Effect of starter culture on taste active amino acids and survival of pathogenic *Escherichia coli* in dry fermented beef sausages

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1 **Abstract**

2 The accumulation of taste active compounds during ripening determines the taste of fermented
3 meats; however, the contribution of defined starter cultures to glutamate during sausage ripening
4 remains unknown. This study investigated the role of lactic acid bacteria and *Staphylococcus*
5 *carnosus* on the accumulation of free amino acids during dry sausage fermentation. A sausage
6 model system was developed to control sausage microbiota throughout ripening. Sausages were
7 produced at the laboratory-scale with defined starter cultures; aseptic controls were fermented
8 without culture addition. *Lactobacillus sakei*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*,
9 *Pediococcus acidilactici* were used as single cultures; *Staphylococcus carnosus* with *L. sakei* or
10 *P. pentosaceus* were used as cocktails. The viable cell counts in aseptic control sausages remained
11 <1 log (CFU / g) throughout 20 d of ripening. The use of the model system demonstrated that
12 bacterial enzymes influenced the release of free amino acids, even during the initial fermentation
13 stage. Ripening time was the most important factors determining the accumulation of free amino
14 acids, and the accumulation of glutamate was not strain specific. The sausage model system was
15 also used for a challenge trial with a cocktail of pathogenic strains of *Escherichia coli*; viable cell
16 counts of pathogenic *E. coli* were reduced by less than 1 log (CFU / g) during ripening. The sausage
17 model for control of ripening microbiota will facilitate further studies on the impact of defined
18 cultures on the safety and quality of fermented meats.

19 **Keywords:** Dry fermented sausage; sterile sausage model; free amino acid; glutamate, Shiga-toxin
20 producing, *E. coli*

21

22 ## Introduction

23 Sausage fermentation preserves meat without thermal processing and provides a
24 characteristic flavor [1]. Dry cured sausages are fermented by lactobacilli including *Pediococcus*
25 spp. (Zheng et al., 2015), and *Micrococcaceae* or staphylococci. Fermentation microbiota develop
26 as natural fermentation microbiota, or are added as starter cultures [1, 2]. The variation of
27 ingredients, caliber, fermentation conditions and fermentation microbiota results in a large
28 diversity of products. Acidification by lactic acid bacteria coagulates meat proteins and reduces
29 microbial risks [1-3]. Micrococci and staphylococci have catalase and nitrate reductase activities
30 that are important for colour formation and stabilization [4]. Proteolysis and lipolysis contribute
31 to flavor and texture of the products by generating taste- or flavor active peptides, amino acids,
32 aldehydes, and organic acids [1, 3, 5, 6]. The endogenous meat protease cathepsin (B, D, H, and
33 L) degrade proteins; lactobacilli release amino acids by intracellular peptidases [3]. Amino acids
34 and short peptides produced by sarcoplasmic and myofibrillar protein hydrolysis contribute
35 directly to meat flavor, or are precursors for the microbial synthesis of flavor compounds [7, 8].
36 *Staphylococcus xylosus* and *Staphylococcus carnosus* metabolize the branched-chain amino acids
37 leucine, isoleucine and valine to flavor-active aldehydes, alcohols, and methyl ketones [1, 2, 9]
38 and thus play an important role in flavor formation during ripening. Lactobacilli convert amino
39 acids predominantly to organic acids that have a different impact on product taste and flavour [10].
40 Accumulation of glutamate during food fermentation depends on proteolysis, and on strain- or
41 species specific conversion of glutamine and glutamate by glutaminase and glutamate
42 decarboxylase, respectively [11-15]. The contribution of the strain specific conversion of
43 glutamine to accumulation of taste active glutamate in meat fermentations, however, requires
44 control of fermentation microbiota and has to date not been investigated.

45 The safety of fermented sausages is mainly dependent on rapid acidification. Bacteriocin
46 producing starter cultures control of *Listeria monocytogenes* in fermented sausages but are not
47 effective against pathogenic *Escherichia coli* O157:H7 [16]. The reduction of viable cell counts of
48 *E. coli* O157 during sausage fermentation and ripening ranges from 1 to 4 log (CFU / g) [17].
49 Survival of pathogenic *E. coli* O157:H7 during the dry sausage manufacture caused several
50 outbreaks and recalls [18]. Biogenic amines in fermented sausages are also of concern; these are
51 generated by microbial decarboxylation of amino acids. Their control relies on competitive and
52 decarboxylase-negative starter cultures, clean raw materials, and processing conditions [19].

53 Elucidation of the role of specific metabolites of fermentation cultures in product quality
54 and safety requires control of fermentation microbiota throughout the fermentation and ripening
55 time. Most studies on dry fermented sausage studies were performed at the pilot scale without
56 comparison to aseptic controls [3]. Past studies with sausage model systems did not provide a
57 comprehensive characterization of fermentation microbiota in fermented products [7, 20, 21]. This
58 study therefore aimed to develop a sausage model allowing control and manipulation of meat
59 microbiota throughout ripening. The sausage model was used to investigate the role of specific
60 strains of lactic acid bacteria that were characterized with respect to their glutamine and glutamate
61 metabolism on the formation of free amino acids, and examine the fate of pathogenic *E. coli*
62 cocktail during sausage fermentation.

63 2. Material and Methods

64 2.1 Strains and growth conditions

65 *L. sakei* FUA3009 and FUA3549, *Lactobacillus plantarum* FUA3073, *Pediococcus acidilactici*
66 FUA3072, and *Pediococcus pentosaceus* FUA 3071 and FUA3550 were cultivated in De Man,
67 Rogosa and Sharpe (MRS) media at 30°C anaerobically for 16 h. *S. carnosus* FUA2133 was

68 routinely grown on MRS at 37°C with 200 rpm agitation for 16 h. *L. sakei* FUA 3009 and
69 FUA3549 and *S. carnosus* FUA2133 are isolates from commercial meat starter cultures; other
70 strains are isolates from retail meat. A five strain cocktail of pathogenic *E. coli* was prepared as
71 described [22] to contain Shiga toxin producing *E. coli* O26:H11, O121:H19, O145:NM 03-6430,
72 O157:H7, O145:NM and the enteropathogenic *E. coli* O145:NM PARC 499. *E. coli* were routinely
73 grown in Luria-Bertani (LB) broth at 37°C with 200 rpm agitation.

74 2.2 Confirmation of strain identity *in silico* and PCR analysis of glutamine and glutamate
75 conversion

76 The identity of starter cultures was confirmed sequencing of genes coding for 16S rRNA. DNA
77 was isolated from 1 mL overnight culture using DNeasy blood and tissue kit according to the
78 instruction provided by the manufacturer (Qiagen, Mississauga, ON, Canada). PCR amplification
79 of 16S rRNA genes was performed in a volume of 25µL containing 1 µL template DNA, 17.5 µL
80 autoclaved water, 2.5 µL 10× buffer, 0.75 µL 50 mM MgCl, 0.5 µL 10mM dNTP, 1.25 µL 10µM
81 27F primer (AGAGTTTGATCMTGGCTCAG), 1.25 µL 10 µM 1492R primer
82 (TACGGYTACCTTGTTACGACTT), and 0.25 µL *Taq* DNA polymerase. The PCR conditions
83 were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45s,
84 annealing at 58°C for 45 s, extension at 72°C for 90 s. Final extension was at 72°C for 7 min, and
85 PCR product was held at 4°C. PCR product was sequenced by service of Macrogen (Rockville,
86 USA).

87 The presence of glutaminase genes in lactobacilli was verified by protein BLAST using each of
88 the three glutaminases of *L. reuteri* as query sequence [14] to determine the presence of
89 glutaminases in all genomes of lactobacilli and pediococci that were available in Dec. 2017
90 (>1500). Genes coding for glutaminases were absent in any of the genomes of *L. plantarum* (279

91 genome sequences), *L. sakei* (32 genome sequences) or *P. acidilactici* (23 genome sequences).
92 The presence of glutamate decarboxylase activity in *L. plantarum*, *L. sakei* and *P. acidilactici* is
93 strain specific; the presence or absence of the gene was therefore determined by PCR. The *gadB*
94 gene in *L. plantarum* was amplified by PCR reaction using primers *gadB* F
95 (CTAAGTATCGGTTACCAAAGCATTC) and *gadB* R
96 (GTGTGTGAATCCGTATTTCTTAGG) under the same condition as described with annealing
97 temperature 54°C for 1 min.

