Autochthonous starter culture selection for Salame Piemonte PGI production

Autochthonous starter culture selection for Salame Piemonte PGI production

Irene Franciosa[a,b], Ilario Ferrocino[a], Maria Rita Corvaglia[a], Manuela Giordano[a], Monika Coton[b], Jérôme Mounier[b], Kalliopi Rantsiou[a] and Luca Cocolin[a*]

[a] University of Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Largo Braccini 2, 10095 Grugliasco, Torino, Italy

[b] Univ Brest, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, F-29280 Plouzané, France

*Corresponding author: Luca Cocolin: lucasimone.cocolin@unito.it

Postal address: Largo Paolo Braccini 2, 10095, Grugliasco, Turin, Italy

Abstract

Salame Piemonte is a dry-fermented meat product typical of the Piedmont region in Italy, manufactured using commercial starter cultures. This study aimed to select autochthonous starter cultures (ASCs) that could be used for sausage fermentation in order to strengthen the link with the geographical area of production and improve the sensory properties of the final product.

A culture-dependent approach was adopted during three different spontaneous sausage fermentation processes to isolate and characterise the main bacterial resources involved. Dominant lactic acid bacteria (LAB) in each batch were *Pediococcus pentosaceus*, *Latilactobacillus sakei*, and *Latilactobacillus curvatus*; *Staphylococcus xylosus* was the most dominant coagulase-negative staphylococci (CNS) in all the studied batches. LAB and presumptive CNS isolates were further evaluated for their physiological properties and biotechnological potential. Thereafter, 11 strains were selected and evaluated for safety. Five selected strains (two *P. pentosaceus*, two *L. sakei*, and one *S. xylosus* strain) were used for pilot-scale Salame Piemonte production with seven different strain combinations. Based on the liking test, three ASC combinations led to the highest liking score compared to industrial products. These three ASCs were then used for the second pilot-scale sausage production confirming the high liking score. In summary, the use of *P. pentosaceus* and *S. xylosus* ASC significantly improved product sensory properties compared with that obtained using commercial starter cultures.

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

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

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*.

Approximately 15 colonies from MRS and MSA media at each sampling point from each batch were randomly isolated, purified, and identified as described by Franciosa *et al.* (2021).Isolates were subjected to DNA extraction, PCR (rep-PCR) with the $(GTG)_5$ primer and cluster analysis as already reported Ferrocino *et al.* (2017). After cluster analysis, 2 isolates from each cluster at 80% of similarity were selected and subjected to identification. LAB and CNS identification was performed by amplifying the 16S rRNA gene (Ferrocino et al., 2017; Weisburg, Barns, Pelletier, & Lane, 1991).

2.2. Strain physiological characterization

Growth curves were reconstructed for each isolate under the following conditions, and spectrophotometric data were processed in the R environment using the package *Growthcurver*. LAB isolates were grown in De Man, Rogosa e Sharpe (MRS) broth at four different temperatures (30, 23, 15, and 10°C) and NaCl concentrations (2, 3, and 4% v/v), whereas presumptive CNS isolates were grown in Brain Heart Infusion (BHI) broth under the same conditions. The acidification rates at different temperatures (30, 23, 15, and 10°C) were also measured. Presumptive CNS isolates were tested for their lipolytic activity on Spirit Blue Agar plates supplemented with a mixture of olive oil and Tween 80 (Ercolini et al., 2010), proteolytic activity on skim milk agar (1% skim milk, 1.5% agar) (Ercolini et al., 2009); nitrate reductase activity by spectrophotometric assay (Casaburi, Blaiotta, Mauriello, Pepe, & Villani, 2005). Lipolytic and proteolytic activity were evaluated by the detection of clear zones around the colonies after 24h at 37°C.

Non-inoculated control samples were included in the readings, and blank data were used for modelling. Each isolate was analysed in triplicates. *L. sakei* and *S. xylosus* strains composing the commercial starter culture currently used by the product manufacturer were analysed and used as controls.

2.3. Safety evaluation

Based on the physiological and molecular results, selected strains (8 LAB and 2 CNS) were evaluated for their safety by detecting biogenic amine genes and screening for antimicrobial resistance as described by Coton *et al.* (2010, 2018). Detection of four biogenic amine genes: histidine decarboxylase (hdc), tyrosine decarboxylase (tyrdc), putrescine decarboxylase (odc) and agmatine deiminase (agdi) genes was performed by multiplex PCR as described by Coton et al. (2010, 2018). An uniplex PCR for each BA gene was performed

to confirm the results of the multiplex. Antibiotic resistance was determined for 12 different antibiotics (kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, neomycin, vancomycin, trimethoprim, oxytetracycline, rifampicin) according to the ISO 10932 IDF 223 International Standard (2010-06-15) (Coton et al., 2018). *Lactiplantibacillus plantarum* (LMG6907) was used as reference strain as specified by the ISO standard. The growth was verified by an automatic Multiskan FC plate reader (Thermo Scientific, Paris, France) set to 620 nm. Each strain was assayed in triplicate in the microplates arranged as explained in the ISO guideline and the MIC was read after 48h as the first well where no growth was visible. The final results were expressed as minimum inhibitory concentrations in micrograms per milliliter and the values were compared to the EFSA breakpoints (European Food Safety Authority, 2012) and literature data (Danielsen and Wind, 2003).

Only strains that did not show the presence of biogenic amine genes were (four LAB and two CNS) were tested for their antimicrobial resistance as decribed above. Strains that showed no antimicrobial resistance beyond known natural ones (four LAB and one CNS strain) were considered safe and used for meat inoculation at a pilot scale.

