1 **Positive effect of combining high pressure and biopreservation treatments on lowering the**

2 **bacterial growth during storage of diced cooked ham with reduced nitrite salt**

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19 **Keywords**

20 food microbiota, nitrite reduction, biopreservation, high pressure, metagenetic

21 **Abstract**

22 We investigated the combined effects of biopreservation and high pressure treatment on bacterial

23 communities of diced cooked ham prepared with diminished nitrite salt. First, bacterial communities

24 of four commercial brands of dice cooked ham from local supermarkets, sampled near the use-by-

25 date, were characterised. The four ham microbiota showed a relative low diversity but harboured

26 quite dissimilar communities. Two ham samples were dominated by different Proteobacteria

27 (*Pseudomonas*, *Serratia* for one, *Psychrobacter* and *Vibrio* for the other one) while the two others

28 were dominated by Firmicutes (*Latilactobacillus* and *Leuconostoc*). Second, sterile diced cooked ham,

29 prepared with reduced level of nitrite was inoculated with the two Proteobacteria-rich microbiota

30 collected from the aforementioned commercial samples together with a *Lactococcus lactis* protective

31 strain. Dices were then treated at 500 MPa for 5 minutes and bacterial dynamics was monitored

32 during storage at 8°C. Applied alone, none of the treatments stabilized durably the growth of hams

33 microbiota. Nevertheless, the combination of biopreservation and high pressure treatment was

34 efficient to reduce the growth of Proteobacteria spoilage species. However, this effect was

35 dependent on the nature of the initial microbiota, showing that use of biopreservation and high

36 pressure treatment as an alternative to nitrite reduction for ensuring cooked ham microbial safety

37 merits attention but still requires improvement.

38   **1. Introduction**

39 Nitrite salts are used since ancient times as curing agents for the production of cured meat products.

40 Nitrite (and eventually nitrate) salts are commonly added in the brine for the manufacturing of

41 cooked ham. Their role is important for the typical pinky/reddish color development of cured meats

42 (Alahakoon et al., 2015). Nitrite salts also participate to the hurdle technology for ensuring microbial

43 safety as bactericidal and bacteriostatic agents against several pathogenic bacteria occurring in meat

44 products, in particular *Clostridium botulinum* (Majou and Christieans, 2018). However, nitrite can

45 potentially lead to carcinogenic nitrosamine production in these products, which rises the concern of

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46   a potential public health risk (Sindelar and Milkowski, 2012). In European Union (EU) all additives

47   authorized before the 20th of January 2009 have to be re-evaluated by 2020. In that context, the

48   European Food Safety Authority (EFSA) re-assessed the safety of nitrite and nitrate salts in 2017. The

49   current acceptable daily intakes for nitrite and nitrate was assessed and experts stated that

50   exposure to nitrites used as food additives was within safe levels for most of the population, except

51   for children (EFSA, 2017). Nevertheless, although experts evaluated the need for further scientific

52   information, a positive link could be established between dietary intake of nitrite (or of processed

53   meat containing both nitrite and nitrate) with some cancers (Mortensen et al., 2017a, 2017b).

54   Therefore, reducing nitrite levels in processed meats appears necessary in order to limit this sanitary

55   issue. Several hurdle technologies can be proposed in order to compensate a higher risk of microbial

56   safety in cooked ham with reduced nitrite salts. Among those, High Pressure Processing (HPP) has

57   been proposed in context of food additive reduction (Pottier et al., 2017) and was shown to

58   efficiently reduce bacterial contaminants in cooked ham (Han et al., 2011; Pietrasik et al., 2016;

59   Pingen et al., 2016). As well, biopreservation, a method using protective cultures, often lactic acid

60   bacteria (LAB) or their metabolites, was described more than 20 years ago for fighting undesired

61   bacterial contaminants (Stiles, 1996). Addition of such LAB protective cultures to cooked meat

62   products, and in particular cooked ham was indeed reported (Vermeiren et al., 2006, 2004). HPP

63   combined to biopreservation has also been studied (Oliveira et al., 2015). HPP treatment of cooked

64   ham treated with bacteriocins has been shown to reduce the population of several bacterial

65   pathogens or to prolong cold storage (Jofré et al., 2008a, 2008b; Liu et al., 2012; Marcos et al., 2008;

66   Teixeira et al., 2018). In the present study, we investigated the combined effect of HPP and

67   biopreservation on the dynamics of bacterial communities of cooked ham with reduced level of

68   nitrite salts. We had previously selected a *Lactococcus lactis* strain able to recover after HPP

69   treatment and producing the bacteriocin nisin (Ramaroson et al., 2018). Here, we first determined

70   and collected bacterial communities present on commercial diced cooked ham. Then the dynamics of

71   these bacterial communities was monitored, following their inoculation in sterile diced cooked ham

72   with reduced nitrite level, in the presence of the bioprotective strain, and after the application of the

73   HPP treatment.

2.   Materials and methods

2.1. Bacterial strains, media and growth conditions

76   *Lactococcus lactis* CH-HP15 (Ramaroson et al., 2018) was first cultivated on M17 agar plates (Biokar

77   diagnostic, Beauvais, France) at 30°C for 72 h. One colony was inoculated for preculture in Medium

78   Modelling Ham (MMH) (Ramaroson et al., 2018, Modugno et al., 2019) at 30°C for 24 h, and then

79   cultured in MMH at 30°C under agitation at 65 rpm for 13h until early stationary phase. Bacterial

80   enumerations were performed by plating serial dilutions of bacterial cultures, microbiota or ham

81   stomached samples. The total viable counts were determined on Plate Count Agar (PCA) (Biokar

82   diagnostic, Beauvais, France). Psychrophilic and mesophilic counts were determined after incubation

83   for 4 days at 15°C or 24 h at 30°C, respectively. LAB were enumerated on Man Ragosa and Sharpe

84   (MRS) agar medium pH 5.2 (AES, France) containing bromocresol green (25 mg·l^{-1}) as previously

85   described after 4 days incubation at 25 °C (Najjari et al., 2008). In ham samples inoculated with *L.*

86   *lactis* CH-HP15, M17 agar plates were used for enumeration after 48 h incubation at 30°C. For

