## Autochthonous starter culture selection for Salame Piemonte PGI production

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1    **Autochthonous starter culture selection for Salame Piemonte PGI production**

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14   **Abstract**

15   Salame Piemonte is a dry-fermented meat product  typical of the Piedmont region in Italy, manufactured using

16   commercial starter cultures. This study aimed to select autochthonous starter cultures (ASCs) that could be

17   used for sausage fermentation in order to strengthen the link with the geographical area of production and

18   improve the sensory properties of the final product.

19   A culture-dependent approach was adopted during three different spontaneous sausage fermentation processes

20   to isolate and characterise the main bacterial resources involved. Dominant lactic acid bacteria (LAB) in each

21   batch were *Pediococcus pentosaceus*, *Latilactobacillus sakei*, and *Latilactobacillus curvatus*; *Staphylococcus

22   xylosus* was the most dominant coagulase-negative staphylococci (CNS) in all the studied batches. LAB and

23   presumptive CNS isolates were further evaluated for their physiological properties and biotechnological

24   potential. Thereafter, 11 strains were selected and evaluated for safety. Five selected strains (two *P.

25   pentosaceus*, two *L. sakei*, and one *S. xylosus* strain) were used for pilot-scale Salame Piemonte production

26   with seven different strain combinations. Based on the liking test, three ASC combinations led to the highest

27   liking score compared to industrial products. These three ASCs were then used for the second pilot-scale

28   sausage production confirming the high liking score. In summary, the use of *P. pentosaceus* and *S. xylosus*

29   ASC significantly improved product sensory properties compared with that obtained using commercial starter

30   cultures.

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## 1. Introduction

The microbiota of fermented meat products is composed of useful and specific microbial communities in which individual members and their interactions positively contribute to the fermentation process by providing their safety and distinct organoleptic properties (Franciosa, Alessandria, Dolci, Rantsiou, & Cocolin, 2018). This typical house microbiota is a source of microorganisms that may participate in the fermentation process and contribute to product uniqueness and quality.

All the different steps and ingredients characterise the fermentation process, which is driven by specific in-house, wild, and inoculated microbes (Baka, Papavergou, Pragalaki, Bloukas, & Kotzekidou, 2011) through complex chemical and physical reactions, providing sensory properties (firmness, flavour, and colour) to the final product (Ammor & Mayo, 2007; Stellato *et al*., 2016).

At the industrial level, the use of starter cultures has replaced spontaneous fermentations performed using unspecified microbiota to provide standardised characteristics for the final product and avoid food losses due to microbial spoilage (Cruxen *et al*., 2019). Most of the available commercial meat starter cultures contain a mixture of lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) (Ammor & Mayo, 2007; Hu *et al*., 2019). The main function of LAB in meat fermentation is to obtain a rapid pH drop, which increases the product safety, stability, and shelf life (Cardinali *et al*., 2018). The role of the CNS is to form the typical sensory characteristics and sausage colour due to their lipase, protease, and nitrate reductase activities (Cardinali *et al*., 2018; Chen, Kong, Han, Xia, & Xu, 2017). This fermented sausage is also a perfect substrate for fungal growth, and many fungal species can colonise dry fermented sausages. *Debaryomyces hansenii* and *Penicillium nalgiovense* are the major species most often used as starter cultures for casing inoculation (Murgia *et al*., 2019; Sunesen & Stahnke, 2003). This mycobiota also plays an important role in the sensory characteristics of sausage.

Strains composing commercial starter cultures are commonly isolated from the wild microbiota of spontaneous fermentations as they are well adapted to the ecological niche in which they are intended for use (Baka *et al*., 2011; Cruxen *et al*., 2019). Accurate strain-level characterisation and selection is also necessary because strains belonging to the same genus can have different impacts on the final product characteristics. Furthermore, regarding safety aspects, strains used in the food industry as starter cultures should not be pathogenic, possess

biogenic amine potential production, or acquire antimicrobial resistance (Álvarez-Cisneros & Ponce-Alquicira,

2018; Laslo, György, & Czikó, 2020).

The use of autochthonous strains significantly improves product sensory properties compared to commercial

ones and native strains contribute to creating distinct final properties of a typical regional fermented product

(Baka *et al*., 2011; Cruxen *et al*., 2019). Therefore, this study aimed to select autochthonous starter cultures

that could be used as a new starter culture for Salame Piemonte manufacturing using an innovative

combinations of methodology. The selection and implementation at industry level of selected autochthonous

starter culture had the goal of improve the organoleptic characteristic of the final products, and at the same

time strengthen the link with the geographical area of production.

To achieve our objectives, we analysed three different batches of spontaneously fermented sausages produced

at different months in the same factory following the same recipe. From each fermentation batch, we isolated

and identified LAB and presumptive CNS, screened them for their technological properties, and evaluated

their safety to select the most suitable strains or strain combinations to compose different starter formulations.

Thereafter, the starter formulations were used to produce different pilot-scale batches of Salame Piemonte,

which were compared in terms of metataxonomic composition, volatilome and sensory properties through

consumer test analyses.

## 2. Materials and methods

### 2.1. Sample collection from spontaneous fermented sausages

Spontaneous fermented sausage samples were collected at time 0 (meat plus seasoning) and after 4, 8, 15, 30,

and 50 days of fermentation from three different batches of Salame Piemonte PGI produced in February,

March, and May 2018, according to the detailed experimental procedure already reported (Franciosa *et al*.,

2021). At each sampling point, water activity ($a_w$) and pH were determined according to the manufacturer's

instructions. Microbial analyses were performed as described by Belleggia *et al.* (2020). The following

microorganisms were counted: lactic acid bacteria (LAB), presumptive coagulase-negative staphylococci

(CNS), *Enterobacteriaceae, Listeria monocytogenes, Enterococci, Salmonella* spp., *Escherichia coli,*

*Clostridium* spp., and *Staphylococcus aureus*.

90     Approximately 15 colonies from MRS and MSA media at each sampling point from each batch were randomly

91     isolated, purified, and identified as described by Franciosa *et al.* (2021).Isolates were subjected to DNA

92     extraction, PCR (rep-PCR) with the $(GTG)_5$ primer and cluster analysis as already reported Ferrocino *et al.*

93     (2017). After cluster analysis, 2 isolates from each cluster at 80% of similarity were selected and subjected to

94     identification. LAB and CNS identification was performed by amplifying the 16S rRNA gene (Ferrocino et

95     al., 2017; Weisburg, Barns, Pelletier, & Lane, 1991).

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97     **2.2. Strain physiological characterization**

98     Growth curves were reconstructed for each isolate under the following conditions, and spectrophotometric

99     data were processed in the R environment using the package *Growthcurver*. LAB isolates were grown in De

100    Man, Rogosa e Sharpe (MRS) broth at four different temperatures (30, 23, 15, and 10°C) and NaCl

101    concentrations (2, 3, and 4% v/v), whereas presumptive CNS isolates were grown in Brain Heart Infusion

102    (BHI) broth under the same conditions. The acidification rates at different temperatures (30, 23, 15, and 10°C)

103    were also measured. Presumptive CNS isolates were tested for their lipolytic activity on Spirit Blue Agar plates

104    supplemented with a mixture of olive oil and Tween 80 (Ercolini et al., 2010), proteolytic activity on skim

105    milk agar (1% skim milk, 1.5% agar) (Ercolini et al., 2009); nitrate reductase activity by spectrophotometric

106    assay (Casaburi, Blaiotta, Mauriello, Pepe, & Villani, 2005). Lipolytic and proteolytic activity were evaluated

107    by the detection of clear zones around the colonies after 24h at 37°C.

