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

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12 13 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.

32 **Keywords:** Fermented sausages; Starter culture selection; Microbiota; Culture-dependent method;
33 Metataxonomic analyses; Volatilome

34

35

36 **1. Introduction**

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

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

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

58 Strains composing commercial starter cultures are commonly isolated from the wild microbiota of spontaneous
59 fermentations as they are well adapted to the ecological niche in which they are intended for use (Baka *et al.*,
60 2011; Cruxen *et al.*, 2019). Accurate strain-level characterisation and selection is also necessary because strains
61 belonging to the same genus can have different impacts on the final product characteristics. Furthermore,
62 regarding safety aspects, strains used in the food industry as starter cultures should not be pathogenic, possess

63 biogenic amine potential production, or acquire antimicrobial resistance (Álvarez-Cisneros & Ponce-Alquicira,
64 2018; Laslo, György, & Czikó, 2020).

65 The use of autochthonous strains significantly improves product sensory properties compared to commercial
66 ones and native strains contribute to creating distinct final properties of a typical regional fermented product
67 (Baka *et al.*, 2011; Cruxen *et al.*, 2019). Therefore, this study aimed to select autochthonous starter cultures
68 that could be used as a new starter culture for Salame Piemonte manufacturing using an innovative
69 combinations of methodology. The selection and implementation at industry level of selected autochthonous
70 starter culture had the goal of improve the organoleptic characteristic of the final products, and at the same
71 time strengthen the link with the geographical area of production.

72 To achieve our objectives, we analysed three different batches of spontaneously fermented sausages produced
73 at different months in the same factory following the same recipe. From each fermentation batch, we isolated
74 and identified LAB and presumptive CNS, screened them for their technological properties, and evaluated
75 their safety to select the most suitable strains or strain combinations to compose different starter formulations.
76 Thereafter, the starter formulations were used to produce different pilot-scale batches of Salame Piemonte,
77 which were compared in terms of metataxonomic composition, volatilome and sensory properties through
78 consumer test analyses.

79

80 **2. Materials and methods**

81 **2.1. Sample collection from spontaneous fermented sausages**

82 Spontaneous fermented sausage samples were collected at time 0 (meat plus seasoning) and after 4, 8, 15, 30,
83 and 50 days of fermentation from three different batches of Salame Piemonte PGI produced in February,
84 March, and May 2018, according to the detailed experimental procedure already reported (Franciosa *et al.*,
85 2021). At each sampling point, water activity (a_w) and pH were determined according to the manufacturer's
86 instructions. Microbial analyses were performed as described by Belleggia *et al.* (2020). The following
87 microorganisms were counted: lactic acid bacteria (LAB), presumptive coagulase-negative staphylococci
88 (CNS), *Enterobacteriaceae*, *Listeria monocytogenes*, *Enterococci*, *Salmonella* spp., *Escherichia coli*,
89 *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).

96

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.

111

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 described 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.

132

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. nalgiovensis*, as

145 classically done for Salame Piemonte production. A control sausage was prepared using a commercial starter
146 culture composed of a mixture of *L. sakei* and *S. xylosum* (10^7 CFU/g) and used in all analyses as a control.
147 Samples were collected after 0, 2, 12, and 15 days of fermentation for physicochemical and microbiological
148 analyses. At each sampling time, three sausages were analysed for each ASC. Microbial analyses were
149 performed as described by Belleggia *et al.* (2020) for the enumeration and isolation of LAB and presumptive
150 CNS. *Listeria monocytogenes* presence was also checked.

151

152 **2.5. Molecular and metataxonomic approach**

153 Approximately nine colonies from MRS and MSA media at each sampling point were isolated and purified.
154 They were used to track the presence of the inoculated strains during fermentation using rep-PCR, as described
155 previously (Iacumin, Comi, Cantoni, & Cocolin, 2006), by comparing their rep-PCR patterns with those of
156 inoculated strains.

157 For the metataxonomic approach, total DNA was extracted from samples at the end of fermentation and used
158 for metataxonomic analyses by amplifying the V3-V4 regions of the 16S rRNA gene following the conditions
159 described elsewhere (Cardinali *et al.*, 2021; Bolyen *et al.*, 2019; Callahan *et al.*, 2016; Klintword *et al.*, 2013).