87   anaerobic conditions, plates were incubated in jars with anaerocult sachets (Anaerocult A, Merck,

88   Germany).

2.2. Ham sampling and microbiota recovery

90   Diced cooked ham (except for one sample consisting of sliced cooked ham) were purchased in 2015

91   and 2016 from local supermarkets in Nantes, France, transported at cold temperature to the

92   laboratory and reconditioned immediately. These samples, sold as ready-to-eat food were

93   conditioned in packs ranging from 120 to 200 g under modified atmosphere or without any indication

94   of any gas mixture used in the packs. At arrival in the laboratory (day 1), bags were opened and dices

95   were immediately reconditioned as 25 g aliquots under air or vacuum packaging and incubated at

96   4°C. Total counts and LAB counts were enumerated at day 1, 7, 14, 21, and 28. When PCA mesophilic

97 counts reached about 7 $\log_{10}$ CFU per gram of ham, bacterial communities were collected by mixing

98 25 g diced cooked ham in 75 ml peptone salt (AES, France) for 2 min in a stomacher (Masticator, IUL

99 Instruments, England). The homogenate was filtered through the bag filter and centrifuged through a

100 filter from Nucleospin Plant II Midi kit (Macherey Nagel, EURL, France) at 8000 × g during 10 min at

101 room temperature. The bacterial pellet was resuspended in 30 ml peptone salt and aliquoted as 1 ml

102 with glycerol 15% and stored at -80°C. In total, eight microbiota were recovered from ham dices

103 either conditioned under air or vacuum.

104 **2.3. Challenge tests**

105 Low-contaminated diced cooked ham containing 18 g/kg sodium chloride and reduced level of nitrite

106 (25 mg/kg; recommended max. level is 120 mg/kg) were used for challenge tests. Those were

107 produced as previously described (Ramaroson et al., 2018). Briefly, pork ham muscles were defatted,

108 trimmed and minced using a 20 mm grid. One kilogram of this grinding was mixed under vacuum

109 with 100 g of brine containing water 74.1 g, nitrite salt 4.6 g (0.6%), sodium chloride salt 15.2 g,

110 sodium ascorbate 0.6 g, and dextrose 5.5 g. The mix was melded and vacuum-packed. Hams (2.5 kg)

111 were cooked in a 100% humidity atmosphere (90 min at 55 °C, 60 min at 60 °C and 235 min at 67 °C),

112 cooled at ambient temperature (18 °C) for 20 min and then stored at 3 °C. Ham cubes of 1 cm × 1 cm

113 were prepared aseptically, aliquoted in 100 g portions, and stored vacuum-packed at −20 °C until

114 use. Prior to microbiota inoculation, cooked ham dices were defrosted at 4 °C for 24 h and first

115 inoculated with microbiota. Two different microbiota were inoculated in low-contaminated diced

116 cooked ham. Each challenge test was performed twice (two independent experiments). Microbiota

117 previously collected were defrosted rapidly at room temperature, diluted to a final concentration of

118 6 log CFU.mL^{-1}, inoculated at 4 log CFU g^{-1} in diced cooked ham (1% v/w inoculation rate), stored

119 overnight at 4°C for allowing bacterial recovery, and then inoculated with a fresh culture of *L. lactis*

120 CH-HP15. *L. lactis* culture in early stationary phase was centrifuged. The bacterial pellet was rinsed in

121 a sterile solution of NaCl 0.9% and resuspended at 9.3 log CFU g^{-1} and 0.5 ml were inoculated in 100

122   g dice cooked ham for an initial concentration of 7 log CFU g^{-1}. Diced cooked ham inoculated with

123   microbiota but without *L. lactis* were also used as controls.

124   **2.4. HP treatments**

125   Cooked ham dices were aliquoted as 20 g portions, vacuum-packed and High-Pressure treated at

126   500 MPa for 5 min. Samples were treated using high pressure in a 3 L vertical high pressure pilot unit

127   (ACB, Nantes, France) equipped with a temperature regulator device. The pressure transmitting fluid

128   used was distilled water. The samples were inserted into the vessel (whose internal temperature was

129   regulated to 20 °C) and processed at a compression rate of 3.4 MPa/s until the targeted pressure was

130   reached. The pressure level was held for 5 min, and decompression was nearly instantaneous (less

131   than 2 s). Water temperature reached 24 °C at 500 MPa because of the adiabatic heating. Once

132   treated, packs were then stored at 8°C and one portion was used at day 1, 5, 12, 30 for bacterial

133   enumeration and bacterial pellet collection for further DNA extraction. Unpressurized samples were

134   also included as controls.

135   **2.5. DNA preparation and amplicon sequencing**

136   Total genomic DNA was extracted from the bacterial pellet as described previously (Poirier et al.,

137   2018) using the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, USA)

138   and the High Pure PCR Template Preparation kit (Roche Diagnostics Ltd, Burgess Hill, West Sussex,

139   UK). For each sample, both DNA extracts were pooled. Then, this DNA sample was used as template

140   for three independent amplifications using either the 16S V3-V4 region of the rRNA encoding gene or

141   an internal 280 bp fragment of the Gyrase B subunit encoding gene *gyrB*, as described previously

142   (Poirier et al., 2018). All PCRs were performed in triplicate. Replicates were pooled and the amplified

143   DNA was purified with a QIAquick kit (Qiagen, Hilden, Germany). Amplicon size, quality, and quantity

144   were checked on a DNA1000 chip (Agilent Technologies, Paris, France). The MiSeq Reagent Kit v2

145   (2x250 paired-end reads, 15 Gb output) was used according to the manufacturer's instructions for

146   library preparation and sequencing on a MiSeq 2 (Illumina, San Diego, USA). The quality of the

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147   obtained sequences was checked with FastQ files generated at the end of the run (MiSeq Reporter

148   Software, Illumina, USA) and additional PhiX Control. The corresponding pairs of sequences were

149   then attributed to their respective samples using the individual multiplexing barcodes. Quality

150   controls indicated a PHRED quality score of at least 30 for 94% of the reads and a median number of

151   65,520 ± 14,180 reads per sample and 95,860 ± 11,300 reads per sample for 16S rDNA and *gyrB*

152   amplicons, respectively.

