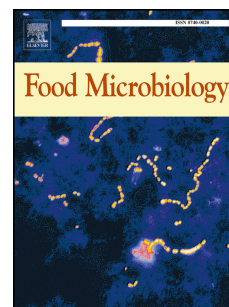| Title	Lactococcus lactis subsp. lactis as a natural anti-listerial agent in the mushroom industry
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Lactococcus lactis subsp. *lactis* as a natural anti-listerial agent in the mushroom industry

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1 <u>*Lactococcus lactis* subsp. *lactis* as a natural anti-listerial agent in the</u>

2 <u>mushroom industry</u>

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26 **Abstract**

27 Mushroom growth substrates from different commercial producers of mushrooms (*Agaricus*

28 *bisporus*) were screened for the presence of bacteria with potential for use as biocontrol

29 agents for controlling *Listeria monocytogenes* in the mushroom production environment.

30 Eight anti-listerial strains were isolated from different sources and all were identified using

31 16s rRNA gene sequencing as *Lactococcus lactis* subsp. *lactis*. Whole-genome sequencing of

32 the *Lc. lactis* isolates indicated that strains from different sites and substrate types were

33 highly similar. Colony MALDI-TOF mass spectrometry found that these strains were Nisin Z

34 producers but inhibitory activity was highly influenced by the incubation conditions and was

35 strain dependant. The biofilm forming ability of these strains was tested using a

36 crystal violet assay and all were found to be strong biofilm formers. Growth of

37 *Lc. lactis* subsp. *lactis* using mixed-biofilm conditions with *L. monocytogenes* on stainless

38 steel resulted in a 4-log reduction of *L. monocytogenes* cell numbers. Additional sampling of

39 mushroom producers showed that these anti-listerial *Lc. lactis* strains are commonly

40 present in the mushroom production environment. *Lc. lactis* has a generally regarded as

41 safe (GRAS) status and therefore has potential for use as an environmentally benign solution

42 to control *L. monocytogenes* in order to prevent product contamination and to enhance

43 consumer confidence in the mushroom industry.

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1 Introduction

Listeria monocytogenes is the causative agent of listeriosis in humans and a foodborne pathogen of significant public health concern. It is ubiquitous in nature and it can contaminate food crops and food processing environments (Ferreira et al., 2014). Although there has been an isolated report of *L. monocytogenes* associated septicaemia in an elderly man linked with the consumption of wild-collected salted mushrooms (*Lactarius rufus,* Junttila and Brander, 1989), there have been, to the author's knowledge, no reports of listeriosis due to the consumption of fresh cultivated mushrooms (*Agaricus bisporus*). However, there is concern in the mushroom industry as studies have shown that *L. monocytogenes* can be found in mushroom production facilities which therefore pose a risk of product contamination (Murugesan et al., 2015; Viswanath et al., 2013). In recent years, multiple recalls occurred in Canada for *L. monocytogenes* contaminated sliced mushroom products, although there were no incidents of listeriosis reported (Canadian Food Inspection Agency, 2012, 2014, 2015). Additionally, there have been eleven notifications of mushrooms contaminated with *L. monocytogenes* since 2013 in the European Commission's Rapid Alert System for Food (RASFF) database. These recalls resulted in an economic and reputational loss for the industry. Thus, it is important to take proactive steps to maintain this industry's current reputation for food safety by exploring novel biocontrol agents to provide enhanced assurance of product quality and safety.

The primary concern in food production and processing environments, including the mushroom industry, is that *L. monocytogenes* biofilms can form on approved materials (e.g. stainless steel, rubber and polymers), including food contact surfaces (Beresford et al., 2001; Bridier et al., 2014; Di Bonaventura et al., 2008). Studies of disinfectants or sanitizing agents that are currently used in the food industry (i.e. quaternary ammonium compounds, chlorine based compounds and oxidising agents) have found that they can reduce biofilm on different surfaces, but they are not consistently effective in dealing with *L. monocytogenes* biofilms, as the reduction levels were dependent on biofilm age, type of surface, and substrate on which the biofilms were formed (Amalaradjou et al., 2009; O'Neill et al., 2015; Pan et al., 2006; Yang et al., 2009). In addition, several studies suggest that listerial biofilms that have been exposed to certain antimicrobials, which did not result in complete removal of the pathogen, induced a change in biofilm structure with increased antimicrobial

83 resistance (Ibusquiza et al., 2011; Pan et al., 2006; Yun et al., 2012). This shows the need for

84 novel agents that can effectively control listerial biofilm and ideally are environmentally

85 benign. As an alternative to chemical disinfectants, biological control (biocontrol) options

86 could be promising alternatives, as they are considered natural, have a "green" image and

87 are environmentally friendly.

