmatine
463 deiminase (EC:3.5.3.12) confer acid resistance in LAB species (Ammor & Mayo, 2007; Lucas et
464 al., 2007). Regarding the metabolome profile, we observed that methyl propionate, butanoic acid,
465 propanoic acid and ethyl propanoate were abundant due to the highest presence of KEGG genes
466 related to propanoate and butanoate metabolism. These specific metabolites confer to the sausages
467 apricot taste (2-butanone), butter and cheese flavor (ethyl propionate, ethyl propanoate, 2-butanol,
468 2,3-pentanedione) (Olivares et al., 2009) and grass, fatty and fruity sweet odor (hexanal) (Casaburi
469 et al., 2015). The dry sausage aroma is often associated with the dominance of 2-methyl ketones,
470 whereas a rancid aroma is associated with high concentration of hexanal (Montel, Masson, & Talon,
471 1998). Diacetyl, acetic acid and hexanal are also associated with buttery, vinegar and green odor
472 notes, respectively (Rimaux et al., 2012). Samples from the batch of March were considered
473 unacceptable from a sensorial point of view, due to the presence of hexanal almost three times
474 higher compared with the other batches. It was already reported that a strong presence of *L.*

475 *curvatus* could be the cause of an unusual smell (off-flavors) linked to the perception of ‘too strong’
476 (Visessanguan et al., 2006). Visessanguan *et al* (2006) reported that the off-flavours observed in
477 sausages inoculated with high concentrations of *L. curvatus* were due to a higher presence of free
478 fatty acids connected with a strong lipid oxidation. On the other hand, it is not easy to link the
479 higher presence of hexanal and of the other VOCs found in March samples only to the *L. curvatus*
480 activity. Some works underline a similar metabolic activity between *L. curvatus* and *L. sakei* strains
481 (Chen, Kong, Han, Xia, & Xu, 2017; Freiding, Gutsche, Ehrmann, & Vogel, 2011; Tabanelli et al.,
482 2012) and consequently the similar development in metabolites. On the contrary, in our study the
483 genes belonging to CAG6 group (genes belonging to butanoate and propanoate pathways) were
484 associated to *L. curvatus*, which was isolated only in March samples. From genome reconstruction
485 we observed the presence of genes related to the propanoate metabolism associated with *L. curvatus*
486 and we can speculate its ability to push the propanoic and butanoic route with the consequential
487 formation of the relative VOCs that we found in high concentration in the March batch especially at
488 the end of ripening.

489 *L. sakei* was positively correlated to ethyl isovalerate production, especially in May samples. In
490 fact, in May samples the highest presence of isobutyric acid and ethyl isovalerate was determined.
491 Isobutyric acids can originate from valine, leucine and isoleucine and their description is related to
492 sweet, sickly and malty odor (Dainty, Edwards,, & Hibbard, 1985). *L. sakei* strains belonging to the
493 May batch showed the lowest occurrence of acetate kinase gene (a key gene in the glycolysis
494 pathway), carbamate kinase (involved in purine, glutamate, arginine and proline and nitrogen
495 metabolism), lactose permease, putrescine carbamoyl-transferase (involved in the agmatine
496 deiminase pathway) and ribokinase (involved in the pentose phosphate pathway). Since all these
497 genes belong to the main route for the production of aromatic VOCs, we can expect less aromatic
498 flavors in the product obtained in May. A confirmation of this hypothesis came from the volatilome
499 profile at the end of fermentation where the presence of 2-butanone, methyl propionate, ethyl
500 alcohol, ethyl propanoate, 2-butanol, propanoic acid, butanoic acid and hexanoic acid were lowest.

501 Our pangenomic analysis highlighted the presence of specific fermentation-driven strain-level
502 profiles of *L. sakei*. The reconstructed genomes from March samples had the higher prevalence of
503 genes like ribokinase and key genes for the glycolysis and pentose phosphate metabolism. On the
504 contrary *L. sakei*-genomes from May samples showed a lower prevalence of these specific genes
505 and a consequently low presence of volatile compounds interesting for the final sensory properties.
506 Our analysis revealed that the majority of the completely reconstructed strains belonged to *L. sakei*
507 and that most of the key enzymes related to volatile compounds metabolism were present in *L. sakei*
508 genomes. This confirms that this species dominates the microbiota of the three analyzed batches of
509 Salame Piemonte and could have a central role for the VOCs formation.