98 2.3 Preparation of inoculum for meat fermentations.

99 *L. sakei* FUA3009, *L. plantarum* FUA3073, *P. pentosaceus* FUA3071, and *P. acidilactici*
100 FUA3072 were prepared as a single strain starter cultures; *S. carnosus* with *L. sakei* FUA3549 or
101 *P. pentosaceus* FUA3550 were used as cocktails. Strains were subcultured twice prior to
102 inoculation. Starter culture inoculum was prepared by harvesting 10 mL overnight culture at
103 4000×g for 15 minutes, washing with sterilized tap water, and re-suspension of the culture in the
104 original volume with sterilized tap water. For the preparation of 10 mL starter culture cocktails, 5
105 mL of each *L. sakei* FUA 3549/*P. pentosaceus* FUA3550 and *S. carnosus* were mixed. The cell
106 count of the inoculum was about 9 log (CFU / ml) for lactobacilli and 8 log (CFU / ml) for *S.*
107 *carnosus.*

108 For the preparation of starter culture with a cocktail of 5 pathogenic strains of *E. coli*, the *E. coli*
109 cocktail was prepared by combining equal volume of washed overnight culture of each of the five
110 strains to form a 5 mL cocktail with a total cell count of about 8 log (CFU / ml). *L. sakei* FUA3549
111 was prepared from a 10 mL overnight culture, which was was washed and resuspended to 5 mL;
112 the cocktail consisting of *L. sakei* FUA3549 and *S. carnosus* was prepared by combining equal
113 volume of washed overnight culture to the final volume of 5 mL. *E. coli* cocktail was then

114 combined with 5mL of either single strain *L. sakei* FUA3549 or cocktail *L. sakei* FUA3549 and *S.*
115 *carnosus* to the 10 mL final volume.

116 2.4 Sausage fermentation

117 The sausage batter contained (% w/w): 86% ground beef (90 g), 3.66% sodium chloride (3.84 g),
118 0.01% sodium nitrite (0.01 g), 0.3% glucose (0.31 g), 0.03% sodium ascorbate (0.03 g), and 10%
119 inoculum (10 mL). For the aseptic control, 10 mL solution containing 100 mg / L each of
120 chloramphenicol, ampicillin, and erythromycin substituted the bacterial inoculum. A total of 6
121 sausages were made from the given recipe. All handling of meat and sausage batters was carried
122 out in a laminar flow biosafety cabinet; and 70% ethanol was used for product contact surfaces
123 and utensils. Ground beef was prepared from a bottom round of beef after removal of the exterior
124 fat, intermuscular fat, and connective tissues with a sterile knife. The lean beef was cut into steaks,
125 vacuum packaged, and stored at -20°C until use. To prepare the sterile seasoned beef batter, frozen
126 steaks were thawed at 4°C overnight, and then minced with the dry ingredients using a food
127 processer. The seasoned beef batter was portioned and packaged in individual sterile stomacher
128 bag, and stored at -20°C until use. To prepare the beef sausages, the seasoned beef batter was
129 thawed at 4°C overnight, mixed with inoculum or with antibiotics, and massaged in a stomacher.
130 The meat batter was stuffed into dialysis tubing (flat width: 32 mm; vol/length 3.3 mL/cm, Fisher
131 Scientific, Canada), cut into 5 cm long segments, and closed by plastic closures. Sausage meat
132 batter was maintained at refrigeration temperature during stuffing. Sausages were sprayed with
133 20% (w/v) potassium sorbate solution, and sausage weight was recorded. Sausages were hung in
134 a sealed plastic container where the relative humidity was controlled at 90% with saturated barium
135 chloride solution at 20°C or at 83% with 2.9 mol kg^{-1} NaCl solution at 18°C [23, 24]. Salt solutions
136 were pre-equilibrated to the designated temperature for 16 h before fermentation. Sausages were

137 incubated at 20°C and aw 0.9 for 3 d and ripened at 18°C and aw 0.83 for 17 d. Sausage
138 fermentations were carried out in at least triplicate independent fermentations. One sausage sample
139 was collected and sampled on day 0, 1, 3, 4, 11, and 20. The sausage weight, water activity
140 (Aqualab, USA), pH and viable cell counts were analyzed immediately after sample collection.
141 The moisture loss (%) was calculated as follows: $\frac{sample\ weight_{day\ 0} - sample\ weight_{day\ 3\ or\ 20}}{sample\ weight_{day\ 0}}\ x\ 100$.

142 Viable cell counts of uninoculated seasoned meat was determined by serial dilution and plating on
143 LB agar, which were incubated at 37°C for 16 h. Lactobacilli were enumerated by surface plating
144 on MRS agar with pH 5.5; staphylococci were enumerated by plating on Mannitol Salt Agar. Total
145 viable and sublethally injured *E. coli* were enumerated by plating on LB agar and Violet Red Bile
146 Agar (VRBA), respectively, and incubated at 45°C. It was verified by plating of pure cultures of
147 lactobacilli and staphylococci that these combinations of medium and incubation conditions allows
148 selective enumeration. Observation of a uniform colony morphology matching the colony
149 morphology of the inoculum was used to confirm the identity of fermentation microbiota with the
150 inoculum. This approach was previously validated by molecular methods allows differential
151 enumeration of strains in model food fermentations with controlled microbiota [13, 14, 37].
152 Sausage samples were freeze dried, powdered using mortar and pestle, and stored at -20°C.

153 2.5 Total α-amino nitrogen in fermented sausages

154 Total α-amino nitrogen was quantified in freeze dried fermented sausages sampled on day 0, 3,
155 and 20 with a modified ninhydrin method [25]. In brief, perchloric acid (300 µL 7%) was added
156 to 50 mg freeze dried sample and vortex thoroughly, and the samples were stored at 4°C for 16 h.
157 Samples were centrifuged at 15,000×g for 10 min, and 20 µL potassium chloride was added to 100
158 µL supernatant. The supernatant was incubated at 21°C for 1 h, and solids were removed by

159 centrifugation at 15,000×g for 10 min. Preparation of reagent 1 and 2 followed the protocol
160 provided by Lie (1973). Reagent 1 (100 μL) and 190 μL sterilized distilled water were added to
161 10 μL supernatant, and incubated at 100°C using water bath for 16 min. Samples were cooled
162 down in room temperature for 20 min, and 500 μL reagent 2 was added into the samples and mixed
163 thoroughly, and the absorbance was read at 570 nm.

164 2.6 Quantitation of free amino acids in fermented sausages

165 Free amino acids were extracted from freeze dried fermented sausages sampled on day 0, 3, and
166 20 as described with modifications [26]. Samples (50 mg) were extracted by shaking with 5 mL
167 0.1M hydrochloric acid at 200 rpm at 4°C for 8 min. The supernatant was collected by
168 centrifugation at 4°C for 20 min, and filtered through 0.45μm filter (Thermo Scientific, 17mm
169 teflon syringe filter). Perchloric acid (5%, 2 mL) was added to 2 mL and centrifuged at 10,000×g
170 at 4°C for 15 min. The supernatant was analysed by HPLC using β-aminobutyric acid as internal
171 standard and derivatization with *o*-phthalaldehyde on a Supelcosil LC-18 column with Varian
172 Prostar UV/Vis detector at 450 nm as described [27]. Histamine, tyramine, cadaverine, putrescine,
173 and 2-phenylethylamine standards were prepared for quantification of biogenic amines. Amino
174 acid concentrations in sausages were analyzed in three independent fermentation batches for starter
175 culture inoculated samples, and nine independent batches for the aseptic control.

