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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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 µl 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 μm film
195 thickness (J&W Scientific Inc., Folsom, CA). Semiquantitative data (μg/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 obtained 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).

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

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

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

3. Results

3.1. Microbiota composition of sausages

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

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

3.2. Functional diversity of sausages

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

3.3. Different distribution of genes repertoire according to season

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

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

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

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

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

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

3.4. Co-Abundance Gene Groups (CAGs)

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

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

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

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

4. Discussion

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

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

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

5. Conclusion

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

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

Authors' contributions: LC, IF and KR conceived and designed the experiment. IFR, IF and MG collected the experiments data. MG performed the metabolomic investigations. IF carried out the bioinformatics analyses and generated the manuscript figures. IFR and IF performed the statistical analysis. LC, K.R and JM supervised the data analysis and contributed to manuscript preparation. IFR and IF wrote the first draft of the manuscript. All authors critically reviewed the manuscript for intellectual content and gave final approval for the version to be published.

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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

Fig 1:

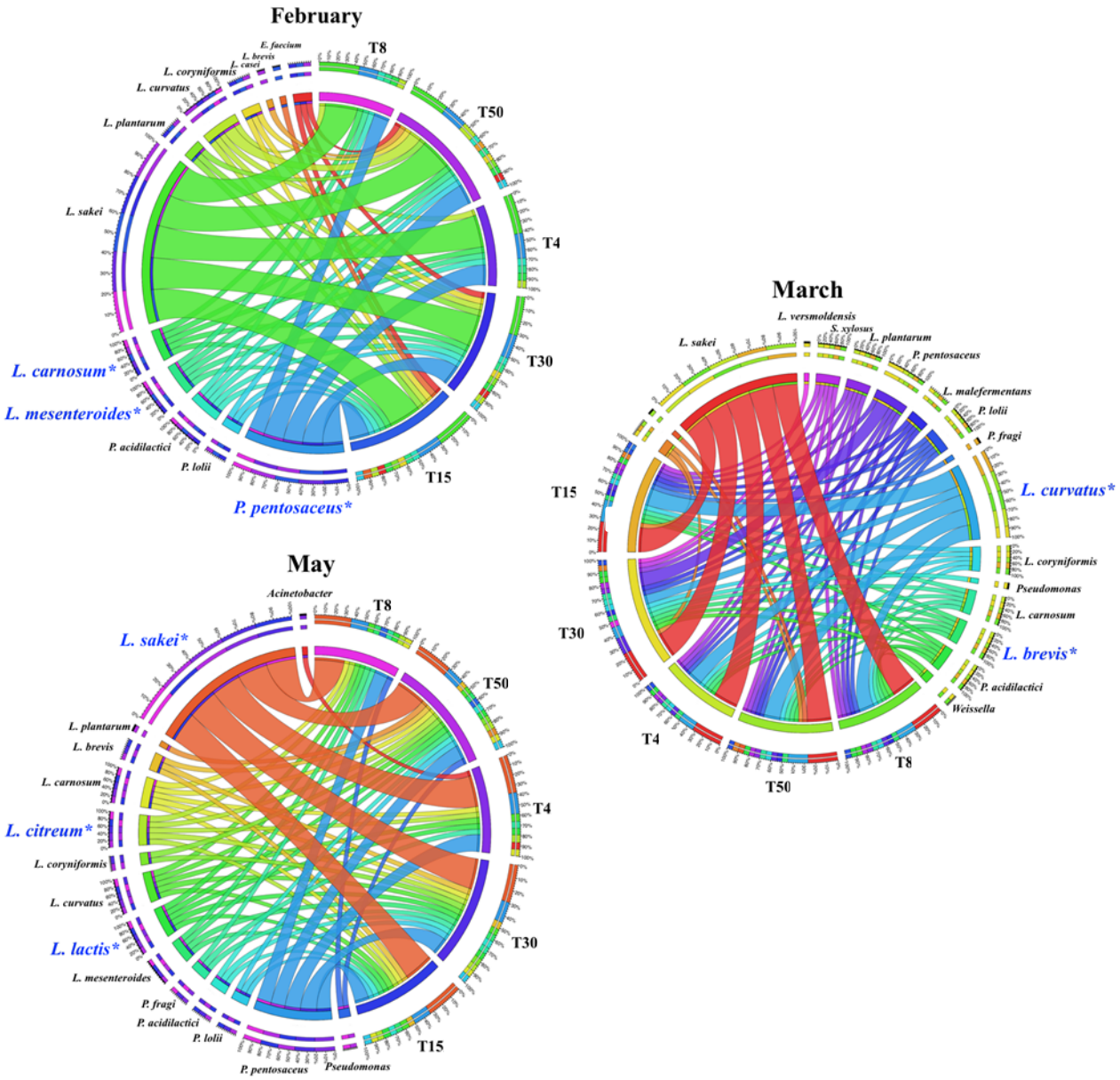

February
E. faecium
L. brevis
L. casei
L. coryniformis
L. curvatus
L. plantarum
L. sakei
L. carnosum*
L. mesenteroides*
P. acidilactici
P. lolii
P. pentosaceus*
T8
T50
T4
T30
T15
March
L. versmoldensis
S. xylosus
L. sakei
L. plantarum
P. pentosaceus
L. malefermentans
P. lolii
P. fragi
L. curvatus*
L. coryniformis
Pseudomonas
L. carnosum
L. brevis*
P. acidilactici
Weissella
T15
T30
T4
T8
T50
May
Acinetobacter
L. sakei*
L. plantarum
L. brevis
L. carnosum
L. citreum*
L. coryniformis
L. curvatus
L. lactis*
L. mesenteroides
P. fragi
P. acidilactici
P. lolii
P. pentosaceus
Pseudomonas
T8
T50
T4
T30
T15

Fig 2:

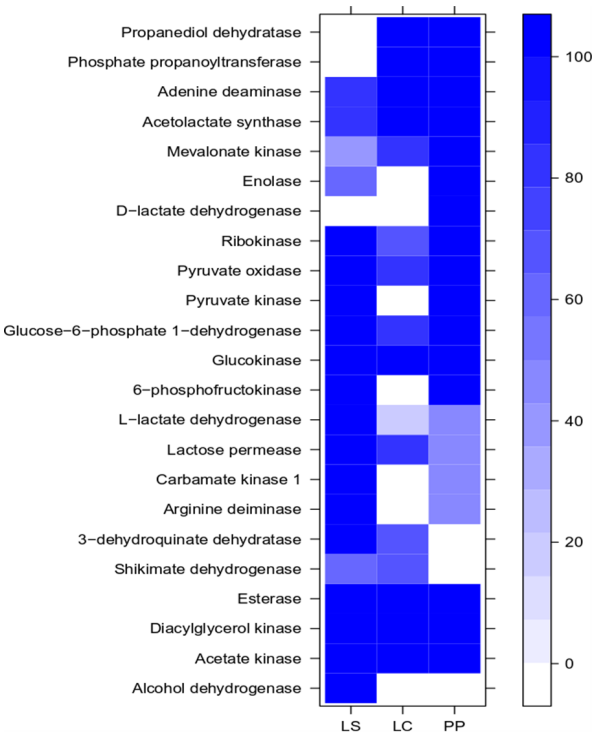

Propanediol dehydratase
Phosphate propanoyltransferase
Adenine deaminase
Acetolactate synthase
Mevalonate kinase
Enolase
D−lactate dehydrogenase
Ribokinase
Pyruvate oxidase
Pyruvate kinase
Glucose−6−phosphate 1−dehydrogenase
Glucokinase
6−phosphofructokinase
L−lactate dehydrogenase
Lactose permease
Carbamate kinase 1
Arginine deiminase
3−dehydroquinate dehydratase
Shikimate dehydrogenase
Esterase
Diacylglycerol kinase
Acetate kinase
Alcohol dehydrogenase
LS
LC
PP
100
80
60
40
20
0

Fig 3:

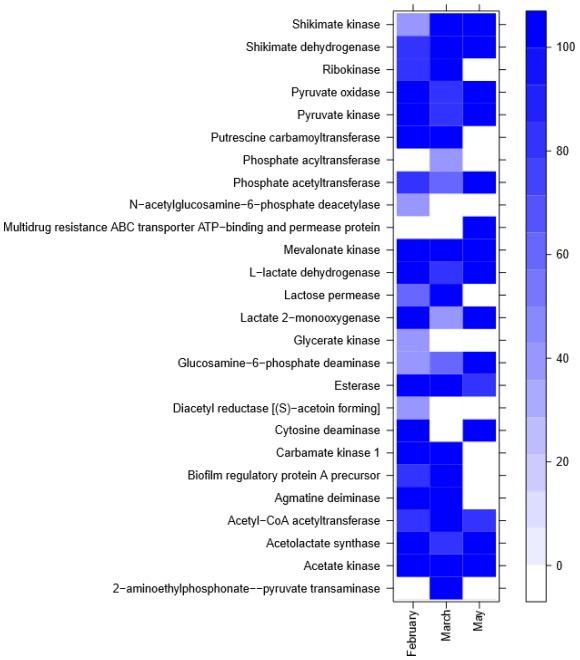
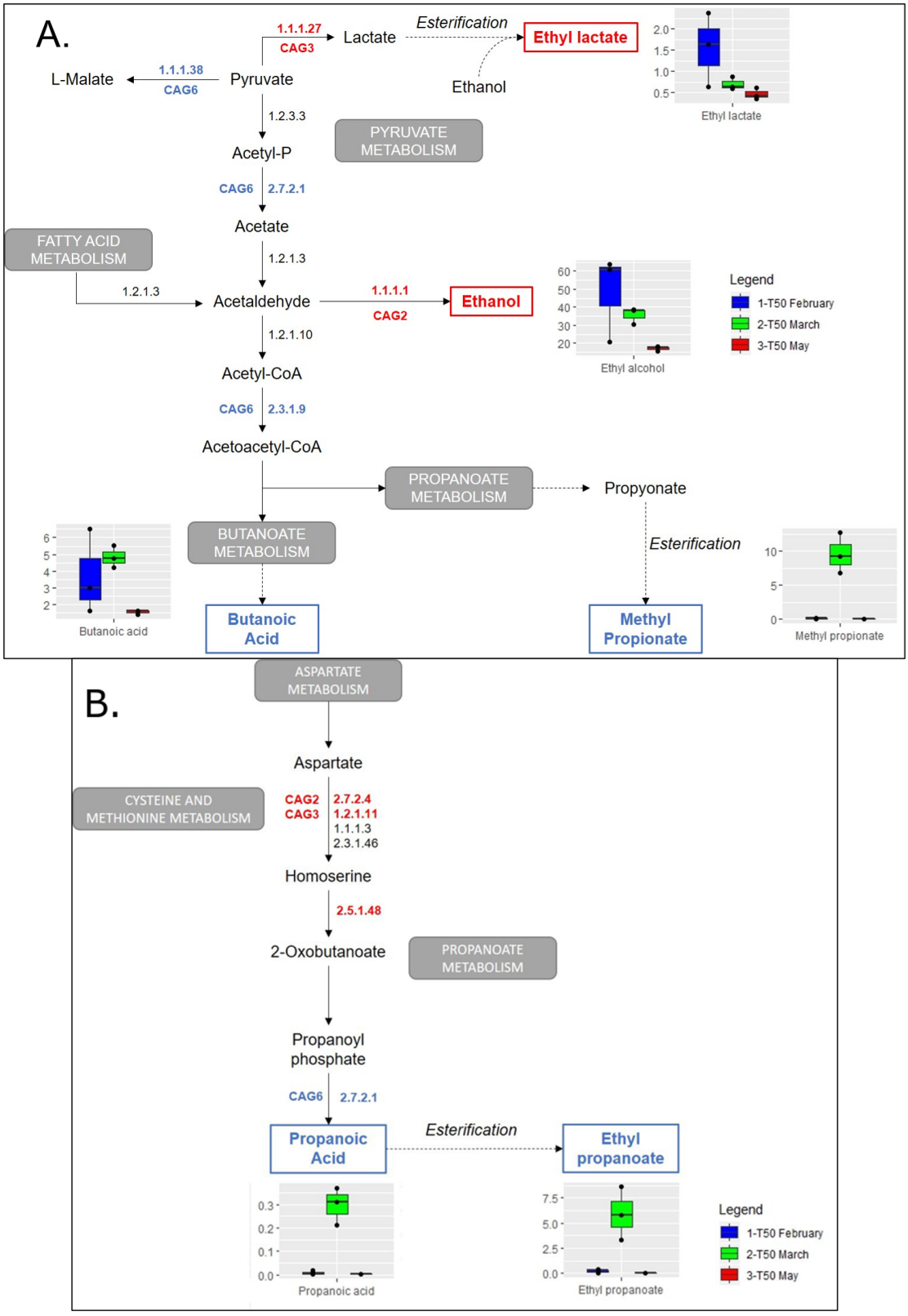
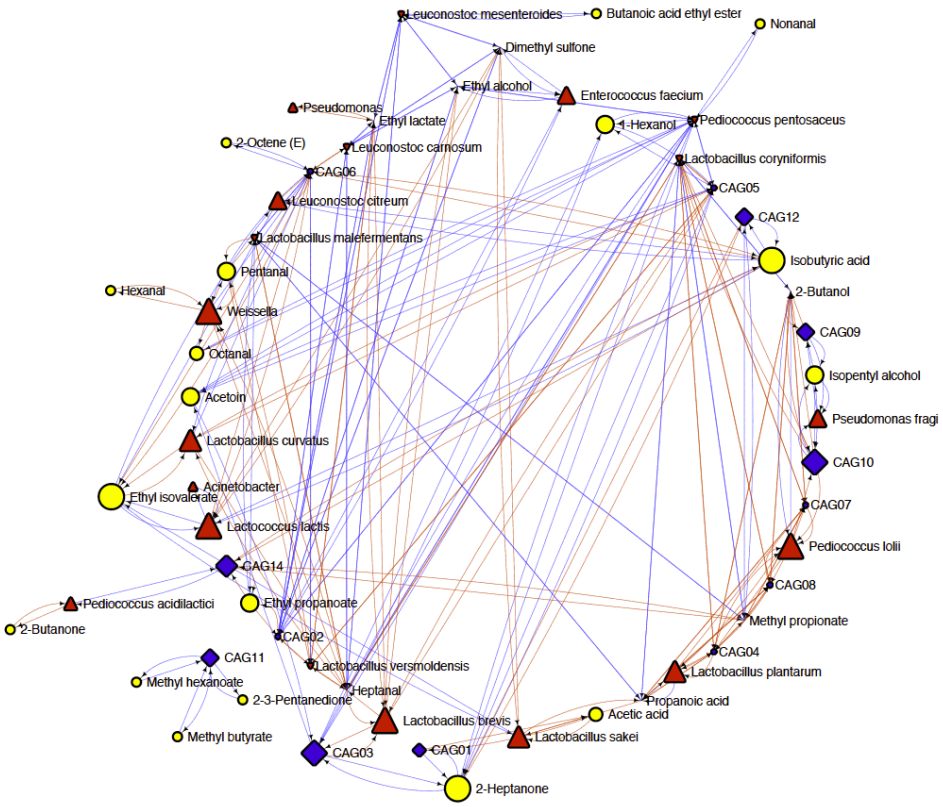