153   **2.6. Operational Taxonomic Unit analysis and accession numbers.**

154   For each sample, paired-end sequences were then loaded in the FROGS (Find Rapidly OTUs with Galaxy

155   Solutions) pipeline (Escudié et al., 2018), checked for quality and assembled. We retained merged

156   sequences with a size of 280 ± 50 bp for *gyrB* and 450 ± 50 bp for 16S rRNA gene. The merged

157   (assembled) sequences have been deposited in the Sequence Read Archive database under the

158   accession numbers SAMN13761947 to SAMN13762161, corresponding to BioProject PRJNA599607.

159   SWARM clustering (Mahé et al., 2014) was applied on the assembled sequences using a maximal

160   aggregation distance of three nucleotides for 16S rRNA gene sequences; for *gyrB* sequences, clustering

161   was more stringent, with a maximal aggregation distance of two nucleotides in order to potentially

162   assign OTUs to the subspecies level. After chimeras and spurious OTUs (low-abundance and low

163   frequency OTUs arising from sequencing artifacts) removal as described previously (Poirier et al.,

164   2018), the dataset comprised 69 OTUs for the 16S rDNA dataset and 252 OTUs for the *gyrB* dataset.

165   Taxonomic assignment of 16S rDNA OTUs was performed with the Blastn+ algorithm (Camacho et al.,

166   2009) on the SILVA 128 SSU database (Quast et al., 2012), using a threshold of 97% identity for species

167   assignment. For *gyrB* OTUs, it has been previously demonstrated that PCR amplification of *gyrB* can

168   also recover the paralogous *parE* gene, which encodes the β subunit of DNA topoisomerase IV. In order

169   to determine which of the two genes had been amplified for each species, the OTU sequences were

170   blasted against the *gyrB/parE* databases established by Poirier and colleagues (Poirier et al., 2018).

171   Both *gyrB* and *parE* OTUs were retained in our diversity analysis. We assigned taxonomy to the species

172   level when 95% of a sequence matched over 90% of length coverage found in the database. The last

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173 step consisted in comparing the taxonomic assignments obtained from the 16S rDNA and the *gyrB*

174 amplicon sequencing. The taxonomic assignment of the 16S rDNA-based OTUs were then

175 homogenized and improved by those obtained from the *gyrB* analysis. This strategy was particularly

176 applied to 16S rRNA gene OTUs with no clear assignment to the species-level (several species included

177 at the threshold of 97% identity).

178   2.7. **Beta-diversity and statistical analysis**.

179 Bacterial diversity analyses were performed using the phyloseq package (v1.24.2) of R (McMurdie

180 and Holmes, 2013). For each of the samples, analysis of diversity was either performed at the OTU

181 level or at the species level. In studies at the species level, OTUs with similar taxonomic assignment

182 (both *gyrB* and 16S rDNA) were merged using the TAX_GLOM function of phyloseq package, and their

183 abundance (number of reads) was averaged. This resulted into a dataset of 56 species with different

184 taxonomic assignment. Similarly, comparative analysis between ham samples with different

185 experimental conditions was performed after technical (16S rDNA/*gyrB*) and biological (biological

186 repetition) data were merged with the phyloseq function MERGE_SAMPLES. In this case, species

187 abundance was averaged between the four replicates. Bacterial diversity was compared among

188 different groups of samples with permutational ANOVA, specifically using the adonis function within

189 the vegan package (Oksanen et al., 2019).

190   **3. Results**

191   3.1. **Diced cooked ham selection for microbiota recovery.**

192 Preliminary tests were performed on diced cooked ham of different brands collected from local

193 supermarkets, as close as possible to the production date. These were then incubated under vacuum

194 or air packaging at 4°C or 8°C. We observed a large variation of initial total viable counts between

195 samples, ranging from 2 to 7 log CFU. g^{-1}. No strong influence of packaging was observed on

196 psychrophilic and mesophilic total counts, or MRS counts. Enumeration on MRS plates containing

197 bromocresol green enabled us to estimate the putative diversity of LAB species present in dices.

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198   Only one to 3 types of colonies were detected indicating a poor diversity among the most dominant

199   LAB species.

200   Since our objective was to recover microbiota for further re-inoculation on cooked diced ham before

201   HPP treatment, we aimed at collecting standardized quantities of bacteria, as diverse as possible, and

202   enough DNA for further amplicon sequencing. For that purpose, we designed the sampling as

203   follows: i) diced cooked ham were sampled from supermarkets as close as possible to their

204   production date; ii) immediately after arrival in the laboratory dices were reconditioned under

205   vacuum packaging and under air to eventually increase the bacterial diversity to be collected; iii)

206   packs were then stored at 4°C and analysed every 7 days; iv) when total viable counts reached about

207   7 log CFU. g^{-1}, microbiota were collected and stored for further analyses and DNA was extracted for

208   amplicon sequencing. Through this strategy, diced cooked ham samples from four different brands

209   out of ten (further referred as HAM_A to HAM_D samples) were obtained with enough bacterial

210   diversity and DNA quality (Table 1). No significant difference in population level was observed

211   between air and vacuum packaging, except in HAM_B for which both PCA and MRS counts were

212   about 1 log higher (respectively 7.97 *vs* 6.53 log CFU g^{-1} and 7.74 *vs* 6.82 log CFU g^{-1}) after storage

213   under air.