2.4. Starter formulation and meat inoculation

A first preliminary pilot-scale production of fermented sausages at a meat plant in the province of Turin (Piemonte Region, Italy) was done and prepared using five different strains combined into seven autochthonous starter cultures (ASC) (Table 1). The sausage formulation (20 Kg of meat mixture for each ASC) included pork meat (lean from the shoulder and fat from the belly), salt (maximum 3%), pepper (maximum 0.4%), aromatic plants and spices, i.e., garlic and cloves, whole, crushed or infused with wine and nutmeg. After chopping and mixing the ingredients, the mixture was divided into batches according to each autochthonous starter formulation. The cutter and filler were cleaned and disinfected before the pilot-scale production and between different batches to avoid contamination. The sausages were placed in the ripening room under the same conditions as that employed for industrial production (Franciosa *et al*., 2021) for 15 days. Each batch was individually inoculated once with approximately 10^7 CFU/g of starter. At the same time, all sausages were also inoculated on the casing surface with a commercial starter culture of *P. nalgiovense*, 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. xylosus* (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 µl 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 (µg/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,

with a glass of water to clean their mouths between each sample. They evaluated external appearance, texture, colour, flavour, and consumers global appreciation. For each sample, consumers completed a table modified from the one described by Chiavari, Coloretti, Ferri, & Nanni (2007). The results were elaborated and plotted using a radar graph generated in an R environment.

2.8. Production process validation

A second pilot-scale production was performed using the combinations of ASC that showed the best sensory results, following the procedure previously described to verify process standardisation. Sausages of the second pilot-scale production were analysed through physicochemical and microbial analyses (see paragraph 2.5.), molecular, and metataxonomic approaches (see paragraph 2.6.) and sensory evaluations (see Section 2.8.).

2.9. Statistical analyses

A one-way analysis of variance (ANOVA) was used to detect significant differences among means followed by Duncan's multiple range test for mean comparison of microbial counts, physicochemical parameters, alpha diversity parameters, and ASV abundance using the SPSS 17.0 program for Windows (SPSS Inc., Chicago, IL, USA) or QIIME2 as appropriate.

3. Results

3.1. Bacterial counts and microbiota composition of spontaneously fermented sausages

The pH values decreased as a function of fermentation time, showing some differences between the three analysed batches (Supplementary Table 1, $P<0.05$). In particular, for the February, March, and May samples, 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 process, respectively. Notably, at the end of the fermentation process, March samples showed the highest pH value and February samples the lowest, despite their highest initial pH values (Supplementary Table 1). No significant differences were observed between the three batches for the a_w values (Supplementary Table 1).

In all analysed batches, LAB counts rapidly increased in the first eight days of ripening and then remained 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).
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 $\log_{10}$ CFU/g. The other four ASC
256 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).
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 *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).
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

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

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

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)

encountered during Salame Piemonte production. Finally, the selected presumptive CNS strains showed proteolytic, lipolytic, and nitrate reductase activity on the assayed agar medium, which was important given the beneficial effects of these activities on texture, flavour, and colour development (Laslo *et al*., 2020). Moreover, before using any strain for pilot-scale production and future industrial applications, the selected strains were evaluated based on biogenic amine gene detection and antimicrobial resistance (MIC determination) to discard any LAB isolates harbouring decarboxylase genes and/or showing intermediate resistance to antimicrobials.

The fermentation process using our ASC satisfied the standard requirements of Salame Piemonte since the directive reported in the disciplinary (Official Gazette of the Italian Republic, nr 184, 10 August 2015) requires the final pH value to be equal to or higher than 5.2. In addition, a correct acidification process favours inhibition of *Listeria* spp. (Zaiko, Bataeva, Yushina, Makhova, & Minaev, 2020).

The sensory characteristics of the final products were related to the different lactic acid bacteria strains used in ASC formulations and the inoculated *S. xylosus* and *P. nalgiovense* strain activities. The difference in the volatile profiles can be linked to the complex synergic interactions between microbiota and microbiome activities. In general, we observed that the sausages that were enriched in aromatic components were those obtained with ASC 1 (*P. pentosaceus* S4XNM and S. xylosusS8HS) and 5 (*L. sakei* S29BM and S. xylosus S8HS), whereas for ASC 7 (*P. pentosaceus* S4XNM and *L. sakei* S29ZEM and S. xylosus S8HS), the volatile profile was similar to that of ASC 4 (*L. sakei* S29ZEM and S. xylosus S8HS). In particular, *L. sakei* S29BM had a significant impact on sausage volatilome. The volatilome of sausages produced with ASC 6 (*L. sakei* S29BM and S29ZEM) was more similar to that of ASC 5 (*L. sakei* S29BM) than to ASC 4 (*L. sakei* S29ZEM) (Fig. 4).

The highest concentrations of diacetyl and acetoin were found in samples in which *P. pentosaceus* strains were used to drive the fermentation (ASC 1). These compounds are products of the carbohydrate catabolism of LAB and staphylococci and are associated with dairy odours, mostly found in fresh meats (Montel, Masson, & Talon, 1998). It is known that *Pediococcus* genera produce more acetoin than *L. sakei*, in particular *P. pentosaceus*, which is often associated with acetoin and diacetyl production (Sunesen, Trihaas, & Stahnke, 2004). Samples inoculated with *P. pentosaceus* strains, which were also characterised by the highest *S. xylosus* population, also showed a high concentration of alcohol compounds, *that is*, ethanol (ethyl alcohol), isopentyl

alcohol, 1-hexanol, and 1-octanol. 1-Octanol has specific odour attributes referred to as waxy, green, citrus, and floral with a sweet and coconut nuance (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015), whereas the attributes related to 1-hexanol are cheese, oxidised fat, rancid, and humidity (Perea-Sanz, Montero, Belloch, & Flores, 2018). Other studies reported that *P. pentosaceus* gives aromatic characteristics to the final product, and the highest aldehyde, alcohol, and acid contents were obtained in samples inoculated with *P. pentosaceus* (Chen, Liu, *et al*., 2015; Cruxen *et al*., 2019).