510 The fundamental role of *Lactobacillus* species during dry fermented meat was already report in
511 literature. Many studies underlined that the presence of specific LAB species can have an impact on
512 the VOCs formation, for example the presence of specific strain of *L. sakei* can increase the
513 presence at the end of the fermentation of some nitrogen and sulfur compounds (Luongo,
514 Giagnacovo, Fiume, Iorizzo, & Coppola, 2001) or specific strains of *Lacticaseibacillus casei* can
515 improve significantly the content of total volatiles and in particular of esters and alcohol (Sidira,
516 Kandylis, Kanellaki, & Kourkoutas, 2015). The presence of *L. plantarum* and *S. xylosum* strains
517 increased the free fatty acids (FFAs) and free amino acids (FAAs) contents with a consequent
518 improving the flavour of sausages (Xiao, Liu, Chen, Xie, & Li, 2020).

519 Our study on naturally fermented sausages confirmed the link between the final volatile profile with
520 a single LAB species: *L. sakei*, *P. pentosaceus* and *L. curvatus*. For example, at the end of the
521 fermentation process, samples obtained from March showed the presence of unpleasant metabolites
522 correlated to the presence of *L. curvatus*.

523 The different metabolomic characteristics of the batches were not only linked to the species level,
524 but also to the strain-level biodiversity, in particular, in case of *L. sakei*. We could suppose that the
525 microbiota composition of the three batches were connected to the starting microbiota from the
526 meat, who was influenced also from the season. The data from culture dependent and independent

527 approach showed a clear association of the LAB species with the three batches analyzed, i.e., *L.*
528 *sakei*, *L. curvatus* and *P. pentosaceus* with May, March and February samples respectively.
529 Obtaining specific information on microbiota composition, not only at the species level, but also on
530 single strains helps to better understand the complex microbial system in fermented foods.

531

532

533 **5. Conclusion**

534 In conclusion, at the end of the fermentation process, samples obtained from March showed the
535 presence of unpleasant metabolites correlated to the presence of *L. curvatus*. The different
536 metabolomic characteristics of the batches were not only linked to the species level, but also to the
537 strain-level biodiversity, in particular, in case of *L. sakei*. We could suppose that the microbiota
538 composition of the three batches were connected to the starting microbiota from the meat,
539 influenced also from the season. We observed a clear distribution of the LAB species in the three
540 batches analyzed. Obtaining information on single strains, but also on the interaction between
541 strains belonging to the same species, helps to better understand the complex microbial system in
542 fermented foods.

543

544 **Data availability:** Sequences have been uploaded to the National Center for Biotechnology
545 Information Sequence Read Archive (Bioproject ID PRJNA636619).

546

547 **Authors' contributions:** LC, IF and KR conceived and designed the experiment. IFR, IF and MG
548 collected the experiments data. MG performed the metabolomic investigations. IF carried out the
549 bioinformatics analyses and generated the manuscript figures. IFR and IF performed the statistical
550 analysis. LC, K.R and JM supervised the data analysis and contributed to manuscript preparation.
551 IFR and IF wrote the first draft of the manuscript. All authors critically reviewed the manuscript for
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553

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561

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563

564 **References**

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819

820 **FIGURE LEGEND**

821

822 **Fig 1:** Circular ideogram showing the microbiota distribution among sausages from February,
823 March and May batch. Taxa and samples are connected with a ribbon, and its thickness is
824 proportional to the abundance of an taxa in the connected sample. The outer circle displays the
825 proportion of each taxa in a given sample and vice versa. For each batch the significant taxa
826 associated were highlighted in blue (FDR < 0.05, pairwise comparisons using Wilcoxon rank sum
827 test).

828

829 **Fig 2:** Occurrence (%) of genes involved in metabolic pathway genes in *Latilactobacillus sakei*
830 (LS), *Latilactobacillus curvatus* (LC), *Pediococcus pentosaceus* (PP) reconstruct genomes. Dark
831 blue color corresponds to the total presence (100%) of gene and white colour to the total absence
832 (0%) of the gene, the intermediate color shades express the occurrence (%) of the gene in the
833 correspondent bacterial species.

834

835 **Fig 3:** Occurrence (%) of genes involved in metabolic pathway genes in reconstructed genomes of
836 *Latilactobacillus sakei* between the three batches. Dark blue color corresponds to the total presence
837 (100%) of gene and white color to the total absence (0%) of the gene, the intermediate color shades
838 express the occurrence (%) of the gene in the correspondent batch.