88 Currently, the mushroom industry uses the commercial product Nemasys® (BASF,

89 Germany) as a biocontrol agent which utilises a nematode called *Steinernema feltiae*, to

90 control sciarid fly larvae during mushroom production cycles. However, such an approach

91 against food-borne pathogens that could contaminate the crop has not been considered.

92 One option for this approach during production would be to utilise the bacteriocin nisin,

93 which is used in the food industry as a food additive that acts as a natural preservative,

94 mainly as an anti-botulinic agent in cheese and liquid eggs, sauces and canned foods

95 (Balciunas et al., 2013). Nisin is produced by certain strains of *Lactococcus lactis* subsp. *lactis*

96 and is the best-studied bacteriocin. Another way of utilising bacteriocin-producing bacteria

97 (BPB) is through competitive exclusion, where the BPB could be used to take over the niche

98 occupied by pathogenic bacteria, such as *L. monocytogenes*, to potentially inhibit the

99 growth of the pathogen. Within a food production environment, bacteria that have GRAS

100 status, such as lactic acid bacteria (LAB) are ideal as they pose low risk to public health.

101 Studies investigating such approaches have found that competitive exclusion bacteria can

102 control *L. monocytogenes* biofilms formed on surfaces found in food processing facilities

103 (García-Almendárez et al., 2008; Zhao et al., 2013). The aim of this study was to isolate and

104 evaluate bacteriocins or bacteriocin-producing bacteria naturally present in mushroom

105 growth substrates, which can prevent or eliminate *L. monocytogenes* and *L. monocytogenes*

106 biofilm formation.

107 **2   Materials and Methods**

108 *2.1   Strains*

109 The main target strain used for activity tests in this study was *L. monocytogenes* 6179,

110 a persistent strain isolated from a cheese processing plant (Fox et al., 2011). The other

111 indicator strains used for activity spectrum tests are listed in Table 1. *L. monocytogenes*

112 strain 2081, isolated from a mushroom production facility, was identified as a strong biofilm

113 former (unpublished results), while strains 1698 and 1721 were identified by Bolocan *et al.*
114 (2016). All bacterial strains were stored on Protect beads (Technical Service Consultants
115 Ltd., UK) with 50% glycerol solution at -80 °C. All the stored cultures from Table 1 were
116 resuscitated by streaking a bead on to Tryptone Soya Agar (TSA, Oxoid UK) and incubating at
117 37°C for 18-24 hours.

118 *2.2   Isolation of anti-listerial bacteria*

119   The three types of mushroom growth substrates that were used for isolating anti-
120 listerial bacteria are as follows: fresh mushroom casing (a nutrient-poor peat based
121 substrate), phase-3 substrate (a nutrient-rich straw and manure based substrate that has
122 been composted, pasteurised and then colonised with mushroom mycelium) and spent
123 mushroom substrate (SMS), which is what remains at the end of the crop. Batches of fresh
124 mushroom casing were obtained from four different farms, phase-3 substrate from one
125 farm and SMS from two farms. All the farms were located in different regions of Ireland. For
126 the preliminary screening for anti-listerial bacteria (ALB), 25 g of substrate was diluted in
127 225 ml of maximum recovery diluent (MRD, Oxoid, UK) and homogenised in a stomacher for
128 five minutes. Ten-fold serial dilutions of the homogenised samples were then carried out
129 and 100 µl of each dilution were spread plated on different media: LB agar (Oxoid, UK), TSA
130 supplemented with 0.6% yeast extract (TSAYE, Oxoid, UK), Brain Heart Infusion (BHI) agar
131 (Oxoid, UK), de Man, Rogosa and Sharpe agar (MRS, Oxoid, UK), GM17 (M17 supplemented
132 with 0.5% glucose, Oxoid, UK), LM17 (M17 supplemented with 0.5% lactose, Oxoid, UK) and
133 Mueller-Hinton agar (Oxoid, UK). The plates were then incubated at a number of
134 temperatures and atmospheres to promote ideal conditions for antagonistic activity:
135 aerobically for 24 hours at 37°C, anaerobically for 24 hours at 37°C, aerobically for 24 hours
136 at 25°C and anaerobically for 24 hours at 25°C. After incubation, the plates were overlaid
137 with soft TSAYE (0.75% agar) seeded with approximately $\log_{10}$ 6 CFU ml^{-1} of *L.*
138 *monocytogenes* 6179 and then incubated again aerobically for 24 hours at 37°C. Positive
139 results for inhibition were identified as visually detectable zones of clearing around a colony
140 or colonies after incubation.