108    Non-inoculated control samples were included in the readings, and  blank data were used for modelling. Each

109    isolate was analysed in triplicates. *L. sakei* and *S. xylosus* strains composing the commercial starter culture

110    currently used by the product manufacturer were analysed and used as controls.

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112    **2.3. Safety evaluation**

113    Based on the physiological and molecular results, selected strains (8 LAB and 2 CNS) were evaluated for their

114    safety by detecting biogenic amine genes and screening for antimicrobial  resistance as described by Coton *et*

115    *al.* (2010, 2018). Detection of four biogenic amine genes: histidine decarboxylase (hdc), tyrosine

116    decarboxylase (tyrdc), putrescine decarboxylase (odc) and agmatine deiminase (agdi) genes was performed by

117    multiplex PCR as described by Coton et al. (2010, 2018). An uniplex PCR for each BA gene was performed

118    to confirm the results of the multiplex. Antibiotic resistance was determined for 12 different antibiotics

119    (kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, neomycin,

120    vancomycin, trimethoprim, oxytetracycline, rifampicin) according to the ISO 10932 IDF 223 International

121    Standard (2010-06-15) (Coton et al., 2018). *Lactiplantibacillus plantarum* (LMG6907) was used as reference

122    strain as specified by the ISO standard. The growth was verified by an automatic Multiskan FC plate reader

123    (Thermo Scientific, Paris, France) set to 620 nm. Each strain was assayed in triplicate in the microplates

124    arranged as explained in the ISO guideline and the MIC was read after 48h as the first well where no growth

125    was visible. The final results were expressed as minimum inhibitory concentrations in micrograms per milliliter

126    and the values were compared to the EFSA breakpoints (European Food Safety Authority, 2012) and literature

127    data (Danielsen and Wind, 2003).

128    Only strains that did not show the presence of biogenic amine genes were (four LAB and two CNS) were tested

129    for their antimicrobial resistance as decribed above. Strains that showed no antimicrobial resistance beyond

130    known natural ones (four LAB and one CNS strain) were considered safe and used for meat inoculation at a

131    pilot scale.

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133    **2.4. Starter formulation and meat inoculation**

134    A first preliminary pilot-scale production of fermented sausages at a meat plant in the province of Turin

135    (Piemonte Region, Italy) was done and prepared using five different strains combined into seven

136    autochthonous starter cultures (ASC) (Table 1). The sausage formulation (20 Kg of meat mixture for each

137    ASC) included pork meat (lean from the shoulder and fat from the belly), salt (maximum 3%), pepper

138    (maximum 0.4%), aromatic plants and spices, i.e., garlic and cloves, whole, crushed or infused with wine and

139    nutmeg. After chopping and mixing the ingredients, the mixture was divided into batches according to each

140    autochthonous starter formulation. The cutter and filler were cleaned and disinfected before the pilot-scale

141    production and between different batches to avoid contamination. The sausages were placed in the ripening

142    room under the same conditions as that employed for industrial production (Franciosa *et al*., 2021) for 15 days.

143    Each batch was individually inoculated once with approximately $10^7$ CFU/g of starter. At the same time, all

144    sausages were also inoculated on the casing surface with a commercial starter culture of *P. nalgiovense,* as

classically done for Salame Piemonte production. A control sausage was prepared using a commercial starter

culture composed of a mixture of *L. sakei* and *S. xylosus* ($10^7$ CFU/g) and used in all analyses as a control.

Samples were collected after 0, 2, 12, and 15 days of fermentation for physicochemical and microbiological

analyses. At each sampling time, three sausages were analysed for each ASC. Microbial analyses were

performed as described by Belleggia *et al.* (2020) for the enumeration and isolation of LAB and presumptive

CNS. *Listeria monocytogenes* presence was also checked.

### 2.5. Molecular and metataxonomic approach

Approximately nine colonies from MRS and MSA media at each sampling point were isolated and purified.

They were used to track the presence of the inoculated strains during fermentation using rep-PCR, as described

previously (Iacumin, Comi, Cantoni, & Cocolin, 2006), by comparing their rep-PCR patterns with those of

inoculated strains.

For the metataxonomic approach, total DNA was extracted from samples at the end of fermentation and used

for metataxonomic analyses by amplifying the V3-V4 regions of the 16S rRNA gene following the conditions

described elsewhere (Cardinali *et al*., 2021; Bolyen *et al*., 2019; Callahan *et al*., 2016; Klintword *et al*., 2013).

### 2.6. Volatilome analyses of fermented sausages

The volatile composition of the final product was determined by headspace (HS) solid-phase microextraction

(SPME) and analysed by gas chromatography-mass spectrometry (GC/MS) following the experimental

procedure reported elsewhere (Franciosa *et al*., 2020). Briefly, 3 g of sample were placed in 20 ml vials with

the internal standard: 10 µl of 2-octanol in ultrapure water (333 ppb as final concentration). We measured the

*m/*z peak area of the quantifier ion in relation to the *m/z* quantifier ion of the added internal standard to obtain

a semiquantitative result (µg/kg) for each identified compound (Franciosa *et al*., 2020; Ferrocino *et al*., 2018).

### 2.7. Liking test of the inoculated sausages

To assess the sensory acceptability of the sausage samples at the end of ripening, a liking test was performed.

A total of 20 sausage consumers (7 male and 13 female participants, aged 28 to 56 years) voluntarily

participated in the evaluation. The consumers were served with a slice of each sausage, randomly numbered,

173    with a glass of water to clean their mouths between each sample. They evaluated external appearance, texture,

174    colour, flavour, and consumers global appreciation. For each sample, consumers completed a table modified

175    from the one described by Chiavari, Coloretti, Ferri, & Nanni (2007). The results were elaborated and plotted

176    using a radar graph generated in an R environment.

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178    **2.8. Production process validation**

179    A second pilot-scale production was performed using the combinations of ASC that showed the best sensory

180    results, following the procedure previously described to verify process standardisation. Sausages of the second

181    pilot-scale production were analysed through physicochemical and microbial analyses (see paragraph 2.5.),

182    molecular, and metataxonomic approaches (see paragraph 2.6.) and sensory evaluations (see Section 2.8.).