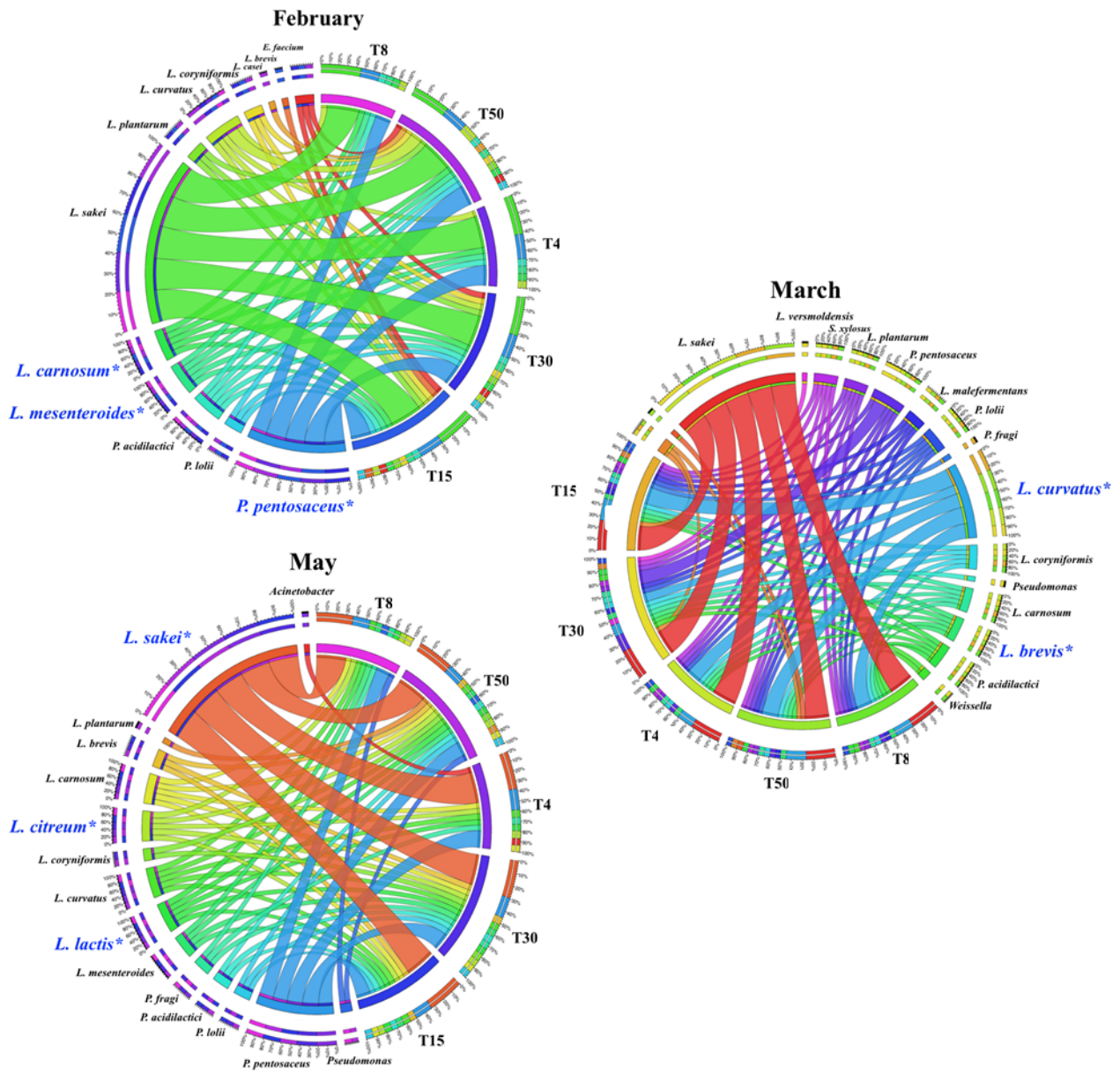
839

840 **Fig 4:** Biosynthesis of volatile compounds from pyruvate (panel **A**) and aspartate (panel **B**). Only
841 KEGG genes identified in the samples analyzed are reported. The graph showed volatile compound
842 after 50 day of fermentation and KEGG gene (and CAG) associated with February production
843 highlighted in red and March production highlighted in blue.