141   *2.3   Identification of anti-listerial agents in cell-free supernatants*

142   Cell-free supernatants (CFS) of isolates with antagonistic activity from the previous

143   test were analysed for their anti-listerial activity using a sensitive well diffusion assay

144   described by Alvarez-Ordóñez *et al.* (2013), with minor modifications. Briefly, two types of

145   solid media were utilised for this assay: an underlay (1% agarose [Sigma-Aldrich, UK], 0.03%

146   TSB [Oxoid, UK] and 0.02% Tween 20 [Sigma-Aldrich, UK]) and overlay (1% agarose, 1X TSB).

147   Wells with a diameter of 8 mm were made on the underlay that was seeded with

148   approximately $\log_{10}$ 6 CFU ml^{-1} of indicator *L. monocytogenes* 6179 strain. Then, 50 µl

149   aliquots of neutralised CFS were added in to the wells and allowed to diffuse for two hours.

150   The overlay was poured over the underlay media, allowed to solidify and incubated at 37°C

151   for 24 hours. Other *L. monocytogenes* strains and foodborne contaminants such as

152   *Escherichia coli* (NCTC 9001), *Staphylococcus aureus* (NCTC 6571) and *Salmonella*

153   Typhimurium (SARB 67) were also tested for inhibition (Table 1). This experiment was

154   repeated three times and the activity was interpreted as the presence or absence of a zone

155   of clearance. The proteinaceous nature of the anti-listerial agents in the CFS was then

156   confirmed using proteolytic enzymes and heat treatments, as shown in Supplementary

157   Table 1. Colonies with anti-listerial activity were selected and the bacteriocins produced

158   were identified using colony mass spectrometry according to the method described by Field

159   et al. (2015). The resulting peptide masses were then compared to a web-based database

160   (BAGEL) to identify putative bacteriocins (de Jong et al., 2006).

161   2.4   Determination of anti-biofilm activity of ALB

162   A modified crystal violet assay was used to test the anti-biofilm efficacy of ALB CFS on

163   pre-formed 72h biofilms of *L. monocytogenes* strains (1698, 1721 and 2081) based on the

164   method used by Bolocan et al. (2016), with minor modifications. In brief, 72h biofilms grown

165   in microtitre plates were treated with ALB CFS for 24h, stained with crystal violet and the

166   absorbance results compared with the biofilms treated without bacteriocins.

167   *2.5   Identification of anti-listerial bacteria*

168   The potential anti-listerial bacteria were initially identified according to their 16S rRNA

169   gene sequence. This was carried out according to the method described by Chopra et al.,

170 (2014), with minor modifications. Genomic DNA was extracted using the DNeasy blood and

171 tissue kit (Qiagen, Germany). Sequencing of the amplicons was carried out by Eurofins

172 (Germany), after which the sequence was   analysed using the BLAST tool at the National

173 Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6   Competitive exclusion assay

175   The method for competitive exclusion (CE) was based on the methodology of García-

176 Almendárez *et al.* (2008), with minor modifications. The CE bacteria, *Lc. lactis* subsp. *lactis*

177 (Ca55), was chosen for this study based on its ability to grow better on plates under aerobic

178 conditions, strong biofilm formation and its anti-listerial activity (Figure 1). The *L.*

179 *monocytogenes* strain used for this study was *L. monocytogenes* 2081. The material chosen

180 to grow the biofilms on was sterile stainless steel coupons (Type 304, measuring 3×0.8×0.1

181 cm)*.* Liquid cultures of *L. monocytogenes* and *Lc. lactis* were prepared in BHIYE and

182 incubated for 24 hours at different temperatures; 37°C for *L. monocytogenes* and 30°C for

183 *Lc. lactis.* Anti-listerial biofilm efficacy was tested by utilising the *Lc. lactis* in mixed biofilm

184 with *L. monocytogenes* and its cell-free supernatant on listerial biofilms. To test the effect of

185 *Lc. lactis*, 100 µl of liquid culture ($\log_{10}$ 8 CFU ml^{-1}) was inoculated on three sterile stainless

186 steel coupons. For testing the anti-biofilm efficacy of the CFS from Ca55, 100 µl of CFS was

187 added to three stainless steel coupons instead. Then, 100 µl of *L. monocytogenes* (LM 2081)

188 liquid culture ($\log_{10}$ 4- $\log_{10}$ 5 CFU ml^{-1}) was added to all of the coupons, including three

189 control coupons with *L. monocytogenes* alone. Cell adhesion was allowed by incubating all

190 coupons for 6 hours at 25°C. Afterwards, the coupons were washed with 5 ml of PBS, 200 µl

191 of BHIYE pipetted on to the surface, placed in sterile petri dishes and incubated for 24 hours

192 at 25°C. For the CFS treated coupons, 100 µl of BHIYE and 100 µl of CFS were added after

193 the washing step. The washing and replacement of media was then repeated every 24 hours

194 for five days.