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184    **2.9. Statistical analyses**

185    A one-way analysis of variance (ANOVA) was used to detect significant differences among means followed

186    by Duncan's multiple range test for mean comparison of microbial counts, physicochemical parameters, alpha

187    diversity parameters, and ASV abundance using the SPSS 17.0 program for Windows (SPSS Inc., Chicago,

188    IL, USA) or QIIME2 as appropriate.

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191    **3. Results**

192    **3.1. Bacterial counts and microbiota composition of spontaneously fermented sausages**

193    The pH values decreased as a function of fermentation time, showing some differences between the three

194    analysed batches (Supplementary Table 1, $P<0.05$). In particular, for the February, March, and May samples,

195    the initial pH values were 6.49, 6.03, and 5.99, reaching 5.29, 5.72, and 5.35 at the end of the fermentation

196    process, respectively. Notably, at the end of the fermentation process, March samples showed the highest pH

197    value and February samples the lowest, despite their highest initial pH values (Supplementary Table 1). No

198    significant differences were observed between the three batches for the $a_w$ values (Supplementary Table 1).

199    In all analysed batches, LAB counts rapidly increased in the first eight days of ripening and then remained

200    constant until the end. Presumptive CNS counts remained relatively constant during ripening at approximately

201    3 $\log_{10}$ CFU/g (Supplementary Table 1). The May samples showed the highest LAB population at the end of

202    fermentation (9.71 $\log_{10}$ CFU/g) as compared with the 2 other batches (Supplementary Table 1, $P<0.05$). The

203    highest presumptive CNS count value was observed at the end of February fermentation (4.23 $\log_{10}$ CFU/g)

204    (Supplementary Table 1, $P<0.05$). *Escherichia coli*, *Enterococcus* spp., *S. aureus*, and *Clostridium* spp. counts

205    were below detection levels (< 2 $\log_{10}$ CFU/g), whereas *L. monocytogenes* and *Salmonella* spp. were not

206    detected in any sample.

207    A total of 443 isolates comprising 224 LAB and 219 presumptive CNS isolates were obtained from the three

208    different batches and further identified (Supplementary Fig 1). As shown in Supplementary Fig 1, LAB isolates

209    were dominated by *L. sakei*, *P. pentosaceus* and *Latilactobacillus curvatus*. For CNS, five species were

210    identified, *that is*, *S. xylosus*, *Staphylococcus succinus*, *Staphylococcus equorum*, *Staphylococcus carnosus*

211    and *Staphylococcus saprophyticus* (Supplementary Fig 1).

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213    **3.2. Physiological characterization of isolates**

214    LAB and CNS isolates were screened for their physiological properties. Although the results showed that

215    isolates harboured different physiological characteristics (Supplementary Table 2), we were able to

216    discriminate the three fermentation batches based only on the acidification rate at all tested temperatures of the

217    isolates from which they originated (Fig. 1). An ANOSIM test confirmed this observation (P<0.001) as isolates

218    from the February batch were separated from the other two batches, whereas isolates of the March and May

219    batches did not differ significantly in their acidification rate as their respective confidence ellipses overlapped

220    (Fig. 1).

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224    **3.3. Isolate selection**

225    Autochthonous starter LAB were selected based on their growth rates at 20°C and 15°C. Regarding

226    acidification properties, selected strains were chosen based on final pH values at 15–10°C, with values

227    comprised between 6.01 and 5.52, since the bacteria show a high acidification rate in the first week of

228    fermentation and a low acidification activity in the later stages (at 15°C). These temperature ranges were

selected according to the PGI product specification (Official Gazette of the Italian Republic, nr 184, 10 August 2015). Among presumptive CNS isolates, we selected those with lipolytic, proteolytic, and nitrate reductase activities and rapid growth at 20°C and 10°C (data not shown). After this selection, rep-PCR fingerprints of selected isolates were compared to select only those with different rep-PCR patterns (data not shown). At the end of the selection process, the selected bacteria were identified as five *P. pentosaceus* strains (C1M, S4NM, S8QM, S4XNM, S45XEM), three *L. sakei* strains (S29BM, S15ZGM, S29ZEM), and two *S. xylosus* strains (S8HS and S29XIS) (Table 2).

### 3.4. Safety evaluation of selected isolates

Safety evaluation of the latter 10 strains was performed by detecting biogenic amine (BA) genes and screening for antimicrobial resistance. None of the CNS strains showed specific bands for targeted biogenic amine genes in multiplex PCR. In contrast, targeted genes (tyramine decarboxylase, ornithine decarboxylase and agmatine deiminase genes) were detected in several LAB strains using both multiplex and simplex PCR (data not shown).

The minimum inhibitory concentration (MIC) for 12 antimicrobials was determined for six strains identified as non-BA-producers (Supplementary Table 3). Overall, intermediate MIC values were found for all antimicrobials and strains (Supplementary Table 3). Based on these results, *P. pentosaceus* S8QM and S4XNM, *L. sakei* S29BM and S29ZEM, and *S. xylosus* strain S8HS were selected for pilot-scale fermented sausage production.

### 3.5. Sausage production, microbial counts and pH of inoculated sausages

Seven ASC were tested for sausage manufacturing during the first pilot-scale production (Table 1). Microbiological analyses showed the absence of *L. monocytogenes* in all the samples. The final pH value of the control sausages (made with commercial starter cultures) was 5.21, and those of sausages produced with the seven ASC were in the same range without any significant differences. However, the seven batches did not follow a similar acidification trend (Supplementary Table 4).

ASC 1, 2, and 3 samples showed CNS counts that were never lower than 8 $\log_{10}$ CFU/g. The other four ASC showed lower values, starting from 7.5 $\log_{10}$ CFU/g with a slight decrease until the end of fermentation to 6.7

257   log$_{10}$ CFU/g. After two days of fermentation, LAB counts in ASC 4, 5, 6, and 7 samples were significantly

258   lower than in the other samples, including the control (Supplementary Table 4, $P<0.05$). After 12 days of

259   fermentation, no significant differences were observed between the inoculated sausages except for ASC 7 (*P.*

260   *pentosaceus* S4XNM and *L. sakei* S29ZEM), for which the LAB count was below 9 log$_{10}$ CFU/g

261   (Supplementary Table 4, $P<0.05$). At the end of the fermentation, the LAB count of ASC 7 remained the lowest

262   (Supplementary Table 4, $P<0.05$).

263   For all tested conditions, the presence of selected autochthonous starters at the end of fermentation was

264   confirmed by rep-PCR (data not shown).

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266   **3.6. Metataxonomic composition**

267   A metataxonomic approach was used in parallel to analyse microbial diversity in the final product of each trial

268   and to investigate the impact of inoculated strains on the fermented meat microbiota. Significant differences

269   in alpha-diversity indices, that is, chao-1, number of observed species, and Shannon index were found between

270   the different samples (data not shown). In particular, samples produced with ASC 4 showed significantly lower

271   values for the Shannon index than the other ASC inoculated samples ($P<0.05$). Alpha-diversity indices of the

272   control samples presented the lowest values when compared to all other tested conditions.

273   Comparison of ASV relative abundances between sausages at the end of the fermentation process and made

274   with different ASC confirmed the dominance of the inoculated species (Fig. 2). Other minor species, including

275   *Lactococcus lactis,* were present at significantly higher relative abundances in samples inoculated with ASC

276   5, whereas *Weisella viridescens* was observed at higher relative abundances in sausages produced with ASC

277   4, 5, and 6 (Fig. 2). *Kocuria rhizophila* and *Listeria* sp. were detected at low relative abundances in samples

278   inoculated with *P. pentosaceus* (ASC 1, 2, and 3). *Weissella hellenica* was more abundant in sausages

279   inoculated with *P. pentosaceus* (ASC 1, 2, and 3) (Fig. 2), whereas *Lactobacillus delbrueckii* was only found

280   in sausages inoculated with ASC 4, 5, and 7 (Fig. 2).