Shikimate kinase
Shikimate dehydrogenase
Ribokinase
Pyruvate oxidase
Pyruvate kinase
Putrescine carbamoyltransferase
Phosphate acyltransferase
Phosphate acetyltransferase
N−acetylglucosamine−6−phosphate deacetylase
Multidrug resistance ABC transporter ATP−binding and permease protein
Mevalonate kinase
L−lactate dehydrogenase
Lactose permease
Lactate 2−monooxygenase
Glycerate kinase
Glucosamine−6−phosphate deaminase
Esterase
Diacetyl reductase [(S)−acetoin forming]
Cytosine deaminase
Carbamate kinase 1
Biofilm regulatory protein A precursor
Agmatine deiminase
Acetyl−CoA acetyltransferase
Acetolactate synthase
Acetate kinase
2−aminoethylphosphonate−−pyruvate transaminase
February
March
May
100
80
60
40
20
0

Fig 4:


A.
1.1.1.27
CAG3
Lactate
Esterification
Ethyl lactate
Ethanol
L-Malate
1.1.1.38
CAG6
Pyruvate
1.2.3.3
PYRUVATE METABOLISM
Acetyl-P
CAG6
2.7.2.1
Acetate
FATTY ACID METABOLISM
1.2.1.3
1.2.1.3
Acetaldehyde
1.1.1.1
CAG2
Ethanol
1.2.1.10
Acetyl-CoA
CAG6
2.3.1.9
Acetoacetyl-CoA
PROPANOATE METABOLISM
Propyonate
BUTANOATE METABOLISM
Esterification
Butanoic Acid
Methyl Propionate
Butanoic acid
Methyl propionate
Ethyl alcohol
Ethyl lactate
Legend
1-T50 February
2-T50 March
3-T50 May
B.
ASPARTATE METABOLISM
Aspartate
CYSTEINE AND METHIONINE METABOLISM
CAG2
CAG3
2.7.2.4
1.2.1.11
1.1.1.3
2.3.1.46
Homoserine
2.5.1.48
2-Oxobutanoate
PROPANOATE METABOLISM
Propanoyl phosphate
CAG6
2.7.2.1
Propanoic Acid
Esterification
Ethyl propanoate
Propanoic acid
Ethyl propanoate
Legend
1-T50 February
2-T50 March
3-T50 May

865 **Fig 5:**


Leuconostoc mesenteroides
Butanoic acid ethyl ester
Nonanal
Dimethyl sulfone
Ethyl alcohol
Enterococcus faecium
Pseudomonas
Ethyl lactate
1-Hexanol
Pediococcus pentosaceus
2-Octene (E)
Leuconostoc carnosum
Lactobacillus coryniformis
CAG06
CAG05
Leuconostoc citreum
CAG12
Lactobacillus malefermentans
Isobutyric acid
Pentanal
Hexanal
2-Butanol
Weissella
CAG09
Octanal
Isopentyl alcohol
Acetoin
Pseudomonas fragi
Lactobacillus curvatus
CAG10
Acinetobacter
Ethyl isovalerate
CAG07
Lactococcus lactis
Pediococcus lolii
CAG14
CAG08
Pediococcus acidilactici
Ethyl propanoate
Methyl propionate
2-Butanone
CAG02
CAG04
CAG11
Lactobacillus versmoldensis
Lactobacillus plantarum
Methyl hexanoate
Heptanal
Propanoic acid
2-3-Pentanedione
Acetic acid
Lactobacillus brevis
Methyl butyrate
Lactobacillus sakei
CAG03
CAG01
2-Heptanone

866