176 2.7 Statistical analysis

177 Total α-amino nitrogen concentrations and free amino acid concentrations were analyzed using
178 nested ANOVA of R (R version 3.5.0) , with strain, time and interactions of strain and time as
179 fixed effect; and fermentation batches as random effect. The principal component analysis (PCA)
180 was carried out by using rotated component correlation matrix model (PASW Statistics 18.0).
181 Results were expressed as mean ± standard error of the means. Significant differences were
182 reported with 5% probability of error ($P < 0.05$).

183 **3. Results**

184 3.1 Establishment of bench-top aseptic sausage fermentation protocol

185 Accumulation of taste-active compounds during sausage ripening influences the taste of the final
186 product; however, sausage fermentation at the pilot scale does not enable the comparison to aseptic
187 controls. The contribution of defined starter cultures and endogenous meat enzymes to
188 accumulation of amino acids during sausage ripening remains thus uncertain. Aseptic sausages
189 prepared in this study had viable cell counts below the detection limit (1 log CFU / g) during 20 d.
190 Spraying of the exterior of sausage with 20% potassium sorbate was necessary to prevent growth
191 of molds during ripening. The development of bench-top sausage model system is a relatively
192 simple model system for meat fermentations with controlled microbiota, which can be manipulated
193 and applied to various objectives.

194 3.2 Characterization of fermented sausages

195 In order to validate the reproducibility of the model system, viable cell counts, pH, water activity,
196 and moisture loss of sausages were monitored during ripening (Fig. 1). The cell counts for the
197 uninoculated seasoned meat were below the detection limit, which provided a clean background

198 for strain specific fermentation. The viable cell counts for the aseptic control remained below the
199 detection limit of 1 log (CFU / g), and the pH remained stable at 5.5 during 20 days. The inoculated
200 samples had comparable viable cell counts for *Lactobacillus* spp. and *Pediococcus* spp. throughout
201 fermentation. Viable cell counts of *S. carnosus* remained stable around 7.3 log (CFU / g) (Fig.
202 2A). The pH values for both single strain and mixed culture batches dropped from 5.5 to 4.7 during
203 the fermentation stage and increased slightly to 4.9 during further ripening (Fig. 2B). The moisture
204 loss of dry fermented sausages including the aseptic control was about 37% and the corresponding
205 water activity was around 0.87 (data shown in online supplemental Table S1).

206 3.3 Total α-amino nitrogen concentrations of sausages

207 Hydrolysis of each peptide bond yields in generation of one primary amine; quantification of
208 primary amines is thus a direct way to quantify proteolysis [25]. Total α-amino nitrogen in aseptic
209 controls and fermented sausages were quantified to assess the proteolytic activity during the
210 sausage fermentation and ripening (Fig. 3). Total α-amino nitrogen concentration for the aseptic
211 control, *P. acidilactici* and *P. pentosaceus* fermented sausages did not change during 20 days,
212 while the amino nitrogen concentration increased in *L. sakei*, *L. plantarum*, and cocktail inoculated
213 sausages on day 20. *Lactobacillus* spp. single strain inoculation increased the content of total
214 amino nitrogen when compared to the aseptic control (Figure 3). Inoculation with *S. carnosus* did
215 not change the proteolytic activity during sausage ripening. Overall, the starter cultures exhibited
216 a strain-dependent contribution to proteolytic activity during sausage ripening.

217 3.4 Free amino acid concentrations of sausages

218 This study also investigated the contribution of controlled fermentation microbiota to the
219 accumulation of free amino acids during sausage fermentation. Relationships between the free
220 amino acids in sausages fermented with different starter cultures and fermentation time were

221 initially assessed by principle component analysis (Fig. 4). Fermentation time strongly influenced
222 the accumulation of free amino acids in inoculated sausages, but not in aseptic controls. In keeping
223 with the effect of the respective cultures on accumulation of amino nitrogen, *Lactobacillus* spp.
224 clustered separately from *Pediococcus* spp. when used as single starter cultures (Fig. 4).
225 Glutamate, valine, isoleucine, and leucine increased in all treatment groups over time (Table 1).
226 Glutamine concentrations were reduced over time, particularly in *L. sakei* single strain or cocktail
227 fermented sausages during the early stage of fermentation. Glutamate concentrations were higher
228 in all fermented sausages when compared to the aseptic control (Table 1). Fermented sausages had
229 significantly higher concentration of branched chain amino acids (valine, isoleucine, and leucine)
230 when compared to the aseptic control. Arginine concentrations (400-450 mg/100g) were not
231 different in sausages fermented with different strains, or after different fermentation times. The
232 gene coding for glutamate decarboxylase, *gadB,* was present only in *L. plantarum*, but the
233 accumulation of γ-amino butyrate (GABA) by *L. plantarum* was below the detection limit. The
234 concentrations of biogenic amines histamine, tyramine, cadaverine, putrescine, and 2-
235 phenylethylamine were also below the detection limit in all samples.

236 3.5 Survival of pathogenic *E. coli* in fermented sausages

237 The effect of starter culture and processing condition on the survival of *E. coli* was investigated
238 with a cocktail of 5 pathogenic strains. *L. sakei* FUA3549 was used as starter culture, alone or in
239 combination with *S. carnosus*. The addition of the *E. coli* strain cocktail did not influence viable
240 cell counts of the starter cultures or the pH of the sausages (data shown in online supplemental
241 Fig. S1). Viable cell counts of *E. coli* were monitored on LB and VRB agars to identify total *E.*
242 *coli* including sublethally injured cells. The initial viable cell counts for *E. coli* cocktail in all
243 treatments were around 6.8 log (CFU / g). Viable cell counts were reduced by less than 0.5 log

244 (CFU / g) on day 20 (Fig. 5). Viable cell counts obtained on LB and VRB agars differed by 0.5-1
245 log (CFU / g) after 20 d of fermentation, indicating sublethal injury of surviving cells. The presence
246 of starter cultures did not affect survival of *E. coli*; the moderate reduction of *E. coli* observed
247 viable cell counts on day 20 was observed in fermented samples as well as the aseptic control.

248 **4. Discussion**

249 Sausage model systems were previously used to study the effect of starter culture on proteolysis
250 [5, 7, 21]. However, the background microbiota in past controls grew to cell counts of up to 10^6
251 CFU/g; this high cell count obscures strain specific contributions to product quality or safety [5,
252 7, 28]. The use of an aseptic sausage model in this study enables unprecedented control of
253 microbiota. Aseptic controls had no detectable microbiota throughout the 20 d ripening period.
254 The use of defined starter cultures revealed strain specific contribution to amino acid turnover. In
255 industrial practice, surface molds are controlled by dipping in potassium sorbate; alternatively,
256 smoking is used to prevent mold development unless products are surface ripened [29]. The
257 ripening conditions used in the model system matched slow fermentation at low temperature [30].
258 Ripening at controlled temperature and humidity provided a consistent product quality. Despite
259 the difference in caliber of the mini-sausages when compared to commercial products, aw and pH
260 values were comparable to commercial dry cured sausages [3, 31].

261 Muscle proteinases and microbial proteases release amino acid and peptides during meat
262 fermentation [3, 8, 32]. The accumulation of total amino nitrogen did not differ between inoculated
263 sausages and aseptic controls after 3 d, however, the accumulation of specific amino acids at 3 d
264 was attributable to bacterial aminopeptidases (Fig. 3, 4, and Table 1). Because the acidity among
265 inoculated samples was comparable throughout 20 d ripening, differences in the concentration of
266 individual amino acids between sausages inoculated with different starter cultures can be attributed

267 to strain specific peptidase activity [10]. Further decomposition of peptides to free amino acids by
268 bacterial peptidases occurred during ripening, resulting in higher accumulation of total α-amino
269 nitrogen in inoculated sausages when compared to the aseptic controls [5, 33]. Peptidases PepC,
270 PepN, and PepM and proline peptidases PepX and PepQ are encoded in genomes of most
271 lactobacilli but the activity of other peptidases is strain or species specific [15, 34]. Dipeptides are
272 preferred over amino acids and tetrapeptides by the cellular transport system and peptidases [10].
273 Control of fermentation microbiota enables the observation that the accumulation of free amino
274 acids in model sausages strongly depended on the ripening time; strain specific accumulation on
275 glutamate, methionine, valine, and leucine was additionally observed (Fig. 4 and Table 1).