The contents of hexanoic acid and octanoic acid, which originate from the oxidation of the corresponding alcohols (Hu *et al*., 2019), were both higher in sausages made with *L. sakei* strains (ASC 4, 5, 6) than in sausages inoculated with *P. pentosaceus* (ASC 1, 2, 3). In other cases, a low concentration of acetic acid has been reported as preferred from a sensory point of view (Iacumin *et al*., 2020). Samples from ASC1 showed also a highest level of 2-pentanone produced by CNS through b-oxidation of saturated fatty acids (Fadda et al. 2002; Engelvin et al. 2000).

In this study, consumers preferred sausages with the highest concentration of acetic acid. The liking test showed that consumers preferred sausages produced using ASC 1 (*P. pentosaceus* S4XNM), which exhibited the highest concentration of acetic acid and 2-pentanone. The preference for this sausage by consumers can be explained by the highest predominance this methyl ketone being highly related to the typical cured aroma of fermented sausages (Berdagué et al. 1993) together with the low saltiness perception ($P<0.05$), high odour intensity ($P<0.05$), and texture characteristics (tenderness $P<0.05$). Consumer preference was also correlated with *S. xylosus* population. *S. xylosus* counts were 2-3 $\log_{10}$ higher in ASC1, 2, and 3 sausages (containing *P. pentosaceus*) than in the other ASC and control sausages (containing *L. sakei* strains). Given the role of the CNS in meat fermentation (colour, lipolysis, proteolysis) (Iacumin, Comi, Cantoni, & Cocolin, 2006), it is likely that *S. xylosus* contributed to a larger extent to the sausage organoleptic properties of ASC1, 2, and 3, and together with *P. pentosaceus* to the metabolic activities that yielded final products preferred by consumers.

The second pilot-scale production underlined the repeatability of the ASC inoculation; rep-PCR fingerprinting of isolates collected during fermentation and metataxonomic analysis confirmed that the inoculated strains were dominant from the beginning to the end of the fermentation process. In samples inoculated with ASC 6 B, *P. pentosaceus* was detected at high relative abundances, despite not being deliberately inoculated. This is possibly due to the high initial load of *P. pentosaceus* in the meat used for sausage production. The raw meat

used for sausage production in the second trial was characterized by the presence of indigenous *P. pentosaceus* and *Pseudomonas fragi*. These two species were found in the final product; thus, they were able to colonise this kind of product and probably grow throughout the fermentation process.

Other subdominant species were identified through metataxonomic analysis; however, their relative abundance was low. Therefore, we can assume that their contribution to the final sensory properties was not major. Therefore, we can affirm that the selected strains significantly contributed to the final product characteristics.

5. Conclusions

In conclusion, to obtain desirable organoleptic characteristics, including the *P. pentosaceus* S4XNM strain during fermentation is the best practice. We demonstrated that the use of autochthonous strains significantly improved the sensory properties of the product as compared to products obtained with commercial starter cultures. However, more work is necessary to improve the standardisation of sausage production with the most promising ASC to avoid or limit the influence of the indigenous microbiota of raw meat.

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

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

Funding: This work, conducted in the framework of the ProMedFoods project 'Promotion of local Mediterranean fermented foods through a better knowledge and management of microbial resources', was supported by the ARIMNet2 2016 Call by the following funding agencies: ANR (France), MERS (Algeria), ELGO-DEMETER (Greece), MIPAF (Italy), INIA (Spain) and MHESR (Tunisia). ARIMNet2 (ERA-NET)

481 has received funding from the European Union's Seventh Framework Programme for research, technological
482 development and demonstration under grant agreement no. 618127.
483
484 **Declarations of interest:** none
485

References

Aina, A. T. (2017). Effect of Sodium Chloride (NaCl) on the growth of *Pediococcus acidilactici* used for the improvement of nutritional and microbial quality of Tsire: a Nigerian grilled meat product. *Journal of Nutrition & Food Sciences*, *07*(04), 7–10. https://doi.org/10.4172/2155-9600.1000614

Álvarez-Cisneros, Y. M., & Ponce-Alquicira, E. (2018). Antibiotic resistance in lactic acid bacteria. In *Antimicrobial Resistance-A Global Threat.* IntechOpen, https://doi.org/10.5772/intechopen.80624

Ammor, M. S., & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production : An update. *Meat science 76*, 138–146. https://doi.org/10.1016/j.meatsci.2006.10.022

Baka, A. M., Papavergou, E. J., Pragalaki, T., Bloukas, J. G., & Kotzekidou, P. (2011). Effect of selected autochthonous starter cultures on processing and quality characteristics of Greek fermented sausages. *LWT - Food Science and Technology*, *44*(1), 54–61. https://doi.org/10.1016/j.lwt.2010.05.019

Belleggia, L., Milanović, V., Ferrocino, I., Cocolin, L., Haouet, M. N., Scuota, S., … Osimani, A. (2020). Is there any still undisclosed biodiversity in Ciauscolo salami? A new glance into the microbiota of an artisan production as revealed by high-throughput sequencing. *Meat Science*, 108128. https://doi.org/10.1016/j.meatsci.2020.108128

Berdagué, J. L., Monteil, P., Montel, M. C., & Talon, R. (1993). Effects of starter cultures on the formation of flavour compounds in dry sausage. *Meat Science*, 35(3), 275-287. https://doi.org/10.1016/0309-1740(93)90033-E