195   To enumerate the biofilms after treatment, the coupons were washed with PBS to

196 remove unattached cells and the biofilms were dislodged by using a 45 kHz ultrasonic bath

197 (VWR, Ireland) for 15 minutes; followed by vortexing for 1 minute and serial dilutions in

198 MRD (Oxoid, UK). *L. monocytogenes* was enumerated from all of the coupons by spread

199 plating on to Oxford selective media (Oxoid, UK), while *Lc. lactis* was pour-plated in MRS

200 agar. The MRS plates were incubated at 30°C for 24h, while Oxford plates were incubated at

201 37°C for 24h. Both experiments were repeated three times and the results expressed in

202 $\log_{10}$ CFU cm^{-2}.

203 *2.7   Whole-genome sequencing and data analysis*

204   In order to assess strain similarity, genomic DNA from the eight *Lc. lactis* subsp. *lactis*

205 strains were prepared using the DNeasy blood and tissue kit (Qiagen, Germany) and were

206 quantified using the NanoDrop ND-1000 (ThermoFisher Scientific, UK). Whole-genome

207 sequence data was generated and assembled by MicrobesNG (https://microbesng.uk) using

208 the Illumina MiSeq platform, including a standard analysis pipeline. Chromosomal

209 organisation of the resulting contigs were predicted by aligning them against the *Lc. lactis*

210 subsp. *lactis* IL1403 reference genome, obtained from the NCBI database

211 (https://www.ncbi.nlm.nih.gov), using the Mauve Contig Mover algorithm (MCM) (Rissman

212 et al., 2009). The progressiveMauve algorithm was then used to align all of the

213 concatenated sequences with the reference genome (Darling et al., 2010). Overall genomic

214 relatedness between the different genomes were then assessed using the Orthologous

215 Average Nucleotide Identity (OrthoANI) tool (Lee et al., 2016), which is the modified version

216 of the average nucleotide identity (ANI) algorithm (Richter and Rossello-Mora, 2009).

217 *2.8   Statistical analysis*

218   One-way ANOVA was used to compare between different groups, followed by the

219 Tukey test to determine significant differences between the means ($P \leq 0.05$). These tests

220 were performed using the IBM SPSS software (version 24.0, SPSS Inc., Chicago IL, USA).

221 **3   Results & Discussion**

222 *3.1   Isolation and identification of anti-listerial bacteria and bacteriocin produced*

223   In this study, LAB with anti-listerial activity were found to exist in growth substrates

224 used in the mushroom production environment. During the initial screening, 234 colonies

225 exhibiting anti-listerial activity were isolated from the different media and growth

226 conditions used. However, after the well diffusion assay experiment, only eight isolates,

227 shown in Table 1, retained their activity; Ca27, Ca29, Ca30, Ca46 and Ca55 were isolated

228   from mushroom casing, while Sp32, Sp34 and Sp41 were isolated from SMS. The high level

229   of false positive inhibition may have been due to zone of inhibition overlap between

230   different colonies and lactic acid production. Molecular identification of the isolates with

231   anti-listerial activity, by 16S rRNA gene sequencing, using the BLAST program, showed that

232   the eight anti-listerial isolates had greatest homology to *Lc. lactis* subsp. *lactis* IL1403. This is

233   similar to the study by Chen *et al.* (2005) that isolated LAB from soil samples in Japan,

234   including *Lc. lactis* subsp. *lactis* strains, but a follow-up study found that those isolates were

235   not bacteriocin-producing strains (Yanagida et al., 2006). Kim and Kwak (2012) also isolated

236   LAB from SMS but they mainly found *Lactobacillus spp.* The eight strains found in this study

237   were able to demonstrate a broad spectrum of inhibition against a bank of *L.*

238   *monocytogenes* strains, including several from the mushroom production environment

239   (Table 1). These *Lc. lactis* isolates were found to have similar anti-listerial activity to strains

240   isolated in other studies (Bolocan et al., 2016; Heo et al., 2012; Noonpakdee et al., 2002;