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282   **3.7. Effect of autochthonous starter cultures on volatilome profiles**

283   Forty-six volatile compounds were identified in the analysed samples and classified according to their most

284   probable origin. Some VOCs possibly originated from spices (2) and were of unknown origin (9). For the other

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molecules, four subgroups were identified based on the origin of their bacterial metabolism: amino acid metabolism (12), lipid metabolism (9), carbohydrate metabolism (8), and esterase activity (6) (Supplementary Table 5).

Dry sausages fermented with the seven ASC and control groups showed several differences in their volatile profiles both qualitatively and quantitatively (Supplementary Table 5). Sausages produced with *L. sakei* strains harboured specific volatile organic compounds (VOCs) at concentrations similar to those of the control, *that is*, hexanal, isopentyl alcohol, 2-methyl-3-octanone, 1-hexanol, acetic acid, 1-octanol, butyrolactone, and hexanoic acid. All such molecules showed a lower concentration ($P<0.05$) in the samples produced with ASC composed of *P. pentosaceus* strains, except for hexanoic acid. Sausages inoculated with *P. pentosaceus* strains did not contain any detectable butanoic acid ethyl ester, butanoic acid methyl ester, hexanoic acid, and octanoic acid (Supplementary Table 5, $P<0.05$). In contrast, acetoin and diacetyl were present at higher concentrations in ASC1, 2, and 3 sausages (all containing *P. pentosaceus* strains) and in control.

Some ASC sausages were also characterised by qualitative and quantitative differences in their volatile profiles (Supplementary Table 5). It is also important to consider that there are important differences in presumptive CNS counts between different ASCs. The *S. xylosus* population showed the highest abundance in ASC1, 2, and 3 as compared to ASC4, 5, 6, and 7 and the lowest in the control. These differences could have a significant impact on the VOC composition.

### 3.8. Effect on sensory attributes of inoculated sausages

The sausages produced with the ASC were evaluated by 20 consumers and compared with the control sausages. In summary, the liking test suggested a higher consumer preference for sausages manufactured with ASC versus the control (Fig. 3).

In particular, sausages produced with ASC 1 (*P. pentosaceus* S4XNM) and ASC 2 (*P. pentosaceus* S8QM) were considered the best in terms of acceptability ($P<0.05$). Comparing all attributes of sausages produced with ASC against the control, they were less bitter, acidic, and the fat and lean parts were less visible, resulting in a more uniform aspect. All ASC inoculated samples also exhibited the highest odour and colour intensity scores ($P<0.05$). Samples produced with ASC 7 (*P. pentosaceus* and *L. sakei)* were evaluated as more balanced for most of the investigated attributes, with the highest salt ($P<0.05$), flavour, and odour intensity. Samples

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produced with ASC 5 showed a medium score for all descriptors and obtained the lowest score for general appreciation ($P<0.05$). All ASC inoculated samples obtained a higher score than the control for the question "Would you buy it?", in particular sausages that were produced with ASC 1 ($P<0.05$). It should be noted that sausages inoculated with ASC 1, ASC 2, and ASC 3, composed of *P. pentosaceus*, showed the highest scores for colour intensity and general appreciation ($P<0.05$) compared to samples with *L. sakei* strains that were perceived as the most acidic ($P<0.05$).

### 3.9. Validation of inoculated sausage production

Based on liking test results, the three most appreciated ASC were used in a second pilot scale production, *i.e.*, ASC 1B (*P. pentosaceus* S4XNM and *S. xylosus* S8HS), ASC 6B (*L. sakei* S29BM, *L. sakei* S29ZEM and *S. xylosus* S8HS) and ASC 7B (*P. pentosaceus* S4XNM, *L. sakei* S29ZEM and *S. xylosus* S8HS). Microbiological analyses showed the absence of *L. monocytogenes* in all analysed samples. No differences in pH were observed between the two pilot-scale productions at the end of fermentation for all ASCs used (Supplementary Table 6, $P<0.05$). However, the initial pH values varied between the ASCs, with ASC 1 showing the lowest pH value (5.88) and ASC 6 the highest value (6.02) (Supplementary Table 6, $P<0.05$). After two days of fermentation, there were no significant differences in the pH of the three products.

The ASC 1 sample showed the highest presumptive CNS counts from the beginning until the end of fermentation (Supplementary Table 6). During fermentation, ASC 1 B, ASC 6 B, and ASC 7 B samples showed lower presumptive CNS counts than their corresponding samples obtained in the first pilot-scale production. Lower LAB initial counts were observed for ASC 6 B and ASC 7 compared with the first pilot-scale trial (Supplementary Table 6, $P<0.05$). The final LAB counts were approximately 9 $\log_{10}$ CFU/g for ASC 1, 6 B, and 6, whereas for ASC 1 B, ASC 7 B and ASC7 LAB counts were slightly lower at 8.85 $\log_{10}$ CFU/g (Supplementary Table 6).

Notably, the presence of selected autochthonous starters at the end of fermentation was confirmed by rep-PCR and the metataxonomic approach (Fig. 2). Metataxonomic data showed the presence of ASCs in the corresponding sausages. However, it should be noted that *P. pentosaceus* reached a relative abundance of 18% in sausages in which it was not deliberately inoculated (Fig. 2). As observed in the first trials, *S. xylosus* was detected at abundances ranging from 6% to 12% (Fig. 2).

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The liking test underlined a lower consumer preference for sausages manufactured with a commercial starter culture (control) than the ASC (Fig. 4). The highest score for global appreciation was assigned to sausages produced by ASCs 1 and 6. Significant differences (*P*<0.05) were observed in tenderness, firmness, fat, and colour intensity parameters. The lowest score for colour intensity and the highest score for tenderness were attributed to the control sample (Fig. 4, *P*<0.05).

## 4. Discussion

Fermented sausages are the result of complex microbiological activities (Belleggia *et al*., 2020). Regarding naturally fermented sausages, we observed that samples belonging to the February batch had a lower pH value at the end of fermentation. This is due to the strong acidification capability of the LAB strains that compose the microbiota of this batch. In contrast, LAB strains that characterised the microbiota of the March batch were characterised by a weak acidification capability. In fact, the microbiota composition of the naturally fermented sausages showed differences among the three studied batches. Based on our findings, we focused on the *L. sakei* and *P. pentosaceus* strains. The latter is known for its high acidification capability, which could be a positive criterion for use as a starter culture in fermented meats with highly acidic products (Chen, Kong, *et al*., 2015; Chen, Liu, *et al*., 2015).