276 The use of the model system provided evidence that bacterial peptidases influenced the release of
277 free amino acids, even during the initial fermentation stage. Among the strains used in this study,
278 the highest level of free amino nitrogen was observed with *L. sakei*. Among amino acids, glutamate
279 is particularly relevant because it imparts umami, savoury flavour with a taste threshold of
280 approximately 1 mmol kg^{-1} [13, 35]. In cheese and cereal fermentations, the conversion of
281 glutamine to glutamate or GABA depends on strain specific enzyme activities of lactobacilli;
282 glutamate accumulation by starter cultures strongly impacts the taste of bread and cheese [13, 35].
283 Glutamate also impacts flavour of fermented meats [36]; however, factors influencing
284 accumulation of glutamate in sausage fermentation remain unclear. The present study
285 demonstrates bacterial activity accumulates glutamate to concentrations that are more than 10 fold
286 in excess of the taste threshold. Different from sourdoughs and cheese, differences in glutamate
287 accumulation between strains used in the sausage model were minor and not attributable to
288 glutaminase activity [13]; typical meat starter cultures including *L. plantarum, L. sakei* and *P.*
289 *acidilactici* generally do not express glutaminases [15, this study]. The strain specific

290 decarboxylation of glutamate by glutamate decarboxylase improves acid resistance of lactobacilli
291 [37]. *L. plantarum* fermented grape beverage had a progressive synthesis of GABA at pH 3.74
292 [38]; moreover, GAD expression and activity in *L. reuteri* required low pH [11]. The lowest pH
293 during dry sausages fermentation, pH 4.7, may have been too high to trigger GAD expression and
294 activity by *L. plantarum*. Several species of lactobacilli utilize arginine as an alternative energy
295 source via arginine-deiminase (ADI) pathway, which contributes to pH homeostasis and acid
296 tolerance of lactobacilli [39]. The current study did not observe a pronounced arginine uptake
297 neither in aseptic nor inoculated sausages. *L. sakei* is ADI positive, but arginine utilization did not
298 confer a competitive advantage to *L. sakei* during sausage fermentation [15, 40].

299 The branched-chain amino acids leucine, isoleucine, and valine are degraded to branched
300 aldehydes, alcohols and acids by *S. xylosus* and *S. carnosus* [9]. Addition of *S. carnosus* to dried
301 sausages accelerated sausage maturation, and the maturity correlated significantly with
302 metabolism of branched chain amino acids metabolism [9]. However, addition of *S. carnosus* to
303 sausages fermented with *L. sakei* and *P. pentosaceus* did not accelerate the utilization of valine,
304 isoleucine, and leucine, which may relate to the short ripening time, or to strain specific differences
305 [41].

306 Biogenic amines in fermented meats are products of microbial decarboxylation of amino acids [42,
307 43]. Their concentration in fermented sausages depends on the hygienic quality of the raw material
308 and the activity of fermentation microbiota [19]. Enterococci and *Enterobacteriaceae* accumulated
309 tyramine, cadaverine and putrescine in fermented sausages [44, 45]. The use of clean meat and
310 decarboxylase negative starter cultures in the present study prevented accumulation of biogenic
311 amines, in keeping with prior knowledge on the role of meat quality and fermentation microbiota
312 on formation of biogenic amines in fermented meats.

313 Pathogenic *E. coli* survive during the processing of dry fermented sausages [17, 18, 46, 47]. *E. coli*
314 O157:H7 has been considered as prototype for enterohaemorrhagic *E. coli* (EHEC) and most
315 studies on EHEC survival in sausages used strains of this serotype [17]. The pathogenic strains
316 used in the present study were selected on the basis of phylogenetic diversity and the resistance of
317 more than 100 strains of STEC to heat and pressure [22, 48]. The use of either single strain or
318 mixed starter cultures did not have a significant impact on the cell reduction of *E. coli* strain
319 cocktail when compared to aseptic controls. Viable cell counts of *E. coli* in sausages were reduced
320 by less than 1 log (CFU / g), a cell count reduction within the range of previous reports on the fate
321 of *E. coli* O157 [17]. Enumerating *E. coli* on selective media only, however, does not account for
322 sublethal injured cells and underestimates the viable cell counts [18, 46]. The aseptic sausage
323 model system enabled the use of both non-selective and selective media to demonstrate that total
324 viable cells of *E. coli* were reduced by 0.5 log (CFU / g) only. This exceptional recovery of
325 pathogenic *E. coli* may relate to the unprecedented use of a strain cocktail in combination with
326 enumeration on non-selective media, which can recover sub-lethally injured cells [17]. A reduction
327 of pathogenic *E. coli* by more than 4 log (CFU / g) is required to meet the regulatory requirements
328 in some countries including Canada and the U.S. [17]. Post-process heating is an effective
329 approach to achieve a 5 log reduction of viable cell counts in dry fermented sausages [47].
330 Alternatively a long time ripening of 5.5 month at 4°C completely eliminated *E. coli* O157:H7 in
331 dry fermented sausages [46]. The aseptic sausage model developed in the present study provides
332 an excellent tool for further studies on the role of starter cultures and process conditions on the
333 survival of pathogenic bacteria during production of dry cured sausages.

334 In conclusion, this study developed a sausage model system, which can be manipulated and used
335 for varies objectives. The uses of aseptic meat and defined starter cultures enabled control of

336 fermentation microbiota throughout 20 d of fermentation and ripening. The accumulation of free
337 amino acids was strongly correlated with ripening time, and we did not observe a significant impact
338 of starter cultures to the accumulation of free amino acids. Consistent with the literature, sausage
339 fermentation and ripening for less than 20 d did not reduce viable cell counts of pathogenic *E. coli*
340 by more than 1 log (CFU / g), necessitating further studies on the control of *E. coli* in fermented
341 meats.

342 **Acknowledgements**

343 This project received funding from the NSERC CREATE and Discovery programs; Michael
344 Gänzle acknowledges support from the Canada Research Chairs program. Weilan Wang is
345 acknowledged for her support with statistical analysis. BITEC Frutarom Savory Solutions
346 (Germany) and Cargill Meat Solutions (Canada) are acknowledged for providing starter cultures
347 and beef respectively.

348 **Conflict of Interest**

349 The authors declare no conflict of interest.

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468 **Fig. 1** Changes in fermentation temperature and moisture loss in aseptic control sausages.
469 Moisture loss data present means of nine independent fermentations. Open circles (○) indicate
470 temperature condition used for the fermentation, and closed circles (●) indicate moisture loss.
471 The standard error of the mean was 4.4% or less for moisture loss data

472 **Fig. 2** Viable cell counts (Panel A) and pH (Panel B) of aseptic and inoculated sausages. Viable
473 cell counts of aseptic controls were below the detection limit of 1 log (CFU / g). Data are presented
474 as means of triplicate biological repeats for inoculated sausages and nine independent
475 fermentations for the aseptic control. Panel A, symbols indicate the viable cell count for sausages
476 fermented with single strain of *L. sakei* FUA3009 (△), *L. plantarum* (▽), *P. pentosaceus*
477 FUA3071 (□), *P. acidilactici* (◇); *L. sakei* FUA3049 and *S. carnosus* (*L. sakei,* ▲; *S. carnosus,*
478 ▲), or *P. pentosaceus* FUA3550 and *S. carnosus* (*P. pentosaceus,* ■; *S. carnosus* ■). The y-axis
479 is scaled to the detection limit of 1 log(cfu/g). Cell counts in the aseptic control remained below
480 the detection limit throughout the 20 d ripening period. The standard error of the mean was 0.7 or
481 less for all viable cell counts. Panel B shows the dry fermented sausages pH, symbols for the single
482 strain are the same as described. The aseptic control is labelled as (○); *L. sakei* and *S. carnosus*
483 cocktail is labelled as (▲); *P. pentosaceus* and *S. carnosus* cocktail is labelled as (■) Standard
484 error of the mean was 0.13 or less for all pH values. (n=3)