241   Olasupo et al., 1999).

242   Whole-genome comparison of the *Lc. lactis* subsp. *lactis* strains, aligned to the

243   reference genome, showed that most of the locally collinear blocks (LCBs) are highly

244   homologous between all of the assemblies (Supplementary Figure 1). The OrthoANI values

245   indicated that there was a high level of similarity between the genomic sequences of the *Lc.*

246   *lactis* isolates (Supplementary Figure 2). Additionally, a cluster can also be observed

247   between Ca29, Ca30, Ca46 and Sp34, with OrthoANI values of 100% similarity between the

248   strains. As a follow-up experiment for this study, LAB were detected and *Lc. lactis* isolates

249   were presumptively identified (data not shown) from five additional mushroom production

250   facilities. This reiterates the results from the initial screening step and the OrthoANI values,

251   which suggests that LAB and bacteriocin-producing *Lc. lactis* are naturally present in the

252   mushroom production environment. To identify if these additional isolates are bacteriocin

253   producers, further analysis would be needed.

254   The proteinaceous nature of the antibacterial agents, present in the CFS of the *Lc. lactis*

255   strains, was confirmed by the effects of enzymes and heat treatment on their activity (as

256   shown in Supplementary Table 1), while colony mass spectrometry detected a molecular

257   mass of 3331 Da, which corresponds to the mass of nisin Z, from all eight *Lc. lactis* subsp.

258   *lactis* mushroom isolates (Supplementary Figure 3). Despite all being nisin Z producers and

259   the high levels of strain similarity, the different strains exhibited varying incubation

260   conditions for optimum levels of antimicrobial activity, with aerobic conditions being the

261   preferable option for possible industry application. Whole-genome sequencing analysis

262   showed no difference in the nisin operon sequence between strains. Bolocan *et al.* (2016)

263   also reported that the growth environment had a differential ability to influence the *Lc.*

264   *lactis* strains' anti-listerial activity. Rattanachaikunsopon and Phumkhachorn (2008)

265   proposed that the sensitivity of the indicator strain used in the activity assays may be a

266   factor, but it could be mainly due to varying concentrations of bacteriocins produced. Kim *et*

267   *al.* (1997) found that, incorporation of an identical nisin-production transposon into

268   different *L. lactis spp.* strains did not yield identical nisin concentrations, as one of the key

269   determinants for final nisin concentrations were the endogenous features of the producing

270   organism. Further studies would be needed to identify these endogenous features that

271   influence bacteriocin production.

272   *3.2   Competitive exclusion*

273   As shown in Table 2, the competitive exclusion treatments had different effects on the

274   biofilms formed by *L. monocytogenes* in either multi-species biofilms or with *Lc. lactis* Ca55

275   supernatant. *L. monocytogenes* was able to form $\log_{10}$ 5 CFU cm^{-2} of mono-culture biofilm

276   on stainless steel. *Lc. lactis* Ca55 exhibited an anti-biofilm effect against *L. monocytogenes,*

277   as it was able to significantly reduce (*P*<0.05) the *L. monocytogenes* biofilm by

278   approximately 4 logs, while exhibiting strong biofilm formation on co-culture biofilm on

279   stainless steel. This is similar to previous studies carried out on *Lc. lactis* subsp. *lactis* strains

280   isolated from floor drains at different food processing plants, which found them to be

281   effective at controlling *L. monocytogenes* biofilms for long periods (28 days) and at low

282   temperatures (4°C) (Zhao et al., 2004, 2013). Guerrieri *et al.* (2009) found that biofilms of

283   other LAB (*Lb. plantarum* 35d) can control *L. monocytogenes* in a small-scale model where

284   they found a 3.9 log reduction of *L. monocytogenes* biofilm during a 10-day experiment.

285   These observed inhibitions of pathogenic bacteria by LAB biofilms cannot only be attributed

286   to the production of bacteriocins but also by possible biosurfactants, such as xylolipid that is

287   produced by a *Lc. lactis* strain found by Saravanakumari and Mani (2010). Additionally,

288   Gómez et al. (2016) observed an anti-biofilm adhesion effect in both bacteriocin producer

289   and non-bacteriocin producer strains of *Lc. lactis.*

290   In contrast to the co-culture study, the supernatant of the *L. lactis* Ca55 strain had no

291   effect on biofilm formed on stainless steel and showed no significant difference on the

292   amount of mono-species biofilm of *L. monocytogenes* 2081. This differs to the results using

293   microtitre plates shown in Figure 1, where the CFS from the different *Lc. lactis* strains

294   caused a significant reduction ($P<0.05$) on most of the 72h biofilms formed by the three *L.*