160

161 **2.6. Volatilome analyses of fermented sausages**

162 The volatile composition of the final product was determined by headspace (HS) solid-phase microextraction
163 (SPME) and analysed by gas chromatography-mass spectrometry (GC/MS) following the experimental
164 procedure reported elsewhere (Franciosa *et al.*, 2020). Briefly, 3 g of sample were placed in 20 ml vials with
165 the internal standard: 10 μ l of 2-octanol in ultrapure water (333 ppb as final concentration). We measured the
166 *m/z* peak area of the quantifier ion in relation to the *m/z* quantifier ion of the added internal standard to obtain
167 a semiquantitative result (μ g/kg) for each identified compound (Franciosa *et al.*, 2020; Ferrocino *et al.*, 2018).

168

169 **2.7. Liking test of the inoculated sausages**

170 To assess the sensory acceptability of the sausage samples at the end of ripening, a liking test was performed.
171 A total of 20 sausage consumers (7 male and 13 female participants, aged 28 to 56 years) voluntarily
172 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.

177

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.).

183

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.

189

190

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 log₁₀ CFU/g (Supplementary Table 1). The May samples showed the highest LAB population at the end of
202 fermentation (9.71 log₁₀ 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 log₁₀ 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 log₁₀ 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 *Lactobacillus 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).

212

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).

221

222

223

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

229 selected according to the PGI product specification (Official Gazette of the Italian Republic, nr 184, 10 August
230 2015). Among presumptive CNS isolates, we selected those with lipolytic, proteolytic, and nitrate reductase
231 activities and rapid growth at 20°C and 10°C (data not shown). After this selection, rep-PCR fingerprints of
232 selected isolates were compared to select only those with different rep-PCR patterns (data not shown).
233 At the end of the selection process, the selected bacteria were identified as five *P. pentosaceus* strains (C1M,
234 S4NM, S8QM, S4XNM, S45XEM), three *L. sakei* strains (S29BM, S15ZGM, S29ZEM), and two *S. xylosus*
235 strains (S8HS and S29XIS) (Table 2).

236

237 **3.4. Safety evaluation of selected isolates**

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

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

248

249 **3.5. Sausage production, microbial counts and pH of inoculated sausages**

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

255 ASC 1, 2, and 3 samples showed CNS counts that were never lower than 8 log₁₀ CFU/g. The other four ASC
256 showed lower values, starting from 7.5 log₁₀ 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).

265

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 *Weissella 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).

281

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

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

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

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

302

303 **3.8. Effect on sensory attributes of inoculated sausages**

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

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

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

319

320 **3.9. Validation of inoculated sausage production**

321 Based on liking test results, the three most appreciated ASC were used in a second pilot scale production, *i.e.*,
322 ASC 1B (*P. pentosaceus* S4XNM and *S. xylosus* S8HS), ASC 6B (*L. sakei* S29BM, *L. sakei* S29ZEM and *S.*
323 *xylosus* S8HS) and ASC 7B (*P. pentosaceus* S4XNM, *L. sakei* S29ZEM and *S. xylosus* S8HS). Microbiological
324 analyses showed the absence of *L. monocytogenes* in all analysed samples. No differences in pH were observed
325 between the two pilot-scale productions at the end of fermentation for all ASCs used (Supplementary Table 6,
326 $P<0.05$). However, the initial pH values varied between the ASCs, with ASC 1 showing the lowest pH value
327 (5.88) and ASC 6 the highest value (6.02) (Supplementary Table 6, $P<0.05$). After two days of fermentation,
328 there were no significant differences in the pH of the three products.

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

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

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

346

347 **4. Discussion**

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

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

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

378 For the CNS group, we did not observe a constant increase in their populations during fermentation, and their
379 abundance was always lower than that of LAB, as previously reported for other naturally fermented sausages
380 (Cardinali *et al.*, 2018; Ferrocino *et al.*, 2018; Rantsiou *et al.*, 2005). *S. xylosus* was the most dominant species
381 in all batches, whereas the other species were randomly isolated. The dominance of *L. sakei* and *S. xylosus* in
382 all batches of spontaneously fermented sausages was in accordance with literature data (Eisenbach, Geissler,
383 Ehrmann, & Vogel, 2019; Iacumin *et al.*, 2020; Pini, Aquilani, Giovannetti, Viti, & Pugliese, 2020; Van
384 Reckem *et al.*, 2019). NaCl tolerance is one of the criteria used to select autochthonous starters (Cruxen *et al.*,
385 2019; Laslo *et al.*, 2020). For most isolates, an increase in salt concentration reduced bacterial growth, as
386 previously reported in the literature (Aina, 2017). The ability of microorganisms to grow under different
387 conditions is species-dependent (Cruxen *et al.*, 2019) and strain-dependent (Casaburi *et al.*, 2011; Ercolini *et*
388 *al.*, 2010), as observed in this study.