844

845 **Fig 5:** Correlation between volatilome data (yellow circle), taxa (orange triangle) and CAGs (blue
846 rhombus). Correlation network showing significant (false-discovery rate FDR <0.05) Spearman's
847 correlations between KEGG genes, VOCs, and taxa. Node sizes are proportional to the numbers of
848 significant correlations. Colours of the edges indicate positive (blue) or negative (red) correlations.
849

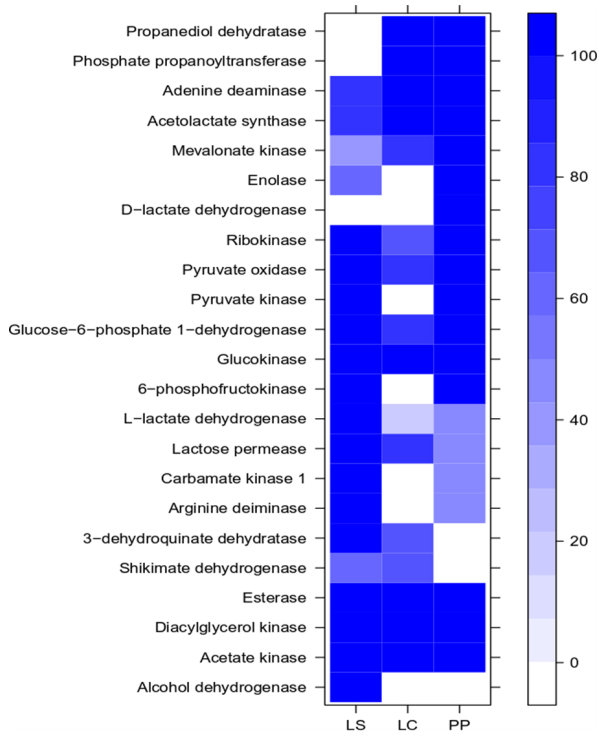
850
851 **Fig 1:**



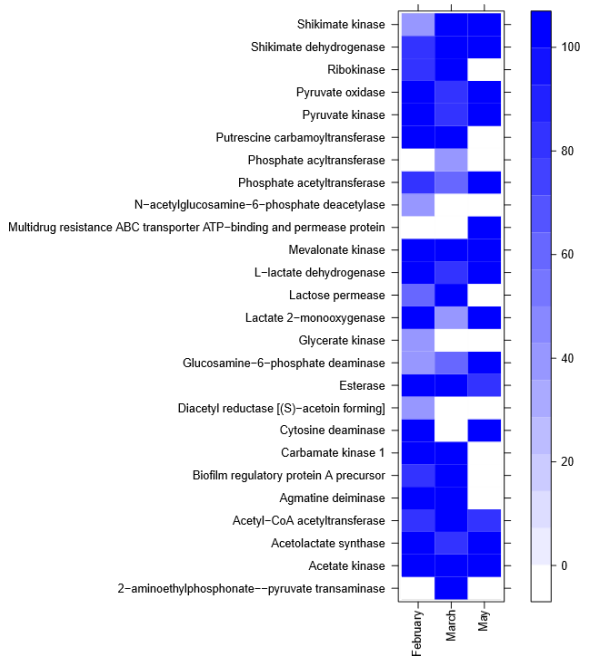
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855 **Fig 2:**