214   **3.2. The four types (brands) of diced cooked ham can be distinguished by the diversity of their**

215   **bacterial communities.**

216   The bacterial diversity was estimated using a combination of two amplicon sequencing strategies,

217   one using V3-V4 region of the 16S rRNA gene and another one using an internal ~280 bp fragment of

218   the gyrase B subunit encoding gene (*gyrB*) in order to improve taxonomic assignment of the OTUs to

219   the species or sub-species level as described previously (Poirier et al., 2018). These two strategies

220   were also used as technical replicates for ensuring that no biases were obtained in the

221   characterization of the four different microbiota as observed in Figure 1A. Only slight differences

222   between both strategies were observed in the clustering of air samples of HAM_C and D. The

223   average number of species detected per sample was 38 ± 5. As shown in Figure 1B, HAM_C and

224   HAM_D samples showed a highly similar microbiota dominated by Firmicutes, in particular

225   *Latilactobacillus sakei* and *Leuconostoc carnosum*, two species commonly found on this type of cured

226   meat products (Raimondi et al., 2019; Vasilopoulos et al., 2010; Zagdoun et al., 2019). Furthermore,

227   storage type under vacuum or air packaging did not influence significantly the overall abundance of

228   the identified species. On the other hand, the bacterial communities characterized from samples of

229   HAM_A and HAM_B were significantly different from each other and from the aforementioned

230   products, in particular with a clear dominance of Proteobacteria over Firmicutes (Figure 1C). Ham_A

231   microbiota was mainly composed of *Pseudomonadaceae* and *Enterobacteriaceae* with *Pseudomonas*

232   *lundensis* and *Serratia grimesii* being the most abundant species for Proteobacteria and

233   *Carnobacterium maltaromaticum* being the most abundant species for Firmicutes. Samples from

234   Ham_B revealed to contain the most original microbiota among the four types of ham with the three

235   most abundant 16S rDNA or *gyrB* OTUs being assigned to unknown species as the identity

236   percentage of these OTUs to *gyrB* gene in databases was lower to the ANI (Average Nucleotide Index)

237   threshold of 95% classically used for bacterial species boundary (Qin et al., 2014). The most

238   abundant Firmicutes *gyrB* OTU was assigned to a *Carnobacterium sp.* showing only 89.0% identity (as

239   best match) to *gyrB* gene of *Carnobacterium funditum* strain DSM 5970. Among Proteobacteria, one

240   OTU was assigned to a *Psychrobacter sp.* with closest match was 89.9% to *gyrB* gene of

241   *Psychrobacter cryohalolentis* FDAARGOS_308, a strain isolated from Siberian permafrost (GenBank:

242   GCF_002208775.2). For the second Proteobacteria OTU, the best identity score found was 91.6 %

243   with *Vibrio sp. SM1977 gyrB* gene, a species isolated from coralline algae surface (GenBank:

244   CP045699.1). It should be noted that the abundance of these two uncharacterized species

245   (*Psychrobacter sp.* and *Vibrio sp.*) varied significantly according to the packaging type used for

246   storage, which may correlate with their 10 times higher counts on air stored samples (Table 1),

247   although we have no evidence for their cultivability in our growth conditions.

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249   **3.3. Combined effect of HPP and biopreservation on bacterial community dynamics.**

250   For the further steps of our analysis dedicated to monitoring the effect of HPP and biopreservation

251   by *L. lactis* CH-HP15 on the ham microbiota dynamics during storage at 8°C, we decided to focus on

252   the two different microbiota from HAM_A and HAM_B. This was based on the fact that these two

253   microbiota showed a community structure enriched in bacterial species with highly spoilage

254   potential (Proteobacteria) in comparison to microbiotas of HAM_C and HAM_D enriched in

255   *Latilactobacillus*; those latter species being generally rather considered as positive microbial

256   component (Vermeiren et al., 2006, 2004). In addition, we chose HAM_B microbiota because of the

257   unexpected presence of the two uncharacterized *Psychrobacter* and *Vibrio* species. The experimental

258   strategy was based on re-inoculation at the level of 4 log CFU. g^{-1} of the original microbiota from both

259   HAM_A and HAM_B on minimally contaminated diced cooked ham with reduced nitrite content (25

260   mg/kg). For both types of microbiota, an equimolar amount mixture of microbiota from air and

261   vacuum storage previously stored at -80°C was used. The *L. lactis* CH-HP15 bioprotective strain was

262   inoculated at a higher level of 7 log CFU. g^{-1} to enable regrowth and ensure efficient biopreservation

263   activity taking into account a possible high inactivation following the HPP treatment (Ramaroson et

264   al., 2018). Table 2 is summarising the estimated bacterial population level at different storage times

265   after the several processing treatments. Similarly, Figure 2 is depicting the relative abundance of

266   each species via 16S rDNA and *gyrB* amplicon sequencing.

267

268   The control samples without any processing treatment revealed that at day 1 after inoculation, the

269   microbial communities were similar to the microbiota from HAM_A and HAM_B analyzed in Figure 1,

270   showing that the dices prepared for the challenge-tests were indeed poorly contaminated and that

271   the microbiota had recovered with no strong bias from freezing. HAM_A microbiota grew rapidly

272   under vacuum from ~4 log CFU. g^{-1} to reach ~7 log CFU. g^{-1} at day 5 and ~8 log CFU. g^{-1} at day 30. In

273   contrast, HAM_B microbiota showed a more reduced growth dynamics, reaching at most ~7 log CFU.

274   g^{-1} during the whole storage period. Interestingly, the structure of the communities also changed

275   during storage: in HAM_A samples, *P. lundensis*, a species abundant at the beginning of storage was

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276   progressively overgrown by *S. grimesii* whereas, *C. maltaromaticum,* the third abundant species,

277   remained at a stable proportion within the community; in HAM_B, the dominant *Vibrio sp.* was also

278   overgrown over time by *Lactococcus piscium*, although this latter species was largely subdominant

279   (<0.1%) in the initial microbiota.

280

281   Processing with biopreservation by *L. lactis* CH-HP15 led to two different effects regarding samples

282   from HAM_A and HAM_B. In HAM_A samples, the addition of *L. lactis* had a strong reduction effect

283   on the abundance of Firmicutes species, in particular on *C. maltaromaticum*, which thus became

284   largely subdominant, whereas *B. thermosphacta* was temporally (up to 12 days of storage) and

285   presumably taking benefit from this change (figure 2A)*.* Meanwhile, the inoculation of *L. lactis* had no

286   obvious effect on Proteobacteria species whose pattern was unchanged overtime compared to

287   control. Indeed, after 30 days of storage, while the microbiota had reached a population level above

288   8.5 log CFU. g^{-1}, *P. lundensis* and *S. grimesii* species displayed a relative abundance of about 50% of

289   the microbiota; the remaining part of the microbiota being covered by *L. lactis,* although this strain

290   had been inoculated thousand-fold higher than the original microbiota. In HAM_B, the growth

291   dynamics of the dominant *Vibrio sp.* was also not affected in comparison to the untreated samples.