The February samples showed a high predominance of *P. pentosaceus*, while May samples were dominated by *L. sakei*. Most *P. pentosaceus* isolates selected for meat inoculation belonged to the February batch. In March, the only batch produced showed isolation of *L. curvatus, L. coryniformis* and *L. plantarum* strains. These results also highlighted the highest acidification capability of *P. pentosaceus* strains, which were dominant in the most acidic batch (February). More acidic fermented meat products, generally from Northern Europe, show a higher prevalence of *P. pentosaceus* in their LAB communities (Van Reckem *et al*., 2019). In contrast, the lower acidification rate of *L. sakei* strains could explain why the March batch (characterised by a high presence of *L. sakei* strains) was the least acidic final product. In addition, the two selected *L. sakei* strains belonging to the March batch showed a lower acidification capability when compared to *P. pentosaceus* isolates from the February batch. We observed that the acidification rates of the different isolates were species dependent. Isolates belonging to February, characterised by a high presence of *P. pentosaceus,* showed a high acidification rate at 20°C. The use of these strains for meat inoculation (ASC 1, 2, and 3) resulted in a rapid

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decrease in pH in the first days of fermentation. The same trend was also observed for control sausages inoculated with *L. sakei*. It has already been reported that *P. pentosaceus* is preferred to other LAB species for producing adequate sausage fermentation because of its acidification properties. Species belonging to the *Pediococcus* genus are used in the food industry as protective cultures against common food spoilage bacteria (Porto, Kuniyoshi, Azevedo, Vitolo, & Oliveira, 2017) and as starter cultures for high acidity sausages (Chen, Kong, *et al*., 2015; Chen, Liu, *et al*., 2015). *P. pentosaceus* is also important for its antioxidant ability (Kim *et al*., 2019), its ability to generate odour precursors that contribute to the formation of specific flavours (Sun, Hu, Chen, Kong, & Liu, 2019), and to prevent excessive lipid oxidation and subsequent off-flavour production (Chen, Kong, *et al*., 2015; Chen, Liu, *et al*., 2015).

For the CNS group, we did not observe a constant increase in their populations during fermentation, and their abundance was always lower than that of LAB, as previously reported for other naturally fermented sausages (Cardinali *et al*., 2018; Ferrocino *et al*., 2018; Rantsiou *et al.*, 2005). *S. xylosus* was the most dominant species in all batches, whereas the other species were randomly isolated. The dominance of *L. sakei* and *S. xylosus* in all batches of spontaneously fermented sausages was in accordance with literature data (Eisenbach, Geissler, Ehrmann, & Vogel, 2019; Iacumin *et al*., 2020; Pini, Aquilani, Giovannetti, Viti, & Pugliese, 2020; Van Reckem *et al.*, 2019). NaCl tolerance is one of the criteria used to select autochthonous starters (Cruxen *et al*., 2019; Laslo *et al*., 2020). For most isolates, an increase in salt concentration reduced bacterial growth, as previously reported in the literature (Aina, 2017). The ability of microorganisms to grow under different conditions is species-dependent (Cruxen *et al*., 2019) and strain-dependent (Casaburi *et al*., 2011; Ercolini *et al*., 2010), as observed in this study.

Regarding the effect of temperature on growth, it can be said that approximately all isolates grew at 30°C, whereas the number of LAB isolates able to grow decreased with a decrease in temperature. Presumptive CNS isolates displayed a higher proportion of isolates able to grow at low temperatures (10°C) than at high temperatures, although with slower growth. This is an unexpected result because it is known that the optimum growth temperature of *S. xylosus* is 30°C (Essid, Ismail, Bel Hadj Ahmed, Ghedamsi, & Hassouna, 2007), although it is able to grow well at temperatures normally used for meat fermentation (10 and 20°C) (Essid, Ismail, Bel Hadj Ahmed, Ghedamsi, & Hassouna, 2007). All the results described above are important for selecting strains able to grow well at the salt concentration (2-3 %) and temperature range (20-10°C)

397 encountered during Salame Piemonte production. Finally, the selected presumptive CNS strains showed

398 proteolytic, lipolytic, and nitrate reductase activity on the assayed agar medium, which was important given

399 the beneficial effects of these activities on texture, flavour, and colour development (Laslo *et al.*, 2020).

400 Moreover, before using any strain for pilot-scale production and future industrial applications, the selected

401 strains were evaluated based on biogenic amine gene detection and antimicrobial resistance (MIC

402 determination) to discard any LAB isolates harbouring decarboxylase genes and/or showing intermediate

403 resistance to antimicrobials.

404 The fermentation process using our ASC satisfied the standard requirements of Salame Piemonte since the

405 directive reported in the disciplinary (Official Gazette of the Italian Republic, nr 184, 10 August 2015) requires

406 the final pH value to be equal to or higher than 5.2. In addition, a correct acidification process favours inhibition

407 of *Listeria* spp. (Zaiko, Bataeva, Yushina, Makhova, & Minaev, 2020).

408 The sensory characteristics of the final products were related to the different lactic acid bacteria strains used

409 in ASC formulations and the inoculated *S. xylosus* and *P. nalgiovense* strain activities. The difference in the

410 volatile profiles can be linked to the complex synergic interactions between microbiota and microbiome

411 activities. In general, we observed that the sausages that were enriched in aromatic components were those

412 obtained with ASC 1 (*P. pentosaceus* S4XNM and S. xylosusS8HS) and 5 (*L. sakei* S29BM and S. xylosus

413 S8HS), whereas for ASC 7 (*P. pentosaceus* S4XNM and *L. sakei* S29ZEM and S. xylosus S8HS), the volatile

414 profile was similar to that of ASC 4 (*L. sakei* S29ZEM and S. xylosus S8HS). In particular, *L. sakei* S29BM

415 had a significant impact on sausage volatilome. The volatilome of sausages produced with ASC 6 (*L. sakei*

416 S29BM and S29ZEM) was more similar to that of ASC 5 (*L. sakei* S29BM) than to ASC 4 (*L. sakei* S29ZEM)

417 (Fig. 4).

418 The highest concentrations of diacetyl and acetoin were found in samples in which *P. pentosaceus* strains were

419 used to drive the fermentation (ASC 1). These compounds are products of the carbohydrate catabolism of LAB

420 and staphylococci and are associated with dairy odours, mostly found in fresh meats (Montel, Masson, &

421 Talon, 1998). It is known that *Pediococcus* genera produce more acetoin than *L. sakei*, in particular *P.*

422 *pentosaceus,* which is often associated with acetoin and diacetyl production (Sunesen, Trihaas, & Stahnke,

423 2004). Samples inoculated with *P. pentosaceus* strains, which were also characterised by the highest *S. xylosus*

424 population, also showed a high concentration of alcohol compounds, *that is,* ethanol (ethyl alcohol), isopentyl

425  alcohol, 1-hexanol, and 1-octanol. 1-Octanol has specific odour attributes referred to as waxy, green, citrus,

426  and floral with a sweet and coconut nuance (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015), whereas

427  the attributes related to 1-hexanol are cheese, oxidised fat, rancid, and humidity (Perea-Sanz, Montero,

428  Belloch, & Flores, 2018). Other studies reported that *P. pentosaceus* gives aromatic characteristics to the final

429  product, and the highest aldehyde, alcohol, and acid contents were obtained in samples inoculated with *P.*

430  *pentosaceus* (Chen, Liu, *et al*., 2015; Cruxen *et al*., 2019).