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cardinali, F., Milanović, V., Osimani, A., Aquilanti, L., Taccari, M., Garofalo, C., … Haouet, M. N. (2018). Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates

514 and nitrites. *International Journal of Food Microbiology, 278*, 61–72.
515 https://doi.org/10.1016/j.ijfoodmicro.2018.04.032
516 Cardinali, F., Ferrocino, I., Milanović, V., Belleggia, L., Corvaglia, M. R., Garofalo, C., … Aquilanti, L.
517 (2021). Microbial communities and volatile profile of Queijo de Azeitão PDO cheese, a traditional
518 Mediterranean thistle-curdled cheese from Portugal. *Food Research International*, *147*(3).
519 https://doi.org/10.1016/j.foodres.2021.110537
520 Casaburi, A., Blaiotta, G., Mauriello, G., Pepe, O., & Villani, F. (2005). Technological activities of
521 *Staphylococcus carnosus* and *Staphylococcus simulans* strains isolated from fermented sausages. *Meat*
522 *Science 71*(4), 643–650. https://doi.org/10.1016/j.meatsci.2005.05.008
523 Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Mauriello, G., Villani, F., & Ercolini, D. (2011). Spoilage-
524 related activity of *Carnobacterium maltaromaticum* strains in air-stored and vacuum-packed meat.
525 *Applied and Environmental Microbiology, 77*(20), 7382–7393. https://doi.org/10.1128/AEM.05304-11
526 Casaburi, A., Piombino, P., Nychas, G. J., Villani, F., & Ercolini, D. (2015). Bacterial populations and the
527 volatilome associated to meat spoilage. *Food Microbiology, 45*, 83–102.
528 https://doi.org/10.1016/j.fm.2014.02.002
529 Chen, Q., Kong, B., Han, Q., Xia, X., & Xu, L. (2017). The role of bacterial fermentation in lipolysis and lipid
530 oxidation in Harbin dry sausages and its flavour development. *LWT - Food Science and Technology*, *77*,
531 389–396. https://doi.org/10.1016/j.lwt.2016.11.075
532 Chen, Q., Kong, B., Sun, Q., Dong, F., & Liu, Q. (2015). Antioxidant potential of a unique LAB culture
533 isolated from Harbin dry sausage: *In vitro* and in a sausage model. *Meat Science, 110,* 180–188.
534 https://doi.org/10.1016/j.meatsci.2015.07.021
535 Chen, Q., Liu, Q., Sun, Q., Kong, B., & Xiong, Y. (2015). Flavour formation from hydrolysis of pork
536 sarcoplasmic protein extract by a unique LAB culture isolated from Harbin dry sausage. *Meat Science,*
537 *100,* 110–117. https://doi.org/10.1016/j.meatsci.2014.10.001
538 Chiavari, C., Coloretti, F., Ferri, G., & Nanni, M. (2007). Proposal of a method for sensory evaluation of italian
539 salami. *Industrie Alimentari, 46*(469).
540 Coton, E., Mulder, N., Coton, M., Pochet, S., Trip, H., & Lolkema, J. S. (2010). Origin of the putrescine-
541 producing ability of the coagulase-negative bacterium *Staphylococcus epidermidis* 2015B. *Applied and*

542 *Environmental Microbiology, 76*(16), 5570–5576. https://doi.org/10.1128/AEM.00441-10
543 Coton, M., Lebreton, M., Leyva Salas, M., Garnier, L., Navarri, M., Pawtowski, A., … Mounier, J. (2018).
544 Biogenic amine and antibiotic resistance profiles determined for lactic acid bacteria and a
545 propionibacterium prior to use as antifungal bioprotective cultures. *International Dairy Journal, 85*, 21–
546 26. https://doi.org/10.1016/j.idairyj.2018.05.001
547 Cruxen, C. E. dos S., Funck, G. D., Haubert, L., Dannenberg, G. da S., Marques, J. de L., Chaves, F. C., …
548 Fiorentini, Â. M. (2019). Selection of native bacterial starter culture in the production of fermented meat
549 sausages: Application potential, safety aspects, and emerging technologies. *Food Research International,*
550 *122*, 371–382. https://doi.org/10.1016/j.foodres.2019.04.018
551 Danielsen, M., & Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International*
552 *Journal of Food Microbiology, 82*(1), 1–11. https://doi.org/10.1016/S0168-1605(02)00254-4
553 Eisenbach, L., Geissler, A. J., Ehrmann, M. A., & Vogel, R. F. (2019). Comparative genomics of *Lactobacillus*
554 *sakei* supports the development of starter strain combinations. *Microbiological Research, 221*, 1–9.
555 https://doi.org/10.1016/j.micres.2019.01.001
556 Engelvin, G., Feron, G., Perrin, C., Mollet, D., Talon, R., 2000. Identification of h-oxidation and thioesterase
557 activities in *Staphylococcus carnosus* 833 strain. *FEMS Microbiology Letters* 190, 115–120.
558 https://doi.org/10.1111/j.1574-6968.2000.tb09272.x
559 Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Ferranti, P., Mauriello, G., & Villani, F. (2010). Different
560 molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers. *International*
561 *Journal of Food Microbiology, 142*(1–2), 120–131. https://doi.org/10.1016/j.ijfoodmicro.2010.06.012
562 Ercolini, D., Russo, F., Ferrocino, I., & Villani, F. (2009). Molecular identification of mesophilic and
563 psychrotrophic bacteria from raw cow's milk. Food microbiology, 26(2), 228-231.
564 Essid, I., Ben Ismail, H., Bel Hadj Ahmed, S., Ghedamsi, R., & Hassouna, M. (2007). Characterization and
565 technological properties of *Staphylococcus xylosus* strains isolated from a Tunisian traditional salted
566 meat. *Meat Science, 77*(2), 204–212. https://doi.org/10.1016/j.meatsci.2007.03.003
567 European Food Safety Authority (EFSA) (2012). Guidance on the assessment of bacterial susceptibility to
568 antimicrobials of human and veterinary importance. *EFSA Journal, 10*(6), 1–10.
569 https://doi.org/10.2903/j.efsa.2012.2740