295   *monocytogenes* strains, when compared to the results from the GM17 controls. Bolocan *et*

296   *al.* (2016) reported similar results, from *L. lactis* ALB79, for the competitive exclusion assay

297   (co-culture and supernatant), where they saw a 3.6 log reduction of *L. monocytogenes*

298   biofilm in co-culture but none from supernatant with *L. monocytogenes.* The possibility of

299   more harbourage sites on stainless steel compared to polystyrene could be promoting

300   increased resistance of *L. monocytogenes* biofilm against nisin but on the contrary,

301   Ibusquiza *et al.* (2011) found that biofilm formed on polystyrene had higher resistance to

302   nisin. Based on the results of Messens *et al.* (2003), this loss of bacteriocin activity could also

303   be attributed to endogenous proteases produced during the growth phase, allowing *L.*

304   *monocytogenes* 2081 to grow and form biofilms during the three 24-hour cycles of washing,

305   media replacement and incubation. Overall, these results suggest that the utilisation of the

306   competitive exclusion strains themselves in the mushroom production environment, where

307   they can continuously produce anti-listerial agents during the mushroom production cycle,

308   may be the most effective approach for minimising *L. monocytogenes*.

309   **4   Conclusion**

310   Based on the results of this study, it was found that *Lc. lactis* subsp. *lactis* strains

311   naturally present in mushroom growth substrates have potential for application as

312   biocontrol agents against *L. monocytogenes* in the mushroom production environment.

313   Their GRAS status, natural presence, and ability to produce bacteriocins and biofilms at

314   industry relevant temperatures make them suitable candidates. They could be either

315   utilised as the strains themselves, their bacteriocins could be isolated and used, or either

316   could be used as part of a hurdle technology to help control *L. monocytogenes* biofilms in

317   the mushroom production environment. Further investigation is required in pilot scale

318   mushroom growing trials.

319 **5   Acknowledgements**

324 **References:**

325 Alvarez-Ordóñez, A., Begley, M., Clifford, T., Deasy, T., Considine, K., and Hill, C. (2013).
326   Structure-activity relationship of synthetic variants of the milk-derived antimicrobial
327   peptide αs2-casein f(183-207). *Appl. Environ. Microbiol.* 79, 5179–5185.
328   doi:10.1128/AEM.01394-13.

329 Amalaradjou, M. A. R., Norris, C. E., and Venkitanarayanan, K. (2009). Effect of octenidine
330   hydrochloride on planktonic cells and biofilms of Listeria monocytogenes. *Appl.
331   Environ. Microbiol.* 75, 4089–4092. doi:10.1128/AEM.02807-08.

332 Balciunas, E. M., Castillo Martinez, F. A., Todorov, S. D., Franco, B. D. G. D. M., Converti, A.,
333   and Oliveira, R. P. D. S. (2013). Novel biotechnological applications of bacteriocins: A
334   review. *Food Control* 32, 134–142. doi:10.1016/j.foodcont.2012.11.025.

335 Beresford, M. R., Andrew, P. W., and Shama, G. (2001). Listeria monocytogenes adhers to
336   many materials found in food-processing environments. *J. Appl. Microbiol.* 90, 1000–
337   1005.

338 Bolocan, A. S., Pennone, V., O'Connor, P. M., Coffey, A., Nicolau, A. I., McAuliffe, O., et al.
339   (2016). Inhibition of Listeria monocytogenes biofilms by bacteriocin-producing bacteria
340   isolated from mushroom substrate. *J. Appl. Microbiol.* 122, 279–293.
341   doi:10.1111/jam.13337.

342 Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J. C., Naïtali, M., and Briandet, R. (2014).
343   Biofilm-associated persistence of food-borne pathogens. *Food Microbiol.* 45, 167–178.
344   doi:10.1016/j.fm.2014.04.015.

345 Canadian Food Inspection Agency (2012). Certain CHAMP'S MUSHROOMS brand SLICED
346   CRIMINI MUSHROOMS may contain Listeria monocytogenes. Available at:
347   http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-
348   warnings/complete-listing/2012-09-27/eng/1357586653536/1357586653551
349   [Accessed April 19, 2016].

350 Canadian Food Inspection Agency (2014). Avina Fresh Mushrooms brand Sliced Crimini
351   Mushrooms recalled due to Listeria monocytogenes. Available at:
352   http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-
353   warnings/complete-listing/2014-08-06c/eng/1407377686071/1407377691861
354   [Accessed April 19, 2016].