389 Regarding the effect of temperature on growth, it can be said that approximately all isolates grew at 30°C,
390 whereas the number of LAB isolates able to grow decreased with a decrease in temperature. Presumptive CNS
391 isolates displayed a higher proportion of isolates able to grow at low temperatures (10°C) than at high
392 temperatures, although with slower growth. This is an unexpected result because it is known that the optimum
393 growth temperature of *S. xylosus* is 30°C (Essid, Ismail, Bel Hadj Ahmed, Ghedamsi, & Hassouna, 2007),
394 although it is able to grow well at temperatures normally used for meat fermentation (10 and 20°C) (Essid,
395 Ismail, Bel Hadj Ahmed, Ghedamsi, & Hassouna, 2007). All the results described above are important for
396 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. xylosum* and *P. nalgiovensis* 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. xylosum* S8HS) and 5 (*L. sakei* S29BM and *S. xylosum*
413 S8HS), whereas for ASC 7 (*P. pentosaceus* S4XNM and *L. sakei* S29ZEM and *S. xylosum* S8HS), the volatile
414 profile was similar to that of ASC 4 (*L. sakei* S29ZEM and *S. xylosum* 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. xylosum*
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 β -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.

466

467 **Data availability:** Sequences have been uploaded to the National Center for Biotechnology Information
468 Sequence Read Archive (Bioproject ID PRJNA669431).

469

470 **Authors' contributions:** LC, IF and KR conceived and designed the experiment. IFR, IF and MRC collected
471 the experiments data. MG performed the metabolomic investigations. IF carried out the bioinformatics
472 analyses and generated the manuscript figures. IFR and IF performed the statistical analysis. LC, KR, MC and
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485

486 **References**

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647

648 **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

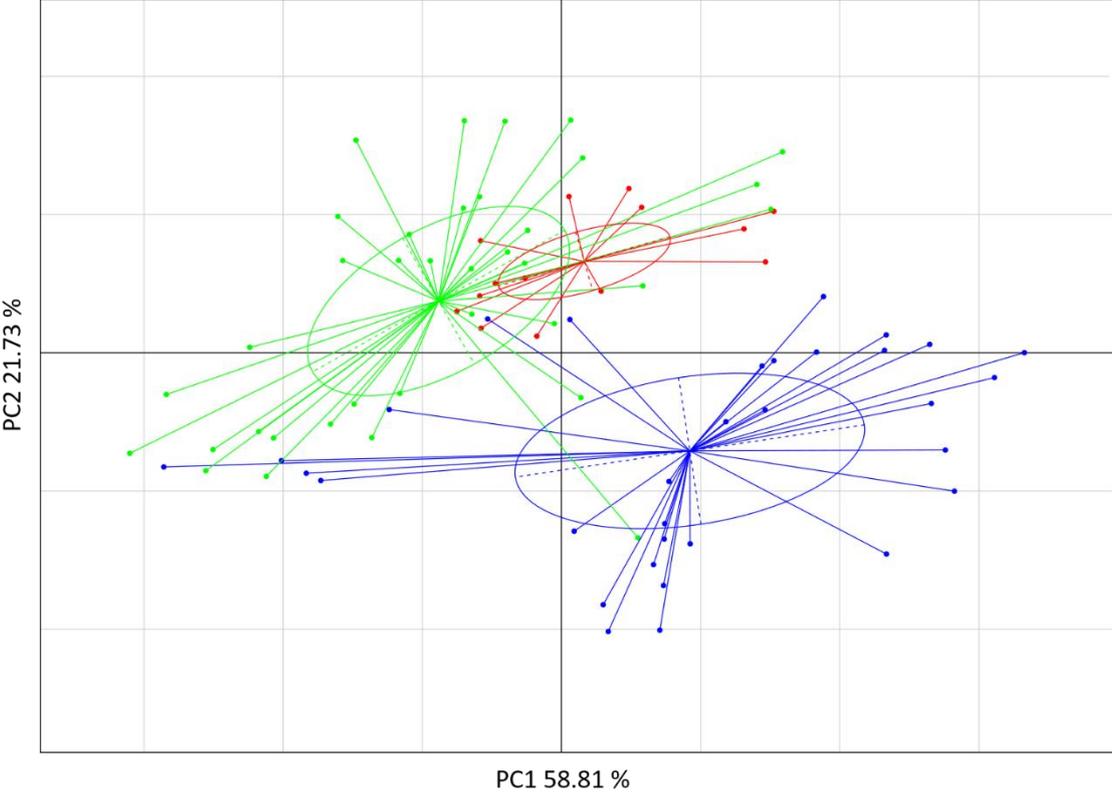
661 **Fig. 4.** Radar graphs displaying the liking of appearance, odor, taste, flavor, and texture and overall liking