570 Fadda, S., Lebert, A., Leroy-Se´trin, S., Talon, R., 2002. Decarboxylase activity involved in methyl ketone
571 production by *Staphylococcus carnosus* 833, a strain used in sausage fermentation. *FEMS Microbiology*
572 *Letters,* 210, 209–214. https://doi.org/10.1111/j.1574-6968.2002.tb11182.x
573 Ferrocino I., Bellio A., Romano A., M. G., & Rantsiou K., Decastell L., C. L. (2017). RNA-Based amplicon
574 sequencing reveals microbiota development during ripening of artisanal versus industrial Lard d'Arnad.
575 *Applied and Environmental Microbiology*, *83*(16), 1–14.
576 Ferrocino, I., Bellio, A., Giordano, M., Macori, G., Romano, A., Rantsiou, K., … Cocolin, L. (2018). Shotgun
577 metagenomics and volatilome profile of the microbiota of fermented sausages. *Applied and*
578 *Environmental Microbiology*, *84*(3), 1–14. https://doi.org/10.1128/AEM.02120-17
579 Franciosa, I., Alessandria, V., Dolci, P., Rantsiou, K., & Cocolin, L. (2018). Sausage fermentation and starter
580 cultures in the era of molecular biology methods. *International Journal of Food Microbiology*, *279*, 26–
581 32. https://doi.org/10.1016/j.ijfoodmicro.2018.04.038
582 Franciosa, I., Ferrocino, I., Giordano, M., Mounier, J., Rantsiou, K., & Cocolin, L. (2021). Specific
583 metagenomic asset drives the spontaneous fermentation of Italian sausages. *Food Research International*,
584 *144*, 110379. https://doi.org/10.1016/j.foodres.2021.110379
585 Hu, Y., Chen, Q., Wen, R., Wang, Y., Qin, L., & Kong, B. (2019). Quality characteristics and flavor profile
586 of Harbin dry sausages inoculated with lactic acid bacteria and *Staphylococcus xylosus*. *LWT - Food*
587 *Science and Technology*, *114*, 108392. https://doi.org/10.1016/j.lwt.2019.108392
588 Iacumin, L., Comi, G., Cantoni, C., & Cocolin, L. (2006). Molecular and technological characterization of
589 *Staphylococcus xylosus* isolated from naturally fermented Italian sausages by RAPD, Rep-PCR and Sau-
590 PCR analysis. *Meat Science*, *74*(2), 281–288. https://doi.org/10.1016/j.meatsci.2006.03.020
591 Iacumin, L., Osualdini, M., Bovolenta, S., Boscolo, D., Chiesa, L., Panseri, S., & Comi, G. (2020). Microbial,
592 chemico-physical and volatile aromatic compounds characterization of Pitina PGI, a peculiar sausage-
593 like product of North East Italy. *Meat Science*, *163*, 108081.
594 https://doi.org/10.1016/j.meatsci.2020.108081
595 Kim, J. S., Kim, J. H., Arunachalam Palaniyandi, S., Lee, C. C., You, J. W., Yang, H., … Lee, K. W. (2019).
596 Yak-Kong soybean (Glycine max) fermented by a novel *Pediococcus pentosaceus* inhibits the oxidative
597 stress-induced Monocyte–Endothelial Cell Adhesion. *Nutrients*, *11*(6).

https://doi.org/10.3390/nu11061380

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), 1–11. https://doi.org/10.1093/nar/gks808

Laslo, É., György, É., & Czikó, A. (2020). Meat starter cultures: Isolation and characterization of lactic acid bacteria from traditional sausages. Acta Universitatis Sapientiae, *Alimentaria, 12*(1), 54–69. https://doi.org/10.2478/ausal-2019-0004

Montel, M. C., Masson, F., & Talon, R. (1998). Bacterial role in flavour development. *Meat Science, 49*. https://doi.org/10.1016/S0309-1740(98)90042-0

Murgia, M.A., Marongiu, A., Aponte, M., Blaiotta, G., Deiana, P., Mangia, N.P., 2019. Impact of a selected *Debaryomyces hansenii* strain's inoculation on the quality of Sardinian fermented sausages. *Food Research International, 121*, 144–150 .

Perea-Sanz, L., Montero, R., Belloch, C., & Flores, M. (2018). Nitrate reduction in the fermentation process of salt reduced dry sausages: Impact on microbial and physicochemical parameters and aroma profile. International *Journal of Food Microbiology, 282*, 84–91. https://doi.org/10.1016/j.ijfoodmicro.2018.06.004

Pini, F., Aquilani, C., Giovannetti, L., Viti, C., & Pugliese, C. (2020). Characterization of the microbial community composition in Italian Cinta Senese sausages dry-fermented with natural extracts as alternatives to sodium nitrite. *Food Microbiology, 89*, 103417. https://doi.org/10.1016/j.fm.2020.103417

Porto, M. C. W., Kuniyoshi, T. M., Azevedo, P. O. S., Vitolo, M., & Oliveira, R. P. S. (2017). *Pediococcus* spp.: An important genus of lactic acid bacteria and pediocin producers. *Biotechnology Advances*, *35*(3), 361–374. https://doi.org/10.1016/j.biotechadv.2017.03.004