485 **Fig. 3** Concentration of total amino nitrogen in dry fermented sausages. Data are presented as
486 means ± standard error of the mean of triplicate biological repeats for the inoculated sausages and
487 nine biological repeats for the aseptic control sausages. Letters A and B denote significant
488 differences ($P<0.05$) of sausages fermented with the same strains at different time points. Letters
489 a, b, and c denote significant differences ($P<0.05$) of sausages fermented with different strains at

490 the same time point. Bar colours and patterns indicate aseptic control (white); *L. sakei* (light grey);
491 *L. plantarum* (dotted light grey); *P. pentosaceus* (dark grey); *P. acidilactici* (dotted dark grey); *L.*
492 *sakei* and *S. carnosus* cocktail (hatched light grey); and *P. pentosaceus* and *S. carnosus* cocktail
493 (hatched dark grey)

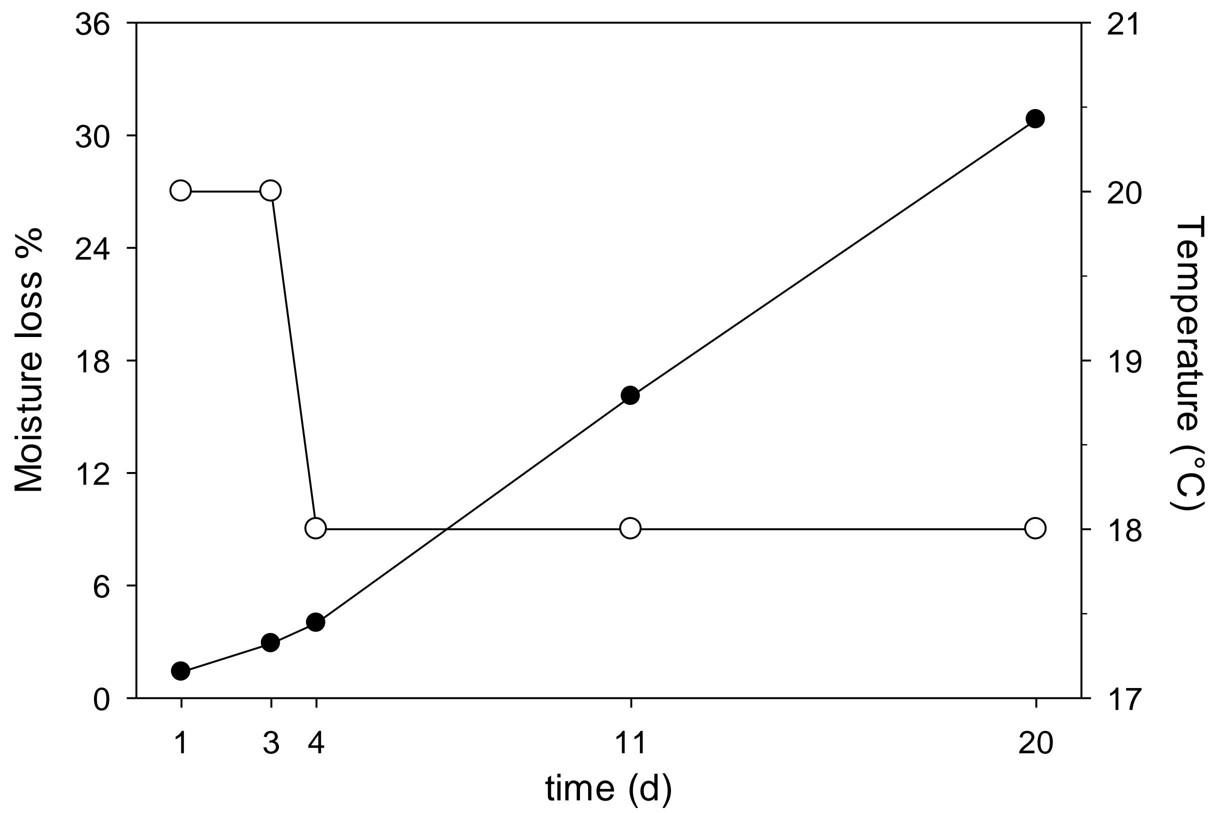
494 **Fig. 4** Principal component analysis of free amino acid concentrations in aseptic and inoculated
495 sausages at day 0 (○), day 3 (●), and day 20 (●)

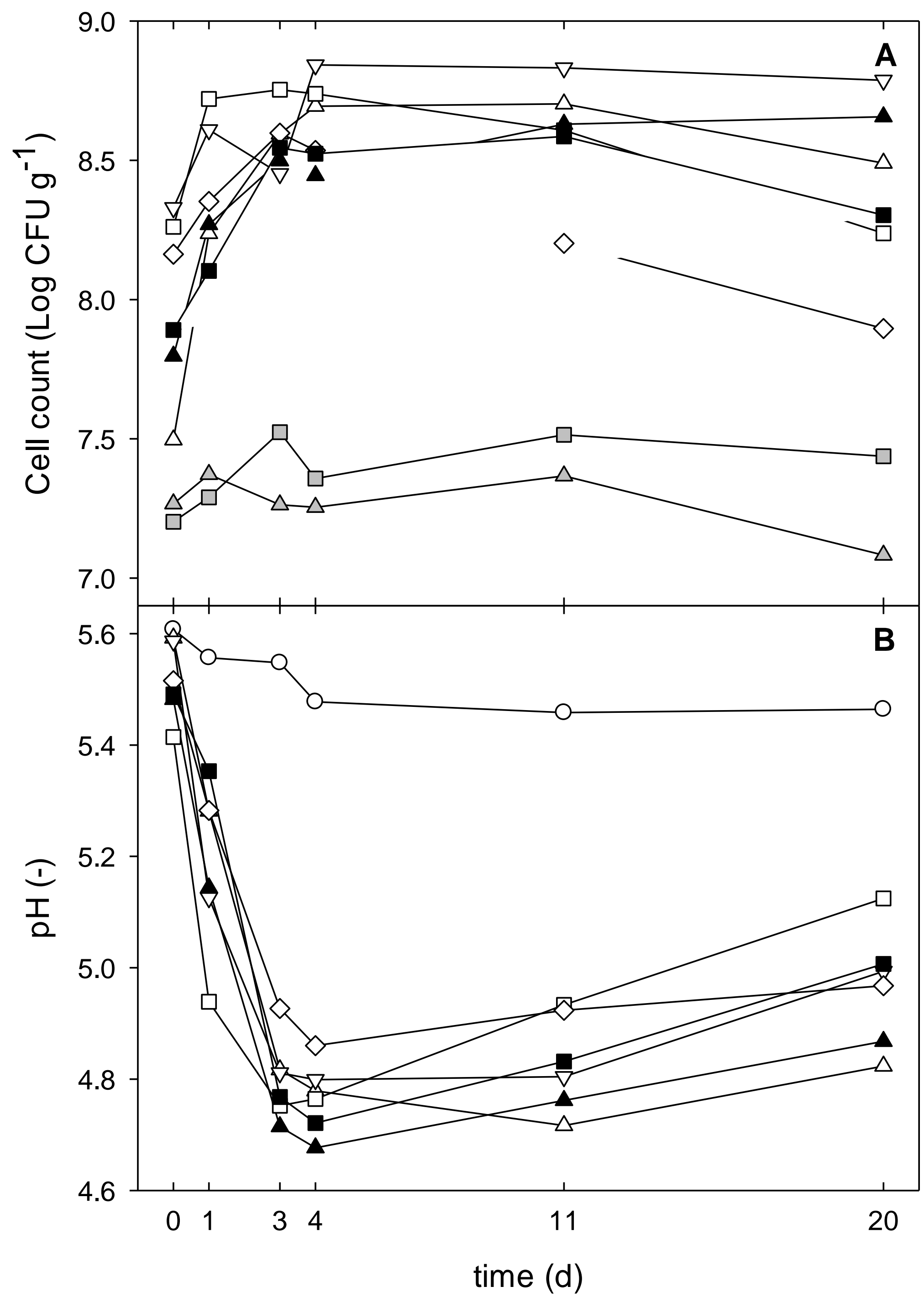
496 **Fig. 5** Viable cell counts of pathogenic *E. coli* in aseptic controls and sausages fermented with
497 *L. sakei* and *S. carnosus*. Data are presented as means ± standard error of the mean of triplicate
498 independent experiments. Bar colours indicate *E. coli* viable cell counts in aseptic controls (white),
499 sausages fermented with *L. sakei* FUA3549 (light grey), or sausages fermented with *L. sakei*
500 FUA3549 and *S. carnosus* (dark grey). Plain bars represent *E. coli* viable cell counts on LB plates;
501 hatched bars represent *E. coli* viable cell count on VRBA plates. Viable cell count and pH for the
502 single strain *L. sakei* or the cocktail *L. sakei* and *S. carnosus* were consistent with results shown in
503 Fig. S1