355 Canadian Food Inspection Agency (2015). Champ's Mushrooms and President's Choice
356   brand sliced mushroom products recalled due to Listeria monocytogenes. Available at:
357   http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-

358   warnings/complete-listing/2015-06-16/eng/1434508769959/1434508761798
359   [Accessed April 19, 2016].

360   Chen, Y.-S., Yanagida, F., and Shinohara, T. (2005). Isolation and identification of lactic acid
361   bacteria from soil using an enrichment procedure. *Lett. Appl. Microbiol.* 40, 195–200.
362   doi:10.1111/j.1472-765X.2005.01653.x.

363   Chopra, L., Singh, G., Choudhary, V., and Sahoo, D. K. (2014). Sonorensin: an antimicrobial
364   peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new
365   marine isolate, Bacillus sonorensis MT93. *Appl. Environ. Microbiol.* 80, 2981–90.
366   doi:10.1128/AEM.04259-13.

367   Darling, A. E., Mau, B., and Perna, N. T. (2010). Progressivemauve: Multiple genome
368   alignment with gene gain, loss and rearrangement. *PLoS One* 5.
369   doi:10.1371/journal.pone.0011147.

370   de Jong, A., van Hijum, S. A. F. T., Bijlsma, J. J. E., Kok, J., and Kuipers, O. P. (2006). BAGEL: A
371   web-based bacteriocin genome mining tool. *Nucleic Acids Res.* 34, 273–279.
372   doi:10.1093/nar/gkl237.

373   Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, a., Conter, M., et al.
374   (2008). Influence of temperature on biofilm formation by Listeria monocytogenes on
375   various food-contact surfaces: Relationship with motility and cell surface
376   hydrophobicity. *J. Appl. Microbiol.* 104, 1552–1561. doi:10.1111/j.1365-
377   2672.2007.03688.x.

378   Ferreira, V., Wiedmann, M., Teixeira, P., and Stasiewicz, M. J. (2014). Listeria
379   monocytogenes persistence in food-associated environments: epidemiology, strain
380   characteristics, and implications for public health. *J. Food Prot.* 77, 150–70.
381   doi:10.4315/0362-028X.JFP-13-150.

382   Field, D., Daly, K., O'Connor, P. M., Cotter, P. D., Hill, C., and Paul Ross, R. (2015). Efficacies
383   of nisin a and nisin V semipurified preparations alone and in combination with plant
384   essential oils for controlling Listeria monocytogenes. *Appl. Environ. Microbiol.* 81,
385   2762–2769. doi:10.1128/AEM.00070-15.

386   Fox, E. M., Leonard, N., and Jordan, K. (2011). Physiological and transcriptional
387   characterization of persistent and nonpersistent Listeria monocytogenes isolates. *Appl.*
388   *Environ. Microbiol.* 77, 6559–6569. doi:10.1128/AEM.05529-11.

389   García-Almendárez, B. E., Cann, I. K. O., Martin, S. E., Guerrero-Legarreta, I., and Regalado,
390   C. (2008). Effect of Lactococcus lactis UQ2 and its bacteriocin on Listeria
391   monocytogenes biofilms. *Food Control* 19, 670–680.
392   doi:10.1016/j.foodcont.2007.07.015.

393   Gómez, N. C., Ramiro, J. M. P., Quecan, B. X. V., and de Melo Franco, B. D. G. (2016). Use of
394   Potential Probiotic Lactic Acid Bacteria (LAB) Biofilms for the Control of Listeria
395   monocytogenes, Salmonella Typhimurium, and Escherichia coli O157:H7 Biofilms
396   Formation. *Front. Microbiol.* 7, 1–15. doi:10.3389/fmicb.2016.00863.

397   Guerrieri, E., de Niederhäusern, S., Messi, P., Sabia, C., Iseppi, R., Anacarso, I., et al. (2009).
398   Use of lactic acid bacteria (LAB) biofilms for the control of Listeria monocytogenes in a
399   small-scale model. *Food Control* 20, 861–865. doi:10.1016/j.foodcont.2008.11.001.

400   Heo, W. S., Kim, E. Y., Kim, Y. R., Hossain, M. T., and Kong, I. S. (2012). Salt effect of nisin Z
401   isolated from a marine fish on the growth inhibition of Streptococcus iniae, a pathogen
402   of streptococcosis. *Biotechnol. Lett.* 34, 315–320. doi:10.1007/s10529-011-0766-6.

403   Ibusquiza, P. S., Herrera, J. J. R., and Cabo, M. L. (2011). Resistance to benzalkonium
404   chloride, peracetic acid and nisin during formation of mature biofilms by Listeria
405   monocytogenes. *Food Microbiol.* 28, 418–425. doi:10.1016/j.fm.2010.09.014.