292   However, the addition of *L. lactis* induced a possible competition with *L. piscium*, resulting over time

293   into equilibrium of both species. More generally, *L. lactis* CH-HP15 was less competitive in microbiota

294   from HAM_B samples than in microbiota from HAM_A samples.

295   Whether or not biopreservation was applied, HPP treatment resulted in major changes in the growth

296   dynamics of both microbiota. HPP treatment alone on HAM_A samples reduced drastically the

297   viability of the original microbiota which remained below 4 log CFU. g^{-1} after 30 days of storage

298   (Table 2). It is likely that bacterial cells were severely injured, indeed no DNA could be recovered

299   from the bacterial pellet despite the use of several extraction protocol trials. Therefore, diversity

300   analysis by amplicon sequencing could not be performed. Nevertheless, samples treated with both

301   HPP and inoculation by *L. lactis* enabled better recovery of DNA and could provide an overview of the

302   HPP treatment on the HAM_A original microbiota.

303   Proteobacteria species were the most impacted by high pressure treatment, whereas *L. Lactis* CH-

304   HP15 could recover rapidly from this treatment reaching progressively a population level above 7.5

305   log CFU. g^{-1} after 30 days, and almost a complete domination of the microbiota. Interestingly, as

306   described above on samples only treated with *L. lactis*, the addition of the bioprotective

307   microorganism increased temporally (up to 12 days of storage) the relative proportion of *B.*

308   *thermosphacta*, thereby indicating that this latter species is quite resistant to HPP treatment. Unlike

309   microbiota from HAM_A samples, that from HAM_B samples recovered rapidly from HPP treatment

310   alone, almost reaching the population level of untreated samples at 30 days (6 log CFU.g in average).

311   The community structure was itself barely affected by the HPP treatment with only a higher

312   proportion of *Psychrobacter sp.* at longer storage time in comparison to untreated samples.

313   Processing with both HPP and *L. lactis* inoculation confirmed these observations as the population

314   level, combining both initial microbiota and *L. lactis* , recovered rapidly (12 days) to 7.0 log CFU. g^{-1}

315   and raised further to ~10 log CFU. g^{-1} at 30 days, which is almost 4 log higher than the level in

316   untreated ham. However, the HPP treatment was more favorable to Firmicutes species in the end,

317   leading to a domination of *L. lactis* CH-HP15 together with *B. thermosphacta.*

318

319   **4. Discussion**

320   The use of an appropriate HPP treatment to preserve perishable food has been the focus of many

321   studies and reviews (Oliveira et al., 2015; Yamamoto, 2017). HPP is being used to enhance food

322   safety by reducing the microbial development in final products, meanwhile maintaining at best their

323   nutritional and sensorial values to a level acceptable to consumers (Pietrasik et al., 2016). However,

324   the effect of HPP on microbial growth dynamic and community structures has not been widely

325   studied and it is not known whether hurdle strategies should be applied in combination with HPP to

326   efficient microbiota inactivation or stabilisation. Indeed, a recent work carried out with a simplified

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327 ham microbiota (five species including *Listeria monocytogenes, L. sakei, B. thermosphacta, C.*

328 *maltaromaticum* and *Leuconostoc gelidum*) showed that HPP treatment is not sufficient to inhibit

329 growth recovery of the microbiota over long storage time (Teixeira et al., 2018). This is pointing out

330 that there is a clear gap in our knowledge on the HPP efficiency towards various microbial

331 communities which may be present on cooked ham. Although the technology could be a very

332 promising strategy to improve the safety of nitrite-reduced cooked ham, it is necessary to investigate

333 whether it should be used as hurdle with the combination of other strategies such as biopreservation

334 (Ramaroson et al., 2018).

335 In this context, our study provides data in link with the following questions: does HPP display a

336 generic effect on microbial inactivation or is this inactivation a microbiota-dependent phenomenon?

337 Is the use of nisin-producing *L. lactis* mediated biopreservation a worth hurdle strategy when

338 combined with HPP, and, is this biopreservation effect also microbiota dependent?

339

340 Our first strategy was to recover microbiota from several types of cooked ham (different brands)

341 stored under air or vacuum. As previously demonstrated by Raimondi and colleagues (Raimondi et

342 al., 2019), the microbiota of the four types of cooked ham was poorly diverse but highly variable.

343 Some cooked hams with poor diversity were dominated by *L. sakei, Latilactobacillus curvatus* and *L.*

344 *carnosum* and their microbiota were not considered further as HPP treatments were previously

345 demonstrated to be efficient against the growth of these species (Han et al., 2011). On the other

346 hand, cooked hams from two other producers were characterized by a more diverse microbiota.

347 These microbiota were composed of a quite different mixture of species from Firmicutes and

348 Proteobacteria phyla, including not yet characterized dominant species. Therefore, these two types

349 of microbiota were chosen as model microbiota to test the efficiency of HPP and biopreservation.

350

351 Firstly, our results demonstrate that the reduction of viable cells population by HPP is slightly more

352 effective on Proteobacteria species than on Firmicutes species resulting in a small shift of the

14

353   dominant population from Proteobacteria to Firmicutes when HPP is applied. This finding

354   corroborates many previous observations made by comparing individual species of Gram-negative

355   and Gram-positive pathogenic bacteria resistance to HPP for instance (see Oliveira et al., 2015 for a

356   review). Although the cell surface morphology of both types of bacteria can explain such difference,

357   it is interesting to notice that the recovery of cells after HPP is species (and *per se* microbiota)

358   dependent. Although both microbiota from HAM_A and HAM_B were re-inoculated at the same

359   population level before HPP treatment, that from HAM_A, composed of *P. lundensis* and *S. grimesii*

360   revealed more sensitive (no or almost no recovery) to HPP than that from HAM_B composed of

361   uncharacterised *Psychrobacter* and *Vibrio* species. The species or even strain-dependent resistance

362   to HPP has already been pointed out for Firmicutes, in particular for *L. monocytogenes* and

363   *Staphylococcus aureus* (Jofré et al., 2008b). The underlying mechanisms are not fully understood as

364   HPP resistance and recovery of bacteria also depend on the cell physiology of bacteria present on the

365   food matrix before the treatment. As well, the matrix composition may influence bacterial recovery

366   (Tassou et al., 2007), as was shown for fat content influencing *L. monocytogenes* recovery from

367   thermal inactivation (Verheyen et al., 2020). Furthermore, the capacity of the bacteria to resist HPP

368   treatment should be dissociated from the ability of the bacterial cells to recover and thrive during

369   the storage conditions. Our data are a good illustration of this phenomenon. For instance, *S. grimesii*

370   revealed more competitive than *P. lundensis* to grow during 30 days at low temperature and perhaps

371   under vacuum packaging, leading to a switch of the two species during storage. Similarly, *L. piscium*,

372   a sub-dominant species in original HAM_B microbiota, outcompeted the initial dominant *Vibrio*

373   species.