Table 1 Concentration of free amino acids (mg/100g) in dry sausages fermented with different combination of starter cultures

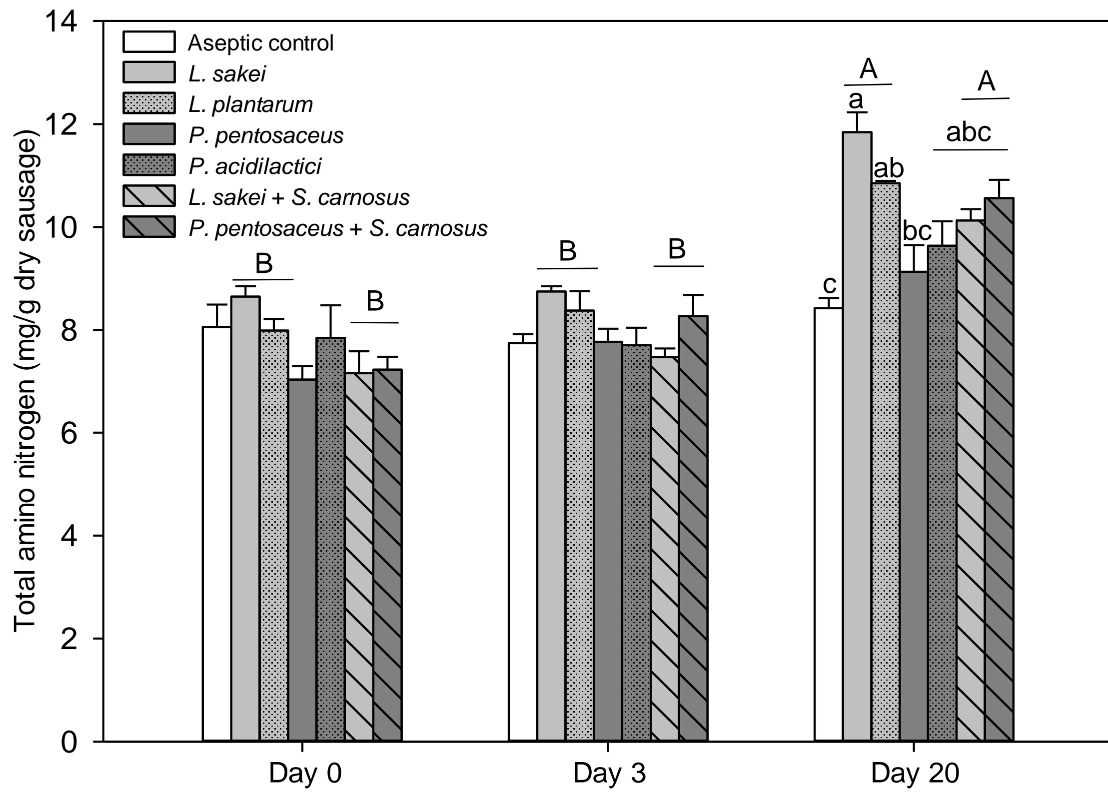
Strain / time (d)	Aseptic control	*L. sakei*	*L. plantarum*	*P. pentosaceus*	*P. acidilactici*	*L. sakei* and *S. carnosus*	*P. pentosaceus* and *S. carnosus*
Glutamate							
0	24±0.40 ^{B}	32±0.80 ^{C}	30±3.1 ^{B}	33±4.6 ^{C}	30±8.1 ^{C}	26±1.0 ^{C}	27±2.7 ^{C}
3	31±1.7 ^{Z,B}	100±5.8 ^{XY,B}	56±6.9 ^{YZ,B}	92±0.98 ^{XY,B}	72±24 ^{XYZ,B}	120±13 ^{X,B}	84±6.4 ^{XYZ,B}
20	57±3.3 ^{Y,A}	170±10 ^{X,A}	140±12 ^{X,A}	140±9.2 ^{X,A}	150±24 ^{X,A}	180±9.2 ^{X,A}	150±1.7 ^{X,A}
Serine							
0	13±1.8 ^{B}	14±1.8	17±1.5	19±0.93	13±3.2	17±1.2 ^{B}	12±1.0
3	16±0.98 ^{B}	6.5±1.5	6.0±1.84	12±0.46	17±4.3	19±2.3 ^{B}	8.5±1.8
20	27±2.5 ^{XY,A}	19±1.5 ^{XY}	10±1.1 ^{Y}	25±4.1 ^{XY}	20±11 ^{XY}	39±4.1 ^{X,A}	24±3.8 ^{XY}
Glutamine							
0	38±2.9	51±3.0 ^{A}	59±3.6	41±10	39±14	35±4.1 ^{A}	27±3.3
3	37±3.9 ^{XY}	29±2.8 ^{XY,B}	48±1.6 ^{X}	26±8.3 ^{XY}	26±11 ^{XY}	11±2.8 ^{Y,B}	20±2.2 ^{XY}
20	29±2.8	25±1.7 ^{B}	42±2.6	22±8.9	23±5.2	11±1.1 ^{B}	12±0.55
Alanine							
0	60±4.9	61±3.3 ^{B}	57±3.4 ^{B}	66±5.5	61±7.5	72±1.8	62±2.5
3	70±11	90±5.6 ^{B}	77±2.6 ^{B}	79±15	69±12	88±6.8	87±8.4
20	85±8.7 ^{Y}	190±11 ^{X,A}	170±21 ^{XY,A}	120±34 ^{XY}	140±62 ^{XY}	150±3.8 ^{XY}	140±1.6 ^{XY}
Tyrosine							
0	22±8.7	12±1.6	12±1.1	47±35	46±35	16±3.6	15±3.5
3	26±6.4	24±3.0	22±0.14	58±38	110±79	32±5.7	29±5.1
20	38±9.2	51±4.1	45±1.7	98±65	120±93	45±1.5	38±4.3
Methionine							
0	18±6.4 ^{AB}	13±3.9 ^{B}	18±4.6 ^{B}	16±2.1	12±1.7 ^{B}	14±1.3 ^{B}	16±1.1 ^{B}
3	18±1.0 ^{B}	22±2.5 ^{AB}	22±2.0 ^{B}	27±1.6	20±3.2 ^{AB}	25±4.2 ^{AB}	26±1.7 ^{AB}
20	35±4.2 ^{A}	49±4.0 ^{A}	53±5.9 ^{A}	42±2.2	41±4.0 ^{A}	51±1.9 ^{A}	47±1.3 ^{A}
Valine							
0	12±0.87 ^{B}	13±1.0 ^{C}	18±2.5 ^{B}	15±2.1 ^{C}	13±2.2 ^{B}	12±2.9 ^{C}	13±0.98 ^{C}
3	17±1.1 ^{Y,B}	30±1.9 ^{XY,B}	24±1.7 ^{XY,B}	31±2.1 ^{XY,B}	25±3.6 ^{XY,B}	28±2.4 ^{XY,B}	32±3.7 ^{X,B}
20	30±1.3 ^{Y,A}	65±5.5 ^{X,A}	59±5.0 ^{X,A}	56±3.8 ^{X,A}	50±4.2 ^{X,A}	56±2.6 ^{X,A}	50±0.66 ^{X,A}
Isoleucine							
0	15±2.2 ^{C}	13±1.4 ^{C}	13±1.7 ^{B}	17±1.1 ^{B}	19±1.9 ^{B}	17±0.99 ^{B}	15±0.73 ^{B}
3	24±1.9 ^{B}	28±0.20 ^{B}	26±3.6 ^{B}	31±2.1 ^{B}	25±3.4 ^{B}	27±1.9 ^{B}	29±1.4 ^{B}
20	40±4.0 ^{Y,A}	63±4.2 ^{X,A}	63±7.7 ^{X,A}	58±4.0 ^{XY,A}	61±3.5 ^{X,A}	56±2.8 ^{X,A}	54±0.94 ^{X,A}
Leucine							
0	28±4.1 ^{C}	26±1.1 ^{C}	29±4.5 ^{C}	35±4.1 ^{C}	38±5.2 ^{B}	33±3.8 ^{C}	32±1.1 ^{C}
3	47±3.2 ^{B}	72±2.0 ^{B}	66±8.8 ^{B}	76±4.0 ^{B}	55±8.9 ^{B}	66±5.4 ^{B}	72±4.5 ^{B}
20	86±9.6 ^{Y,A}	150±4.7 ^{X,A}	150±17 ^{X,A}	140±8.0 ^{X,A}	130±1.8 ^{X,A}	140±4.6 ^{X,A}	130±1.1 ^{X,A}