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.

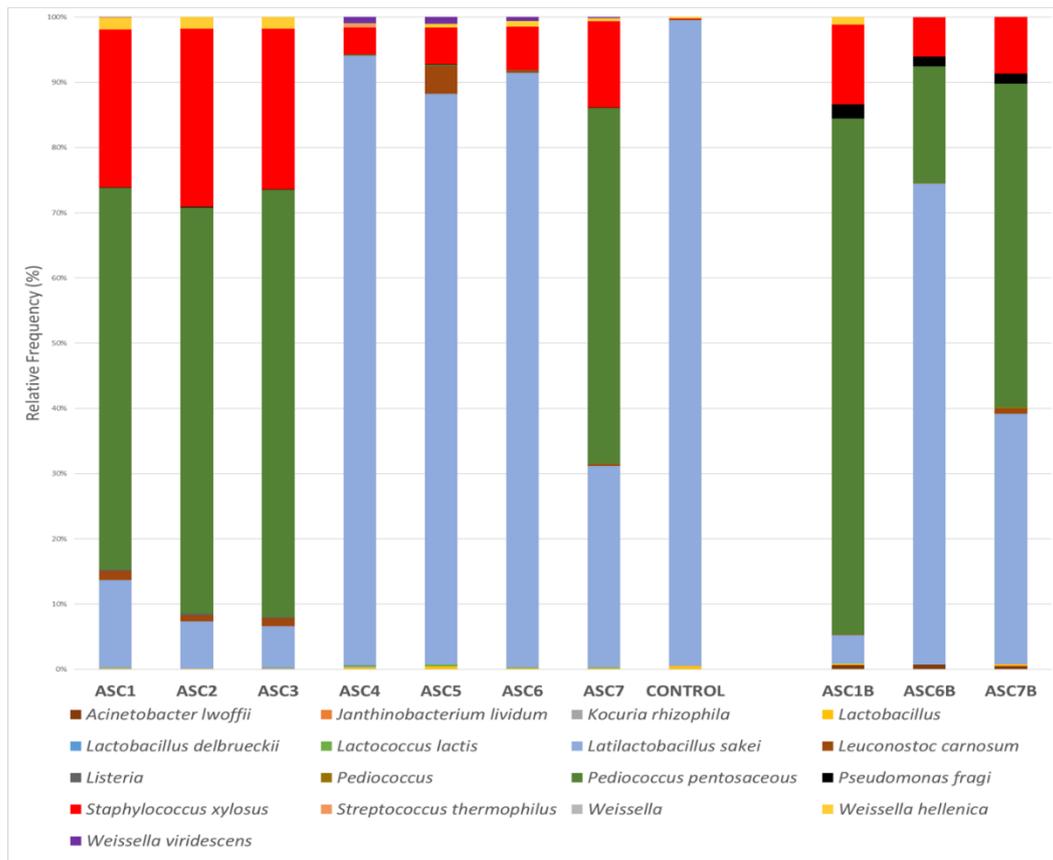


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669 **Fig. 2.**

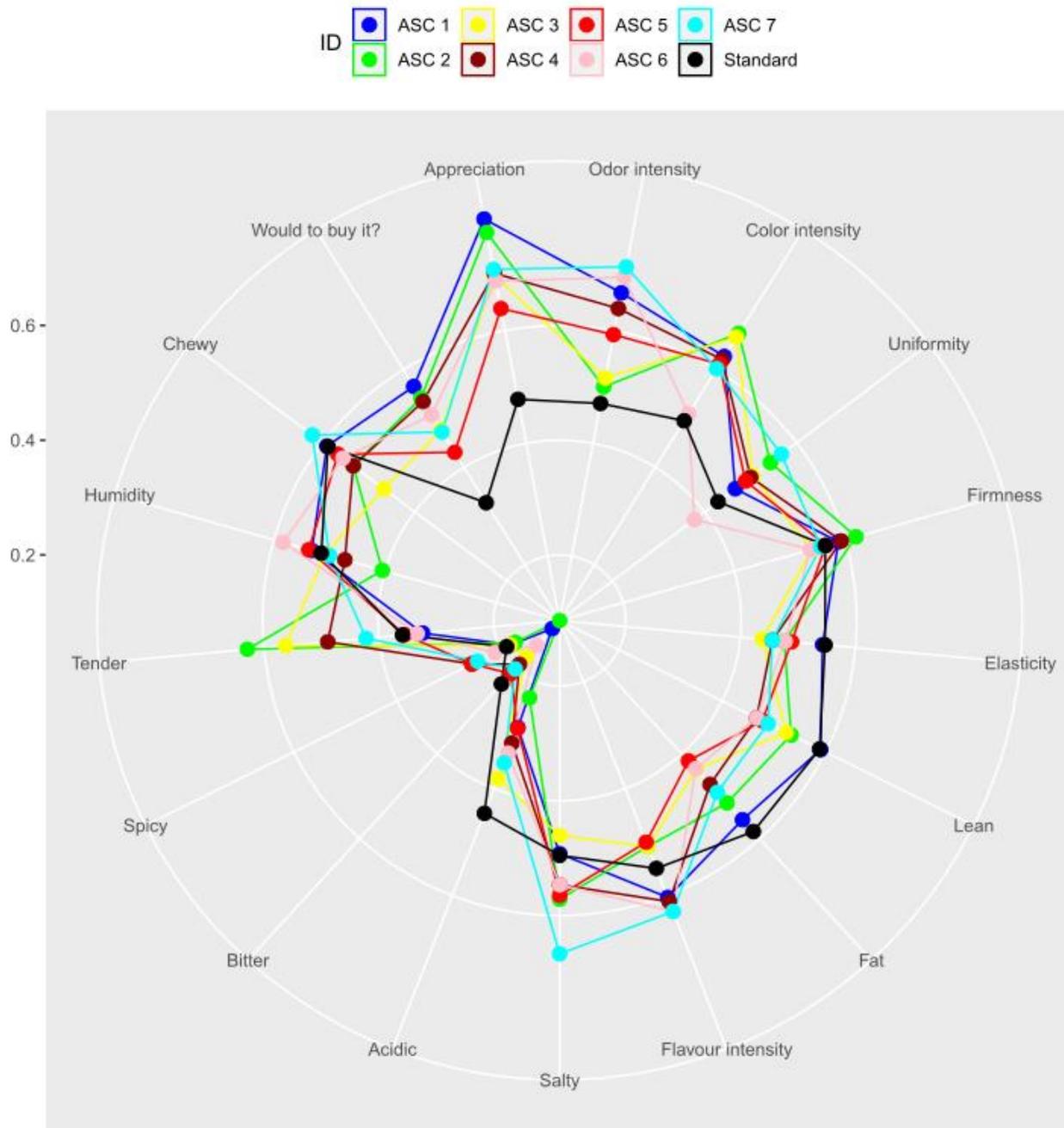


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673 **Fig. 3.**

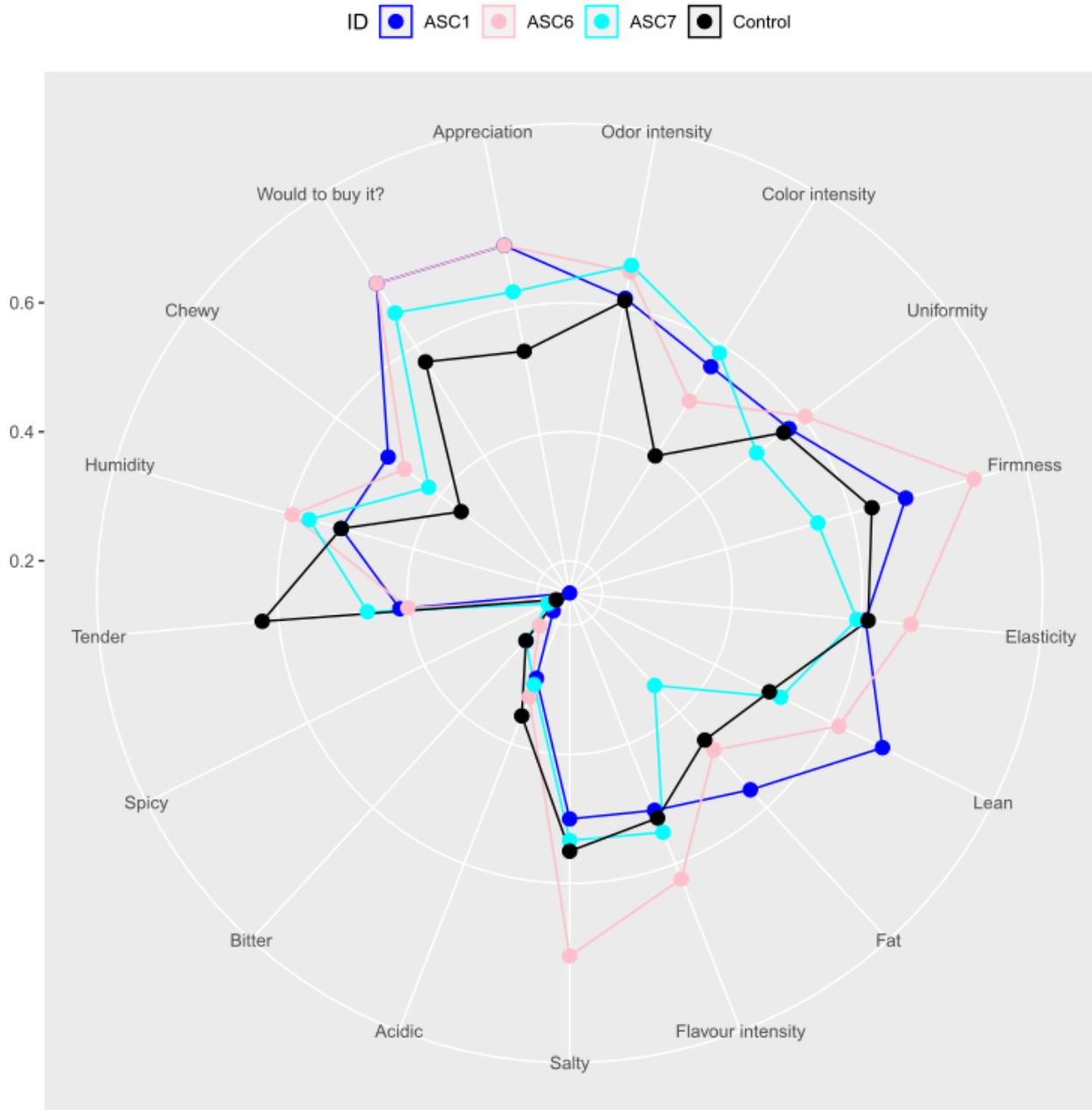


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677 **Fig 4.**



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680

681 **Table legends**

682

683 **Table 1.**

	<i>P. pentosaceus</i>	<i>L. sakei</i>	<i>S. xylosus</i>
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

684

685

686

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

Species	Sample Code	Time (days)	Batch	Growth rate in different NaCl concentrations (μ max)			pH (after 24 h)			pH (after 8 h)			Growth rate at different temperatures (μ max)				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	10°C	15°C				
<i>P. pentosaceus</i>	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	\	\	-	-		
<i>P. pentosaceus</i>	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	\	\	-	-		
<i>P. pentosaceus</i>	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	\	\	-	-		
<i>P. pentosaceus</i>	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	\	\	-	-		
<i>P. pentosaceus</i>	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	\	\	-	-		
<i>L. sakei</i>	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	\	\	-	-		
<i>L. sakei</i>	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	\	\	-	-		
<i>L. sakei</i>	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	\	\	-	-		
<i>S. xylosois</i>	S8HS	8	February	0,43	0,38	0,36	\	\	\	\	0,04	0,07	0,19	0,52	+	+	+	\		
<i>S. xylosois</i>	S29XIS	30	May	0,59	0,65	0,58	\	\	\	\	0,03	0,07	0,17	0,00	-	+	+	\		