431  The contents of hexanoic acid and octanoic acid, which originate from the oxidation of the corresponding

432  alcohols (Hu *et al*., 2019), were both higher in sausages made with *L. sakei* strains (ASC 4, 5, 6) than in

433  sausages inoculated with *P. pentosaceus* (ASC 1, 2, 3). In other cases, a low concentration of acetic acid has

434  been reported as preferred from a sensory point of view (Iacumin *et al*., 2020). Samples from ASC1 showed

435  also a highest level of 2-pentanone produced by CNS through b-oxidation of saturated fatty acids (Fadda et al.

436  2002; Engelvin et al. 2000).

437  In this study, consumers preferred sausages with the highest concentration of acetic acid. The liking test

438  showed that consumers preferred sausages produced using ASC 1 (*P. pentosaceus* S4XNM), which exhibited

439  the highest concentration of acetic acid and 2-pentanone. The preference for this sausage by consumers can be

440  explained by the highest predominance this methyl ketone being highly related to the typical cured aroma of

441  fermented sausages  (Berdagué et al. 1993) together with the low saltiness perception ($P<0.05$), high odour

442  intensity ($P<0.05$), and texture characteristics (tenderness $P<0.05$). Consumer preference was also correlated

443  with *S. xylosus* population. *S. xylosus* counts were 2-3 $\log_{10}$ higher in ASC1, 2, and 3 sausages (containing *P.*

444  *pentosaceus*) than in the other ASC and control sausages (containing *L. sakei* strains). Given the role of the

445  CNS in meat fermentation (colour, lipolysis, proteolysis) (Iacumin, Comi, Cantoni, & Cocolin, 2006), it is

446  likely that *S. xylosus* contributed to a larger extent to the sausage organoleptic properties of ASC1, 2, and 3,

447  and together with *P. pentosaceus* to the metabolic activities that yielded final products preferred by consumers.

448  The second pilot-scale production underlined the repeatability of the ASC inoculation; rep-PCR fingerprinting

449  of isolates collected during fermentation and metataxonomic analysis confirmed that the inoculated strains

450  were dominant from the beginning to the end of the fermentation process. In samples inoculated with ASC

451  6 B, *P. pentosaceus* was detected at high relative abundances, despite not being deliberately inoculated. This

452  is possibly due to the high initial load of *P. pentosaceus* in the meat used for sausage production. The raw meat

453  used for sausage production in the second trial was characterized by the presence of indigenous *P. pentosaceus*

454  and *Pseudomonas fragi*. These two species were found in the final product; thus, they were able to colonise

455  this kind of product and probably grow throughout the fermentation process.

456  Other subdominant species were identified through metataxonomic analysis; however, their relative abundance

457  was low. Therefore, we can assume that their contribution to the final sensory properties was not major.

458  Therefore, we can affirm that the selected strains significantly contributed to the final product characteristics.

459

460  **5. Conclusions**

461  In conclusion, to obtain desirable organoleptic characteristics, including the *P. pentosaceus* S4XNM strain

462  during fermentation is the best practice. We demonstrated that the use of autochthonous strains significantly

463  improved the sensory properties of the product as compared to products obtained with commercial starter

464  cultures. However, more work is necessary to improve the standardisation of sausage production with the most

465  promising ASC to avoid or limit the influence of the indigenous microbiota of raw meat.

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484    **Declarations of interest:** none

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646

647

**FIGURE LEGEND**

649

650 **Fig. 1.** PCA based on acidification rate for LAB rep biotype (February in blue, March in green, May in red).

651 The first component (horizontal) accounts for 58.81% of the variance and the second component (vertical)

652 accounts for 21.73%.

653

654 **Fig. 2.** Amplicon sequence variant relative abundance (%) in Salame Piemonte sausages inoculated with

655 different autochthonous starter culture (ASC) using a metabarcoding approach.

656

657 **Fig. 3.** Radar graphs displaying the liking of appearance, odor, taste, flavor, and texture and overall liking

658 expressed by consumers for the sausages made by Standard starter cultures (Control) and inoculated

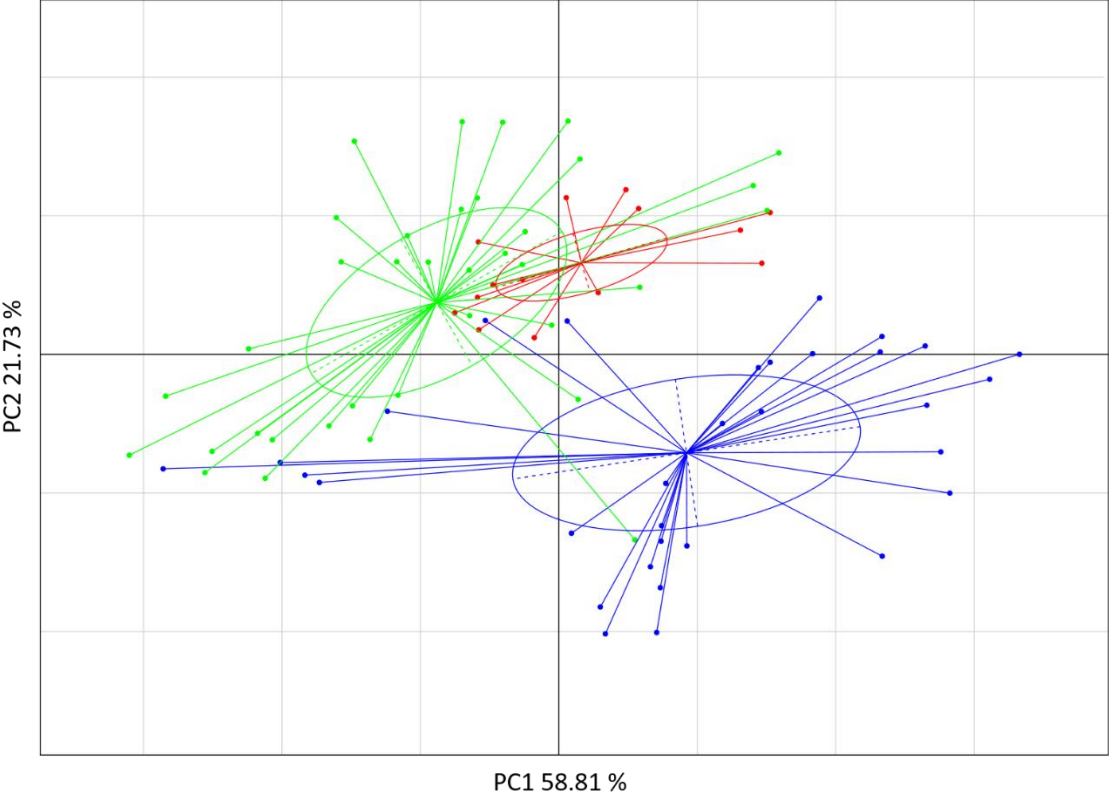659 fermentation of the first pilot-scale production.