Data represent means ± standard error of mean of triplicate independent fermentations. Superscripts X, Y, and Z denote significant differences ($P < 0.05$) among concentrations of the same free amino acid in dry sausages fermented with different strains at the same fermentation time; A, B, and C denote significant differences ($P < 0.05$) among concentrations of the same amino acid in dry sausages fermented with the same strain over time. Superscripts are not indicated if values were not significantly different. n=9 for aseptic control; n=3 for the rest

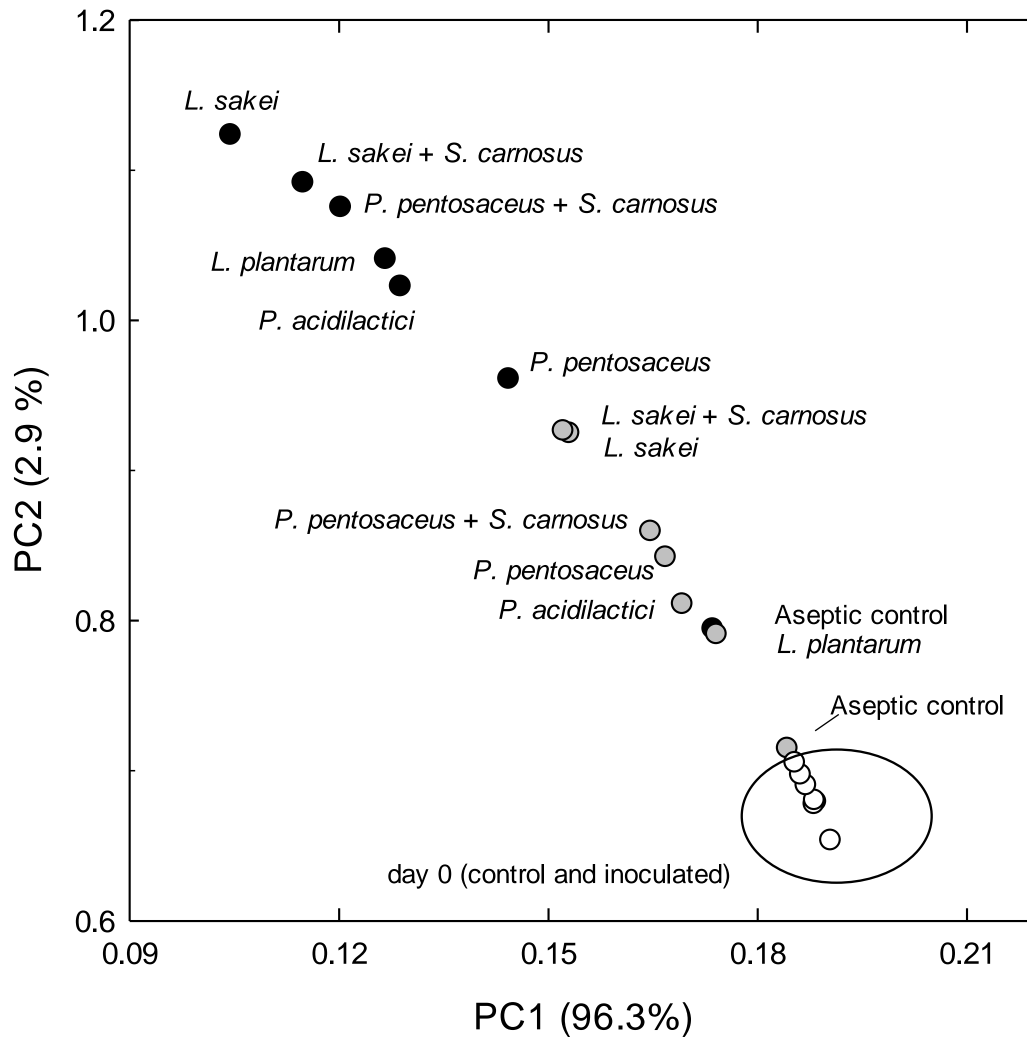

36
30
24
18
12
6
0
Moisture loss %
21
20
19
18
17
Temperature (°C)
1
3
4
11
20
time (d)



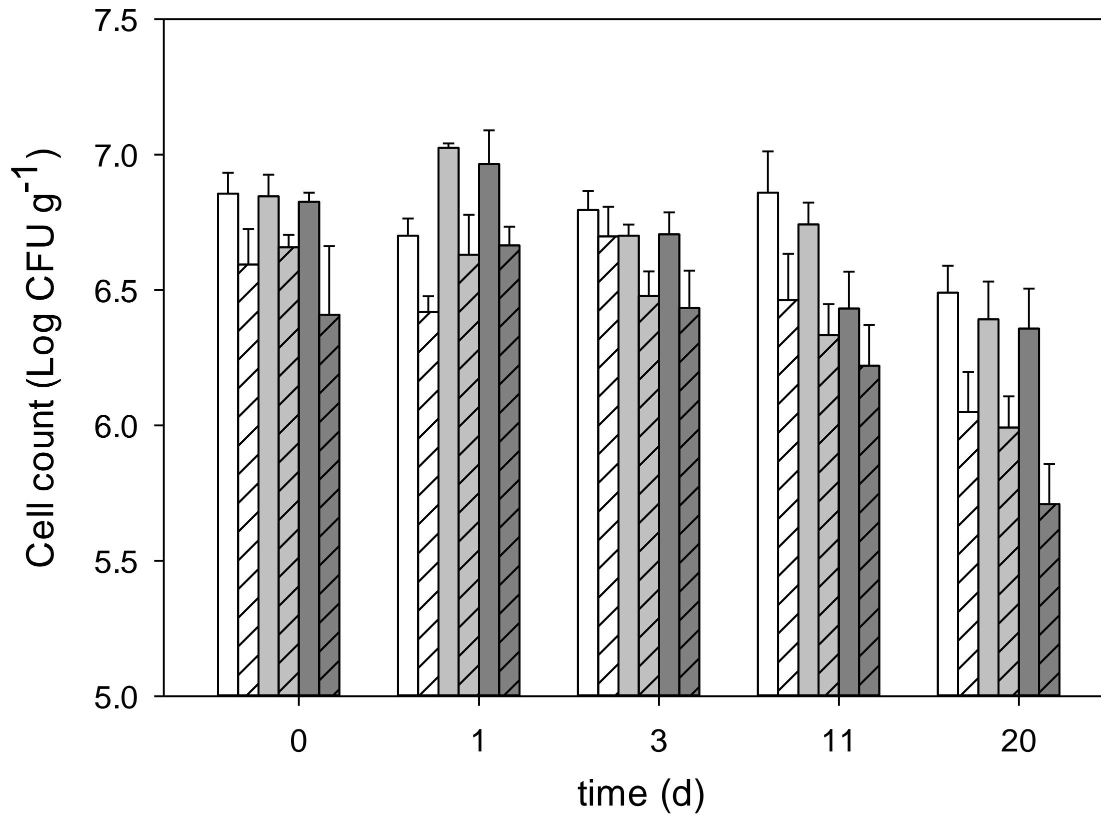
A
B
Cell count (Log CFU g-1)
pH (-)
time (d)
9.0
8.5
8.0
7.5
7.0
5.6
5.4
5.2
5.0
4.8
4.6
0
1
3
4
11
20