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., & Cocolin, L. (2005). Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. *Applied and Environmental Microbiology, 71*(4), 1977–1986. https://doi.org/10.1128/AEM.71.4.1977-1986.2005

Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F., & Ercolini, D. (2016). Overlap of spoilage-associated microbiota between meat and the meat processing environment in small-scale and large-scale

626 retail distributions. *Applied and Environmental Microbiology*, *82*(13), 4045–4054.
627 https://doi.org/10.1128/AEM.00793-16
628 Sun, F., Hu, Y., Chen, Q., Kong, B., & Liu, Q. (2019). Purification and biochemical characteristics of the
629 extracellular protease from *Pediococcus pentosaceus* isolated from Harbin dry sausages. *Meat Science*,
630 *156*, 156–165. https://doi.org/10.1016/j.meatsci.2019.05.030
631 Sunesen, L. O., Trihaas, J., & Stahnke, L. H. (2004). Volatiles in a sausage surface model-influence of
632 *Penicillium nalgiovense*, *Pediococcus pentosaceus*, ascorbate, nitrate and temperature. *Meat Science*,
633 *66*(2), 447–456. https://doi.org/10.1016/S0309-1740(03)00133-5
634 Sunesen, L.O., & Stahnke, L.H., (2003). Mould starter cultures for dry sausages - Selection, application and
635 effects. *Meat Science*, *65*, 935–948. https://doi.org/10.1016/S0309-1740(02)00281-4
636 Van Reckem, E., Geeraerts, W., Charmpi, C., Van der Veken, D., De Vuyst, L., & Leroy, F. (2019). Exploring
637 the link between the geographical origin of European fermented foods and the diversity of their bacterial
638 communities: the case of fermented meats. *Frontiers in Microbiology*, *10*, 1–11.
639 https://doi.org/10.3389/fmicb.2019.02302
640 Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for
641 phylogenetic study. *Journal of Bacteriology*, *173*(2), 697–703. https://doi.org/10.1128/jb.173.2.697-
642 703.1991
643 Zaiko, E. V., Bataeva, D. S., Yushina, Y. K., Makhova, A. A., & Minaev, M. Y. (2020). Antibiotic resistant
644 of microorganisms in fermented sausages. *IOP Conference Series: Earth and Environmental Science*,
645 *421*(5). https://doi.org/10.1088/1755-1315/421/5/052013
646
647

FIGURE LEGEND

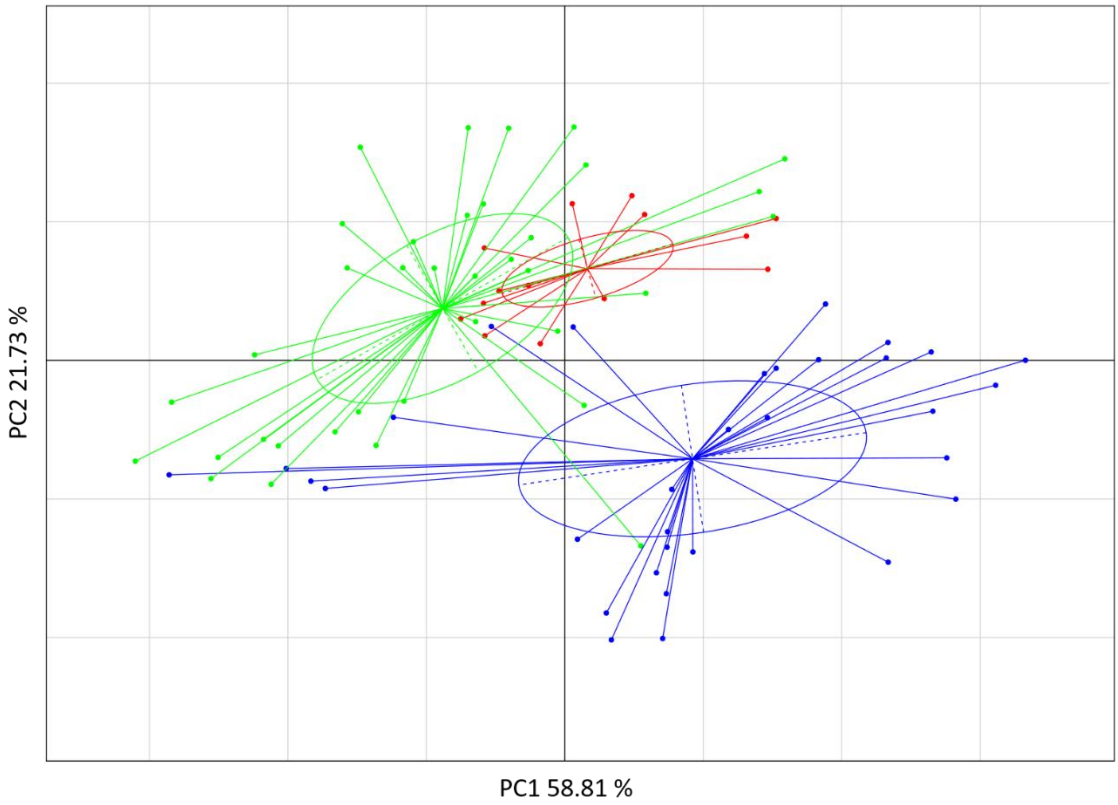
Fig. 1. PCA based on acidification rate for LAB rep biotype (February in blue, March in green, May in red). The first component (horizontal) accounts for 58.81% of the variance and the second component (vertical) accounts for 21.73%.

Fig. 2. Amplicon sequence variant relative abundance (%) in Salame Piemonte sausages inoculated with different autochthonous starter culture (ASC) using a metabarcoding approach.