374   Another finding from our work is that the *L. lactis* strain used for biopreservation is not competitive

375   towards ham original microbiota. Although, we noticed that it has perceivable effect towards the

376   reduction of other Firmicutes species (*e.g*. *C. maltaromaticum*), perhaps due to the production of

377   nisin. Indeed, this bacterium has been shown in previous studies to harbour sensitivity to this

378   bacteriocin in vacuum packed meat (La Storia et al., 2012; Teixeira et al., 2018). Albeit the *L. lactis*

379   CH-HP15 strain was inoculated with a level three orders of magnitude higher than the original ham

380   microbiota, the strain was found between 25% to 50% of overall relative abundance in HAM_A and

381   HAM_B, respectively. We previously observed that the inactivation level of this strain after HPP was

382   more important than that of other LAB species, which was compensated by its better ability to

383   recover and rapidly regrow after the treatment (Ramaroson et al., 2018). The lack of *L. lactis*

384   competitiveness is probably due to the specific ecology of ham. Indeed, *L. lactis* CH-HP15 was shown

385   able to grow in sterile diced cooked ham at 8°C, reaching 9 log CFU g^{-1} within 5 days with an initial

386   inoculum of 6 log CFU g^{-1} (Ramaroson et al., 2018). In the present study, the presence of species

387   belonging to the natural microbiota and thus potentially better adapted may explain the lack of

388   fitness of *L. lactis* CH-HP15. Nevertheless, the combination of high level of inoculation of *L. lactis* with

389   HPP treatment leads to an efficient stabilisation of the original cooked HAM_A microbiota, by limiting

390   the growth of *P. lundensis* and *S. grimesii*. Unlike this situation, results were different for HAM_B

391   samples for which the hurdle strategy could not trigger, over the storage time, the outcompetition of

392   *L. lactis* versus the original HAM_B microbiota. Therefore, it can be concluded that for microbiota of

393   HAM_B, the hurdle strategy failed in stabilizing and inactivating the microbial growth.

394   Furthermore, our results show that *B. thermosphacta* is a species with a very strong recovery

395   dynamics following the HPP treatment. Such observation has already been made by Teixiera and

396   colleagues using the simplified ham microbiota described above (Teixeira et al., 2018). This finding

397   raises the question of HPP treatment benefit on *B. thermosphacta* containing microbiota as this

398   species is a well-known meat spoilage micro-organism (Illikoud et al., 2019).

399

400   **5. Conclusion**

401   From our work, we thus conclude that both HPP treatment and *L. lactis*-based biopreservation is

402   strongly microbiota dependent and thus, the value of this strategy requires a specific assessment for

403   each type of cooked ham production. We recommend that HPP treatment should be evaluated not

404   only for pathogenic bacteria but also on putative spoilage bacteria in order to estimate the specific

405   selection of these undesirable micro-organisms, in particular *B. thermosphacta.*

406

407   **Legends to the figures.**

408   **Figure 1. Comparative bacterial community composition between the four brand of cooked ham**

409   **analysed.**

410   **Panel A:** Cooked ham samples unsupervised clustering tree based on Bray-Curtis distance and Ward

411   algorithm. Samples are colored according to the cooked ham brand. Both air (AIR) and vacuum

412   packed (VPA) samples as well as 16 S rDNA-based or *gyrB* –based analysis are shown. **Panel B and C**

413   show a bar plot composition of the top 10 species identified among Firmicutes and Proteobacteria

414   phyla, respectively. Species are plotted according to their relative abundance in percentage of the

415   whole microbiota. Novel genus nomenclature was applied for *Latilactobacillus* species (*L. sakei* and *L.*

416   *curvatus*) as recently proposed by Zheng and colleagues (Zheng et al., 2020).

417   **Figure 2. Time course dynamic barplot composition of bacterial communities in nitrite-reduced**

418   **cooked ham samples upon various treatments.**

419   Data for HAM_A and HAM_B samples are shown in panel A and B, respectively. Species are plotted

420   according to their relative abundance in percentage of the whole microbiota. The *L. lactis* strain CH-

421   HP15 used for biopreservation is highlighted in green in the left legend. Each bar plot is the mean of

422   two biological replicates and each replicate value being the average of both 16S rDNA and *gyrB*

423   amplicon sequencing data. As described in Figure 1 for *Latilactobacillus* species, novel genus

424   nomenclature was applied for *Dellaglioa algida* (former *Lactobacillus algidus*) as recently proposed

425   by Zheng and colleagues (Zheng et al., 2020).

426

427   **Conflict of interest**

428   None. The funders had no role in the study's design, data collection, and interpretation, or the

429   decision to submit the work for publication.

430   **Funding**

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571

Table 1 Description of ham specificities

Sample name according to brands	Sampling date	Additives[a]	Total counts[b] log CFU g^{-1}	LAB counts[c] log CFU g^{-1}
HAM_A	UBD + 6 days Dec 2 2015	Dextrose Sodium nitrite E250 Potassium chloride E508 Sodium erythorbate E316 Potassium lactate E326 Sodium ascorbate E301 Sodium acetate E262 Diphosphates E450 Triphosphates E451 Polyphosphates E452	8.6 ± 0.4	8.2 ± 0.0
HAM_B[d]	UBD + 5 days Mai 18 2016	Sugar Lactose Nitrite salt Common salt Spices	7.2 ± 0.7	7.3 ± 0.5
HAM_C	UBD − 1 day Nov 6 2015	Dextrose Sodium nitrite E250 Potassium chloride E508 Potassium lactate E326 Sodium acetate E262 Sodium ascorbate E316 Polyphosphates E452	8.6 ± 0.1	9.0 ± 0.1
HAM_D	UBD − 4 days Aug 21 2015	Sodium nitrite E250 Potassium chloride E508 Potassium lactate E326 Sodium diacetate E262 Sodium ascorbate E316	10.3 ± 0.1	8.1 ± 0.1

[a]: from information provided on the packaging. [b]: PCA count mean values from portions stored under vacuum and air packaging . [c]: MRS count mean values from portions stored under vacuum and air packaging. [d]: brand sold as organic.