Total amino nitrogen (mg/g dry sausage)
14
12
10
8
6
4
2
0
Aseptic control
L. sakei
L. plantarum
P. pentosaceus
P. acidilactici
L. sakei + S. carnosus
P. pentosaceus + S. carnosus
B
B
B
B
A
a
ab
bc
c
abc
A
Day 0
Day 3
Day 20



1.2
1.0
0.8
0.6
0.09
0.12
0.15
0.18
0.21
PC2 (2.9 %)
PC1 (96.3%)
L. sakei
L. sakei + S. carnosus
P. pentosaceus + S. carnosus
L. plantarum
P. acidilactici
P. pentosaceus
L. sakei + S. carnosus
L. sakei
P. pentosaceus + S. carnosus
P. pentosaceus
P. acidilactici
Aseptic control
L. plantarum
Aseptic control
day 0 (control and inoculated)


Cell count (Log CFU g-1)
7.5
7.0
6.5
6.0
5.5
5.0
0
1
3
11
20
time (d)

Online supplementary material

Effect of starter culture on taste active amino acids and survival of pathogenic *Escherichia coli* in dry fermented beef sausages

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Table S1. Changes of moisture loss and water activity during sausage fermentation.

Fig. S1 Cell counts (Panel A) and pH (Panel B) of model sausages inoculated with fermentation organisms, EHEC, or both.

Table S1. Changes of moisture loss and water activity during sausage fermentation.

Moisture loss (%)					
Time (d)	1	3	4	11	20
Aseptic control	1.38	2.90	3.99	16.08	30.83
L. sakei	1.39	2.88	4.49	14.55	35.99
L. plantarum	1.45	2.66	3.36	21.01	38.81
P. pentosaceus	2.07	4.15	6.93	19.66	41.00
P. acidilactici	2.08	4.76	5.87	19.34	39.84
L. sakei and *S. carnosus*	0.96	3.17	4.22	11.11	32.24
P. pentosaceus and *S. carnosus*	1.56	2.96	4.28	9.63	34.78
Water activity					
Time (d)	0	1	3	4	20
Aseptic control	0.94	0.94	0.95	0.94	0.87
L. sakei	0.94	0.94	0.95	0.95	0.87
L. plantarum	0.94	0.94	0.93	0.92	0.88
P. pentosaceus	0.95	0.94	0.94	0.94	0.83
P. acidilactici	0.95	0.94	0.95	0.94	0.86
L. sakei and *S. carnosus*	0.95	0.95	0.95	0.94	0.90
P. pentosaceus and *S. carnosus*	0.94	0.95	0.95	0.94	0.90

Data represent means of triplicate independent fermentations.

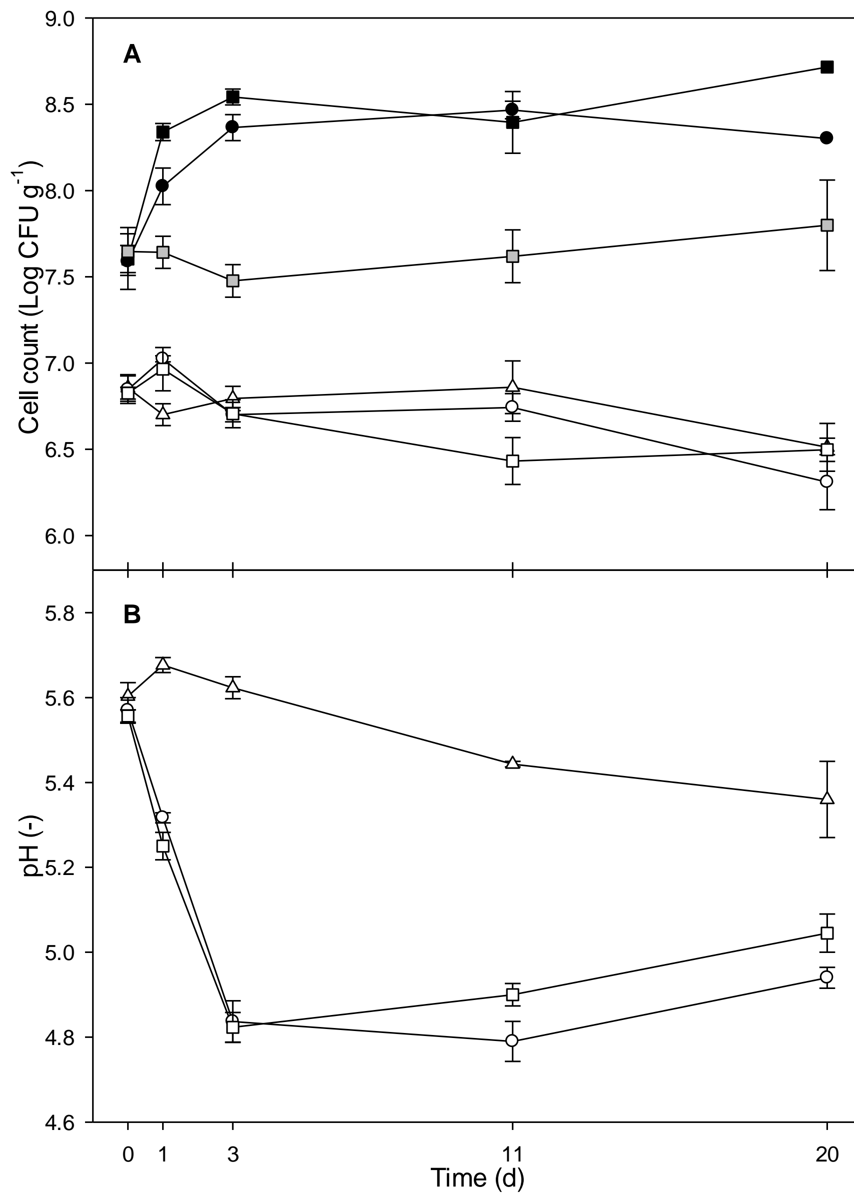

Fig. S1 Cell counts (Panel A) and pH (Panel B) of model sausages inoculated with fermentation organisms, EHEC, or both. Data are presented as means of triplicate biological repeats with the standard error of the mean. Panel A, symbols indicate the EHEC cell counts on LB agar in the positive control (△), *L. sakei* sausages (○), *L. sakei* and *S. carnosus* sausages (□); cell counts of *L. sakei* in the single strain fermented sausages (●) and *L. sakei* and *S. carnosus* cocktail fermented sausages (■); cell counts of *S. carnosus* in *L. sakei* and *S. carnosus* cocktail fermented sausages (■). Panel B, symbols indicate the pH of the positive control sausages (△), *L. sakei* fermented sausages (○), and *L. sakei* and *S. carnosus* cocktail fermented sausages (□).