660

661 **Fig. 4.** Radar graphs displaying the liking of appearance, odor, taste, flavor, and texture and overall liking

662 expressed by consumers for the sausages made by Standard starter cultures (Control) and inoculated

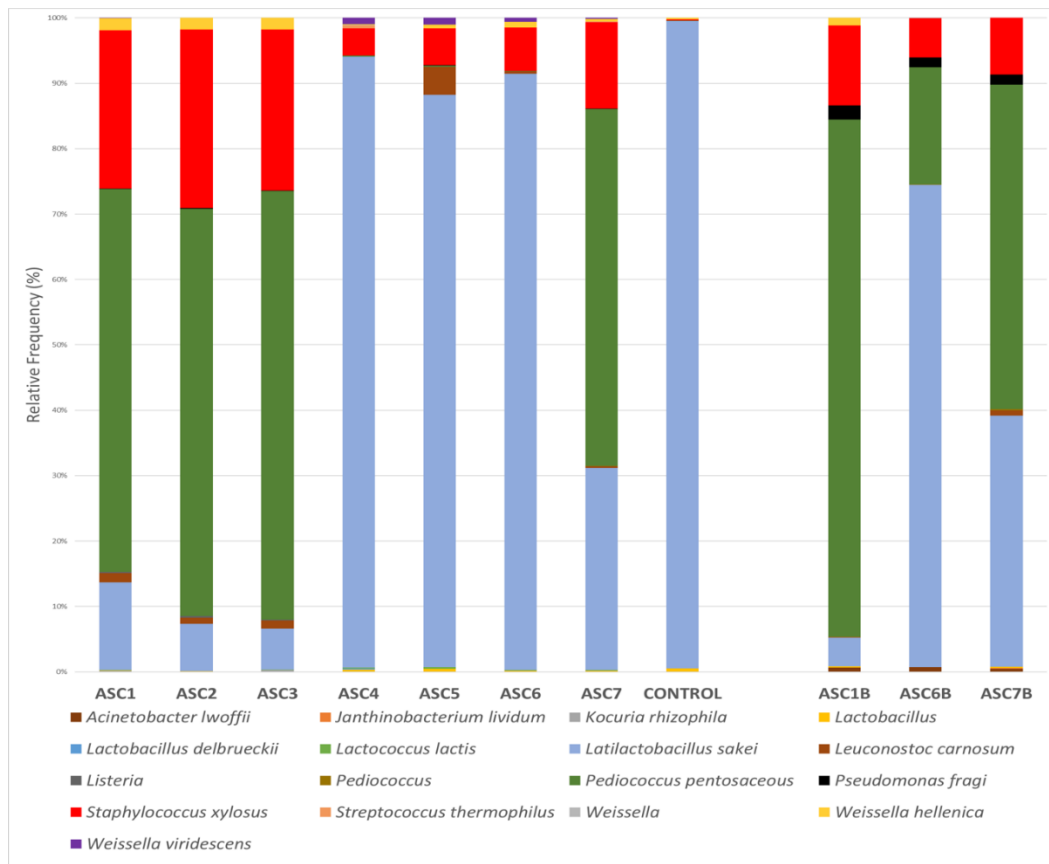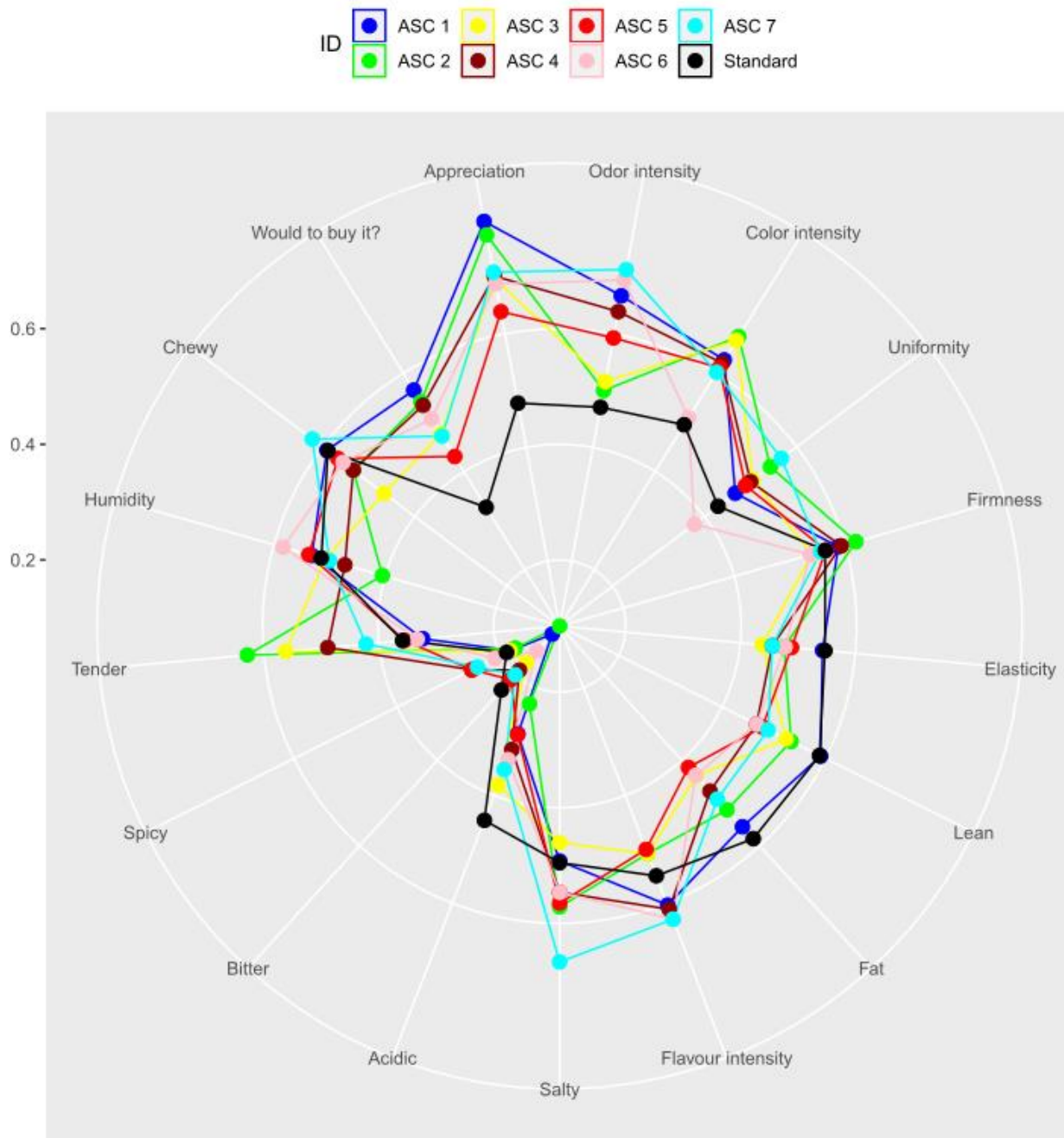663 fermentation of the second pilot-scale production.