Table 2.

|	Population level in log CFU.g^{-1} of nitrite-reduced diced cooked ham			
	Storage time in days^A			
HAM_A samples	D01	D05	D12	D30
Without treatment	4.63 ± 0.09	7.32 ± 0.14	7.84 ± 0.10	8.15 ± 0.15
With HPP	1.20 ± 0.14	3.74 ± 0.01	3.00 ± 1.00	4.02 ± 0.59
With L. lactis	7.21 ± 0.62	9.18 ± 0.02	8.39 ± 0.12	8.87 ± 0.87
With L. lactis and HPP	2.54 ± 0.45	3.97 ± 0.26	5.42 ± 1.27	7.60 ± 0.03
HAM_B samples	D01	D05	D12	D30
Without treatment	5.24 ± 0.18	6.10 ± 0.13	6.90 ± 0.33	6.50 ± 0.20
With HPP	1.39 ± 0.09	ND^B	5.33 ± 0.34	6.23 ± 0.42
With L. lactis	7.25 ± 0.51	8.29 ± 0.01	7.82 ± 0.21	8.50 ± 0.50
With L. lactis and HPP	2.75 ± 0.44	3.64 ± 0.07	7.11 ± 0.13	10.2 ± 0.20

^{A} Population data were calculated and averaged from the two biological replicates and from two plating culture condition enumerating both total mesophilic bacteria and lactic acid bacteria (see M&M).

^{B} Not Determined.