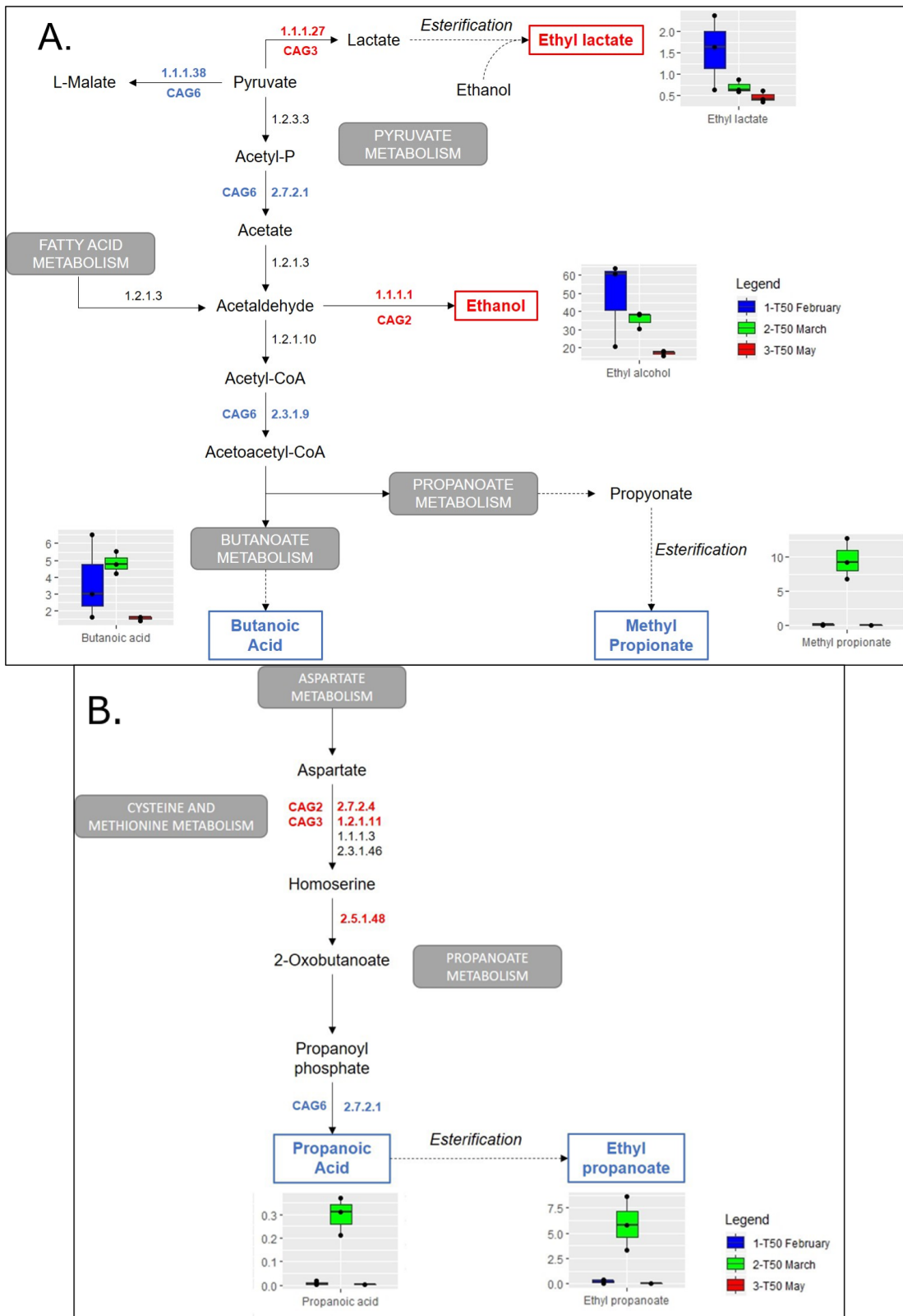


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857 **Fig 3:**



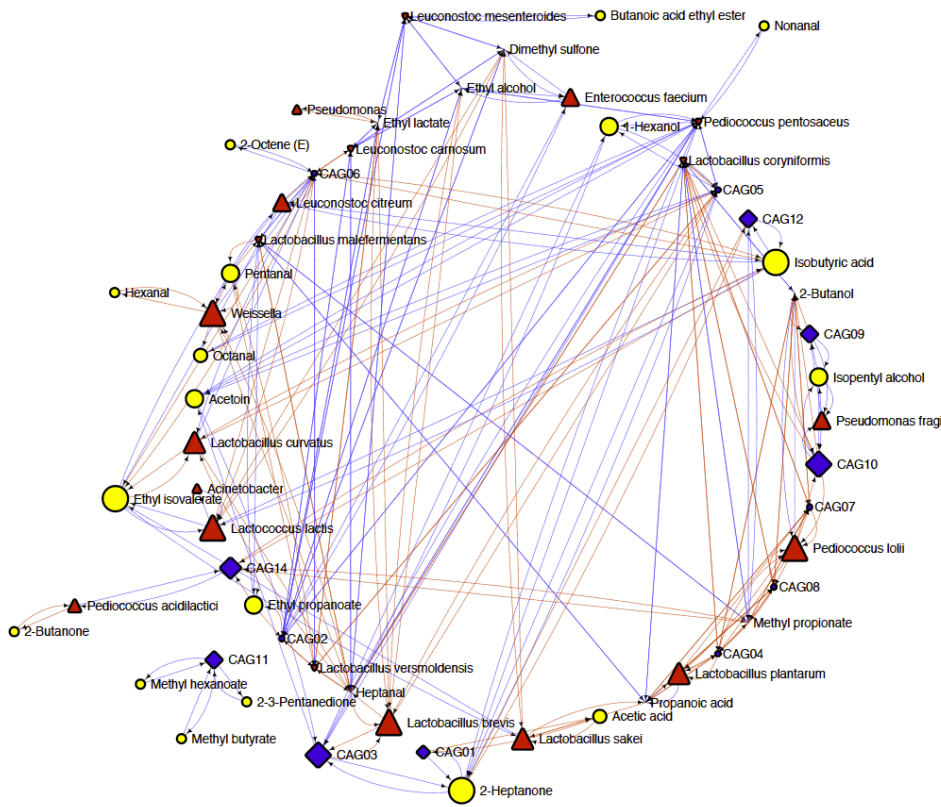
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861 **Fig 4:**



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865 Fig 5:



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