Fig. 3. Radar graphs displaying the liking of appearance, odor, taste, flavor, and texture and overall liking expressed by consumers for the sausages made by Standard starter cultures (Control) and inoculated fermentation of the first pilot-scale production.

Fig. 4. Radar graphs displaying the liking of appearance, odor, taste, flavor, and texture and overall liking expressed by consumers for the sausages made by Standard starter cultures (Control) and inoculated fermentation of the second pilot-scale production.

665 **Fig. 1.**

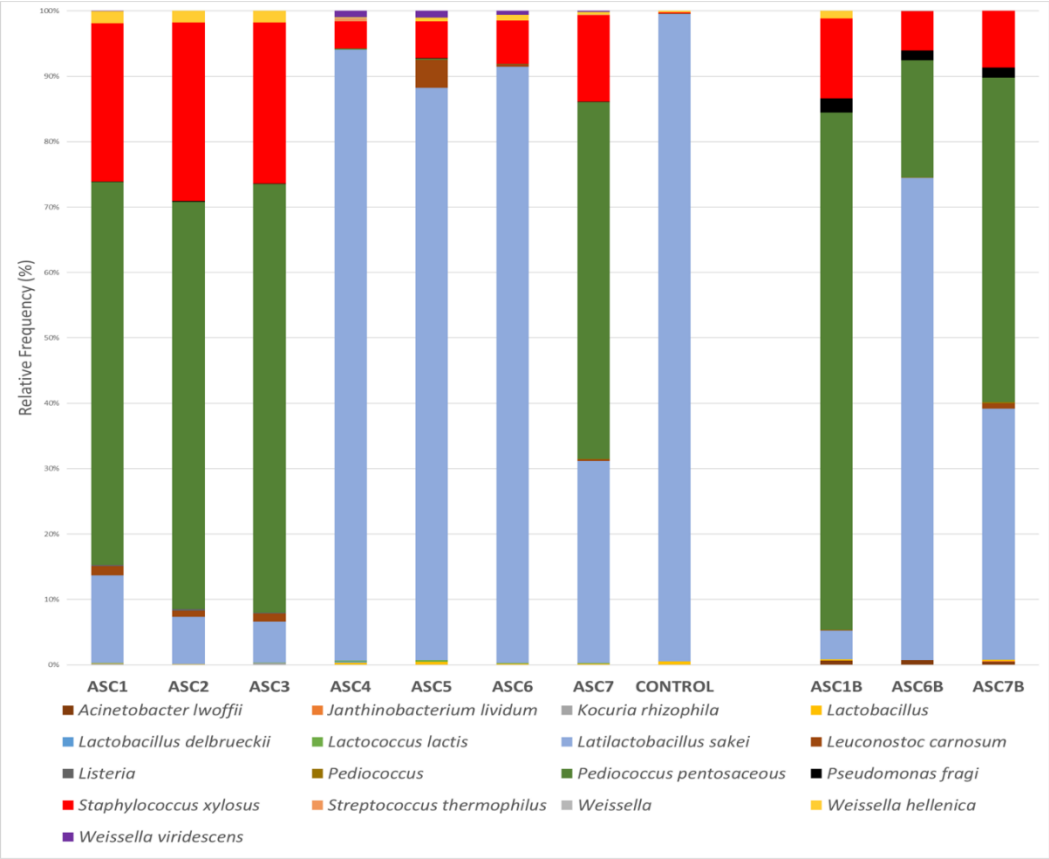


666

667

668

669 **Fig. 2.**

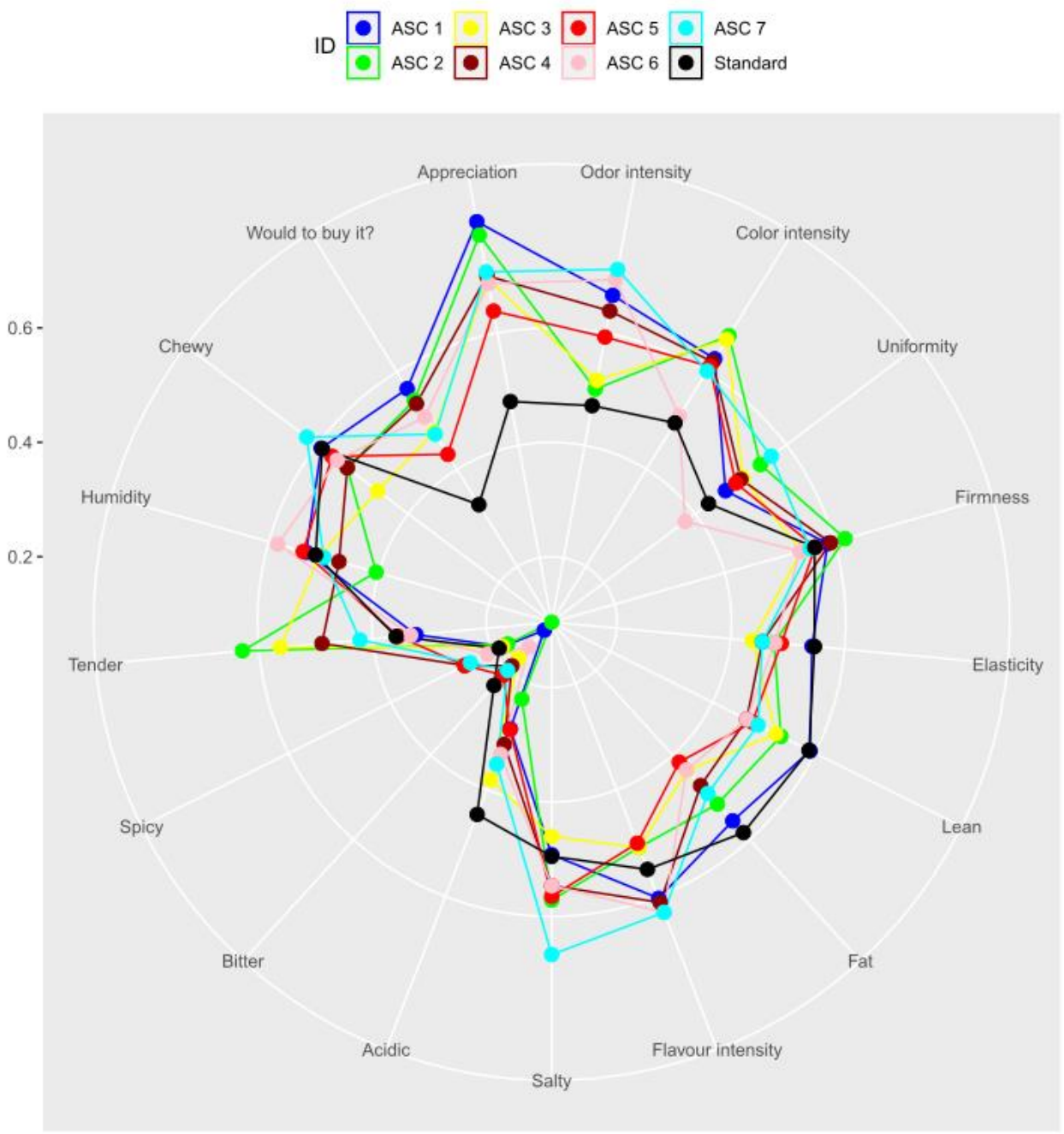


670

671

672

673 **Fig. 3.**