Composition of top10 Firmicutes Species
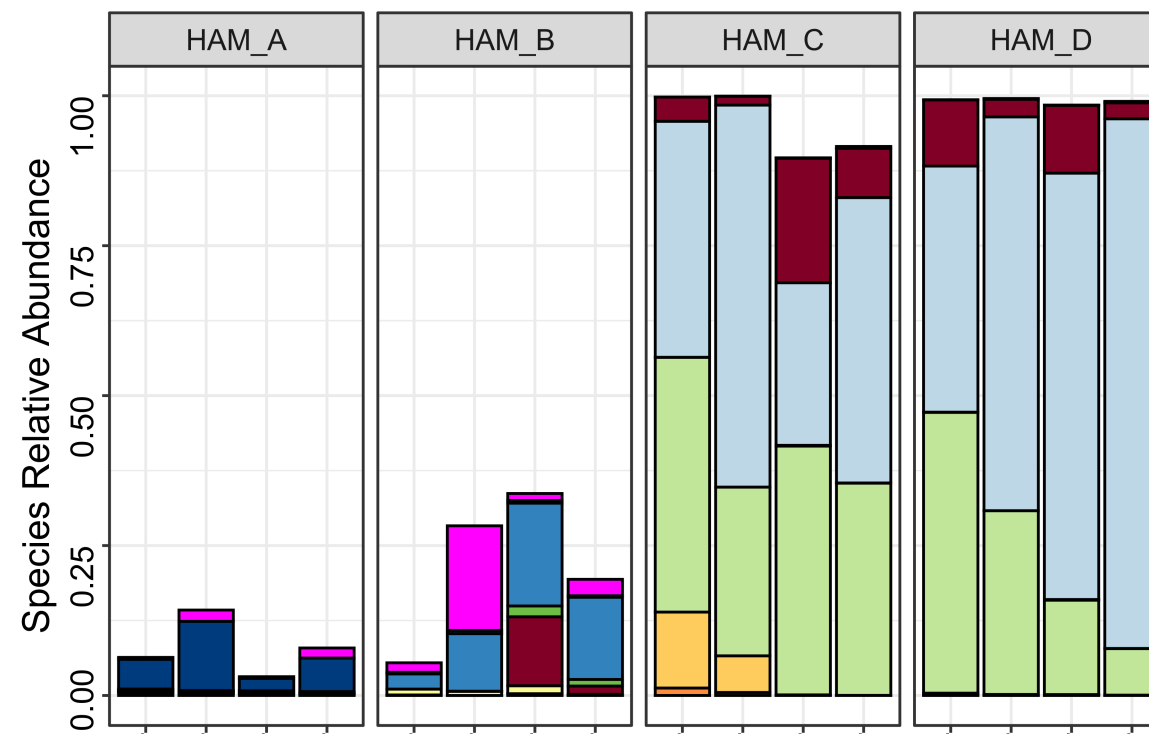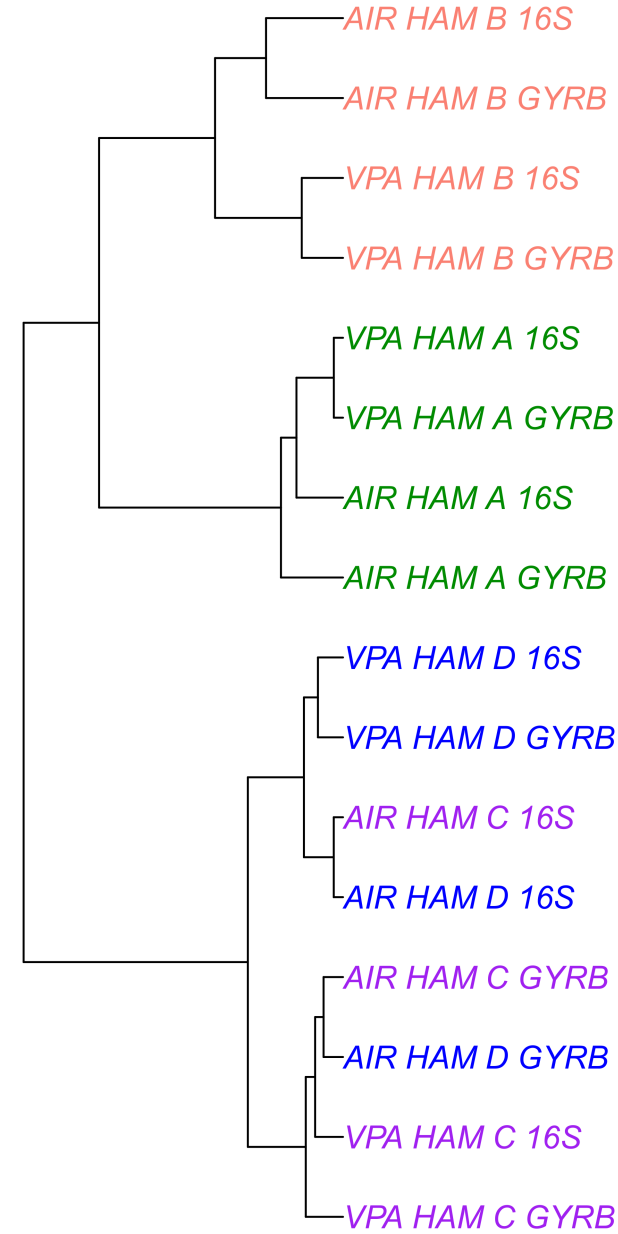
Composition of top10 Proteobacteria Species
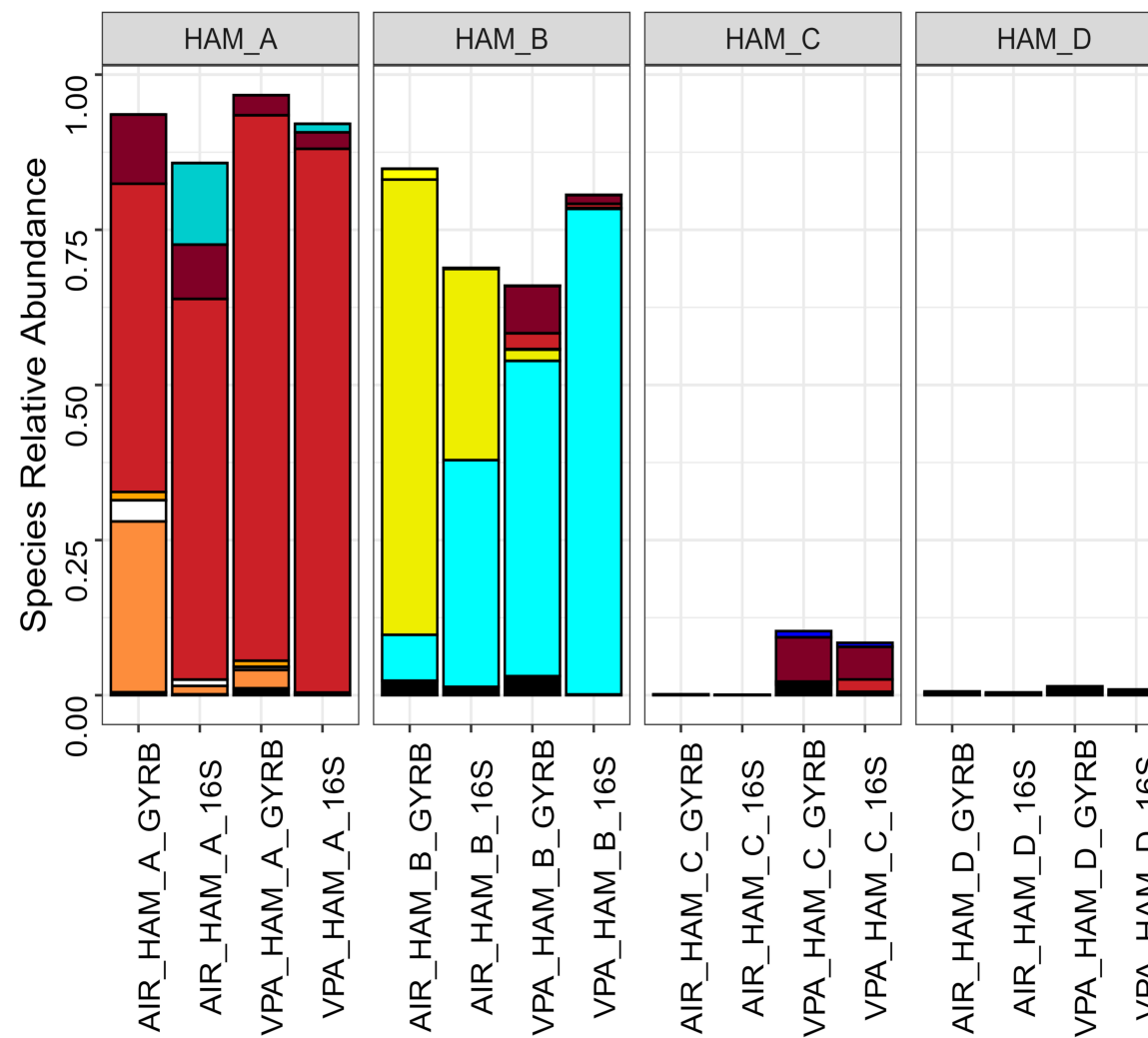
A Composition of top10 species in HAM_A samples

| | without treatment | with HPP | with *L. lactis* | with *L. lactis* & HPP |

Species
- *Brochothrix thermosphacta*
- *Carnobacterium maltaromaticum*
- *Dellaglioa algida*
- *Latilactobacillus sakei*
- *Lactococcus lactis subsp lactis*
- *Leuconostoc carnosum*
- *Pseudomonas fragi*
- *Pseudomonas lundensis*
- *Serratia grimesii*
- *Serratia proteamaculans*
- *Other*

Relative abundance of species — Storage Time in Days

B Composition of top10 species in HAM_B samples

Species
- *Brochothrix thermosphacta*
- *Carnobacterium maltaromaticum*
- *Dellaglioa algida*
- *Latilactobacillus sakei*
- *Lactococcus lactis subsp lactis*
- *Lactococcus piscium*
- *Leuconostoc carnosum*
- *Morganella psychrotolerans*
- *Psychrobacter sp.*
- *Vibrio sp.*
- *Other*

Relative abundance of species — Storage Time in Days