664

665    **Fig. 1.**



PC2 21.73 %

PC1 58.81 %

666
667

668

669 **Fig. 2.**

670

671

672

**Fig. 3.**



674
675

676

**Fig 4.**



678
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680

**Table legends**

682
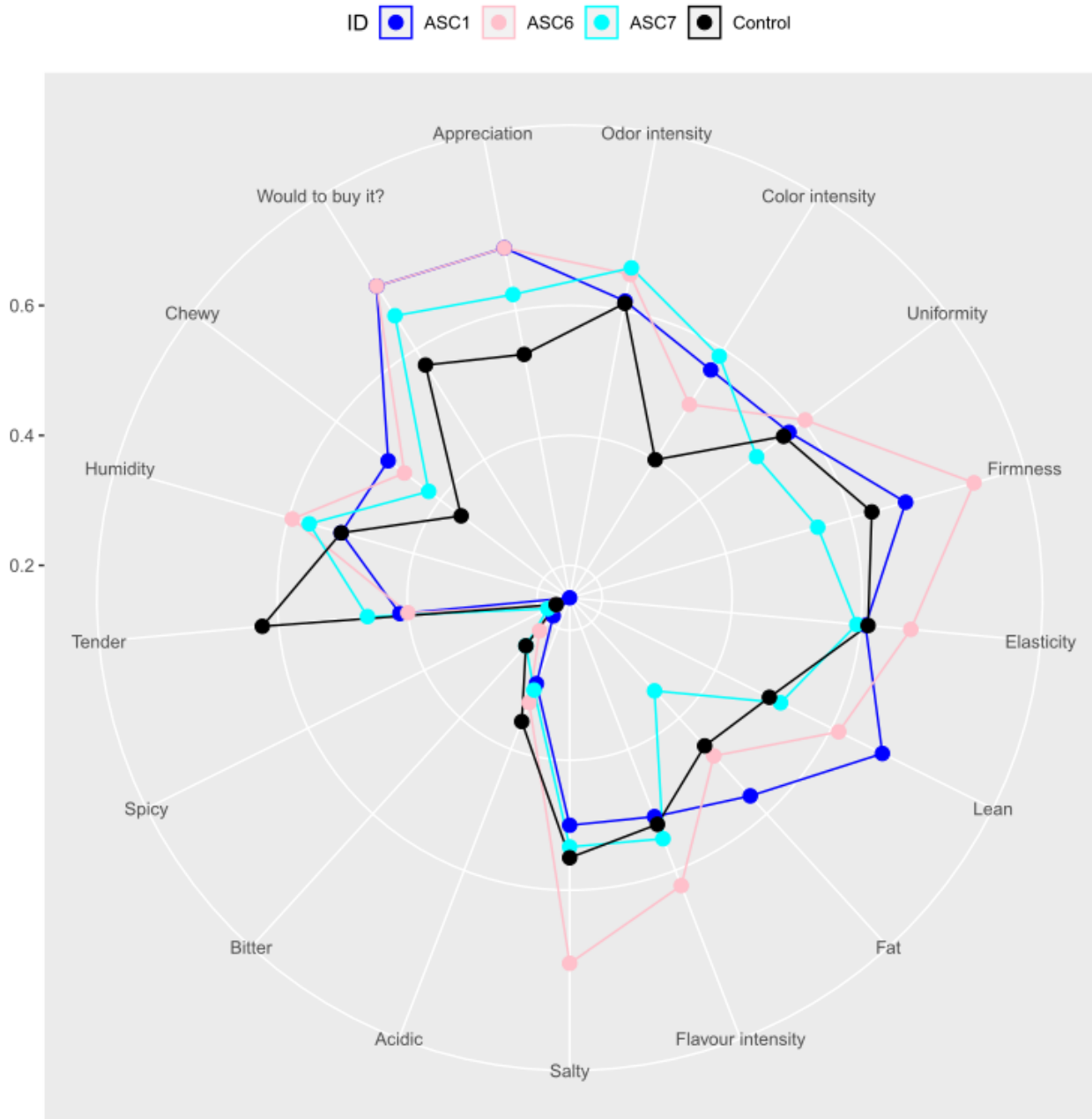
**Table 1.**

|  | *P. pentosaceus* | *L. sakei* | *S. xylosus* |
|---|---|---|---|
| **ASC 1** | S4XNM | - | S8HS |
| **ASC 2** | S8QM | - | S8HS |
| **ASC 3** | S8QM+S4XNM | - | S8HS |
| **ASC 4** | - | S29ZEM | S8HS |
| **ASC 5** | - | S29BM | S8HS |
| **ASC 6** | - | S29BM+S29ZEM | S8HS |
| **ASC 7** | S4XNM | S29ZEM | S8HS |

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686

**Table 2.** Physiological values of the ten selected strains.

| Species | Sample Code | Time (days) | Batch | Growth rate in different NaCl concentrations (µmax) | | | pH (after 24 h) | | pH (after 8 h) | | Growth rate at different temperatures (µmax) | | | | Lipolytic Activity | Nitrite Reductase Activity | Proteolytic Activity | Catalase Activity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 4% | 3% | 2% | 10°C | 15°C | 20°C | 30°C | 10°C | 15°C | 20°C | 30°C | | | | |
| *P. pentosaceus* | C1M | 0 | February | 0,37 | 0,00 | 0,01 | 6,13 | 5,72 | 5,92 | 4,96 | 0,03 | 0,10 | 0,20 | 0,48 | / | / | - | - |
| *P. pentosaceus* | S4NM | 4 | February | 0,56 | 0,00 | 0,01 | 6,05 | 5,70 | 5,92 | 4,97 | 0,04 | 0,10 | 0,21 | 0,46 | / | / | - | - |
| *P. pentosaceus* | S8QM | 8 | February | 0,56 | 0,00 | 0,01 | 6,04 | 5,83 | 5,92 | 4,89 | 0,03 | 0,10 | 0,18 | 0,45 | / | / | - | - |
| *P. pentosaceus* | S4XNM | 4 | May | 0,39 | 0,64 | 0,52 | 6,18 | 5,66 | 6,08 | 5,36 | 0,00 | 0,00 | 0,29 | 0,22 | / | / | - | - |
| *P. pentosaceus* | S45XEM | 50 | May | 0,42 | 0,39 | 0,53 | 6,17 | 5,73 | 6,05 | 4,84 | 0,03 | 0,10 | 0,20 | 0,44 | / | / | - | - |
| *L. sakei* | S29BM | 30 | February | 0,43 | 0,59 | 0,65 | 6,23 | 5,97 | 6,06 | 5,90 | 0,00 | 0,16 | 0,24 | 0,46 | / | / | - | - |
| *L. sakei* | S15ZGM | 15 | March | 0,44 | 0,61 | 0,65 | 6,02 | 6,09 | 6,19 | 5,11 | 0,04 | 0,10 | 0,17 | 0,42 | / | / | - | - |
| *L. sakei* | S29ZEM | 30 | March | 0,44 | 0,58 | 0,50 | 6,29 | 5,64 | 6,17 | 6,03 | 0,00 | 0,00 | 0,20 | 0,38 | / | / | - | - |
| *S. xylosus* | S8HS | 8 | February | 0,43 | 0,38 | 0,36 | / | / | / | / | 0,04 | 0,07 | 0,19 | 0,52 | + | + | + | / |
| *S. xylosus* | S29XIS | 30 | May | 0,59 | 0,65 | 0,58 | / | / | / | / | 0,03 | 0,07 | 0,17 | 0,00 | - | + | + | / |