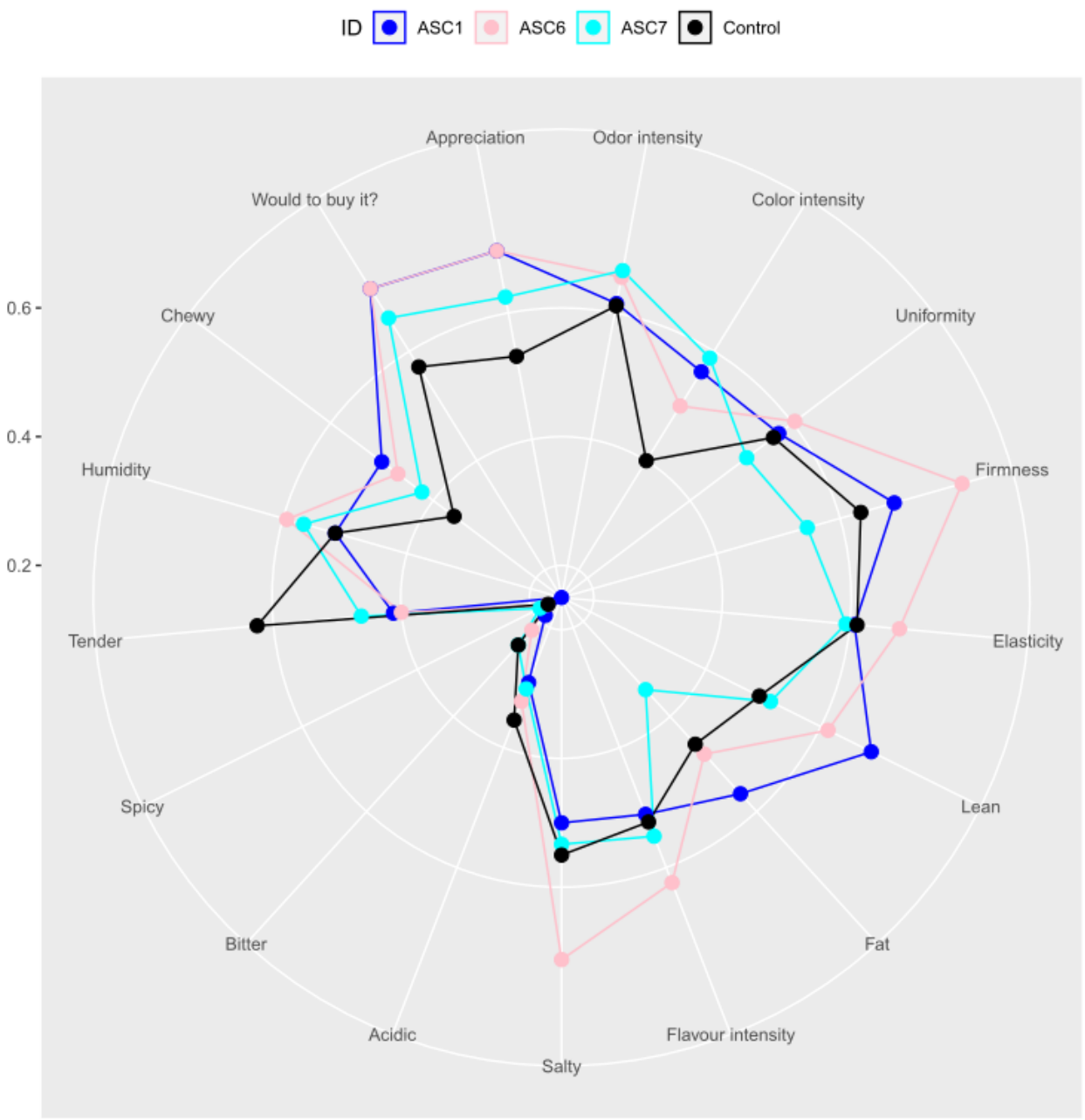


674

675

676

677 **Fig 4.**



678

679

Table legends

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

687 **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	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	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	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	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	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	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	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	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	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	-	+	+	\

688