406   Junttila, J., and Brander, M. (1989). Listeria monocytogenes septicemia associated with
407   consumption of salted mushrooms. *Scand. J. Infect. Dis.* 21, 339–342.
408   doi:10.3109/00365548909035707.

409   Kim, W. S., Hall, R. J., and Dunn, N. W. (1997). Host specificity of nisin production by
410   Lactococcus lactis. *Biotechnol. Lett.* 19, 1235–1238. doi:10.1023/A:1018450223732.

411   Kim, Y.-I., and Kwak, W. S. (2012). Isolation and identification of lactic acid bacteria from
412   spent mushroom substrate for silage making and determination of optimal medium
413   conditions for growth. *J. Anim. Sci. Technol.* 54, 435–442.
414   doi:10.5187/JAST.2012.54.6.435.

415   Lee, I., Kim, Y. O., Park, S. C., and Chun, J. (2016). OrthoANI: An improved algorithm and
416   software for calculating average nucleotide identity. *Int. J. Syst. Evol. Microbiol.* 66,
417   1100–1103. doi:10.1099/ijsem.0.000760.

418   Messens, W., Verluyten, J., Leroy, F., and De Vuyst, L. (2003). Modelling growth and
419   bacteriocin production by Lactobacillus curvatus LTH 1174 in response to temperature
420   and pH values used for European sausage fermentation processes. *Int. J. Food
421   Microbiol.* 81, 41–52. doi:10.1016/S0168-1605(02)00168-X.

422   Murugesan, L., Kucerova, Z., Knabel, S. J., and LaBorde, L. F. (2015). Predominance and
423   Distribution of a Persistent Listeria monocytogenes Clone in a Commercial Fresh
424   Mushroom Processing Environment. *J. Food Prot.* 78, 1988–98. doi:10.4315/0362-
425   028X.JFP-15-195.

426   Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., and Panyim, S. (2002).
427   Isolation of nisin-producing Lactococcus lactis WNC 20 strain from nham, a traditional
428   Thai fermented sausage. *Int. J. Food Microbiol.* 81, 137–145. doi:10.1016/S0168-
429   1605(02)00219-2.

430   O'Neill, T., Lole, M., Drakes, D., Baars, J., and Grogan, H. (2015). Use of chemical
431   disinfectants in mushroom production. Agriculture and Horticulture Development
432   Board Available at: https://www.teagasc.ie/media/website/publications/2015/Use-of-
433   chemical-disinfectants.pdf [Accessed January 1, 2017].

434   Olasupo, N. A., Schillinger, U., Narbad, A., Dodd, H., and Holzapfel, W. H. (1999). Occurrence
435   of nisin Z production in Lactococcus lactis BFE 1500 isolated from wara, a traditional
436   Nigerian cheese product. *Int. J. Food Microbiol.* 53, 141–152. doi:10.1016/S0168-
437   1605(99)00146-4.

438   Pan, Y., Breidt, F., and Kathariou, S. (2006). Resistance of Listeria monocytogenes biofilms to
439   sanitizing agents in a simulated food processing environment. *Appl. Environ. Microbiol.*
440   72, 7711–7717. doi:10.1128/AEM.01065-06.

441   Rattanachaikunsopon, P., and Phumkhachorn, P. (2008). Incidence of nisin Z production in

442   Lactococcus lactis subsp. lactis TFF 221 isolated from Thai fermented foods. *J. Food*
443   *Prot.* 71, 2024–2029.

444   Richter, M., and Rossello-Mora, R. (2009). Shifting the genomic gold standard for the
445   prokaryotic species definition. *Proc. Natl. Acad. Sci.* 106, 19126–19131.
446   doi:10.1073/pnas.0906412106.

447   Rissman, A. I., Mau, B., Biehl, B. S., Darling, A. E., Glasner, J. D., and Perna, N. T. (2009).
448   Reordering contigs of draft genomes using the Mauve Aligner. *Bioinformatics* 25, 2071–
449   2073. doi:10.1093/bioinformatics/btp356.

450   Saravanakumari, P., and Mani, K. (2010). Structural characterization of a novel xylolipid
451   biosurfactant from Lactococcus lactis and analysis of antibacterial activity against multi-
452   drug resistant pathogens. *Bioresour. Technol.* 101, 8851–8854.
453   doi:10.1016/j.biortech.2010.06.104.

454   Viswanath, P., Murugesan, L., Knabel, S. J., Verghese, B., Chikthimmah, N., and Laborde, L. F.
455   (2013). Incidence of Listeria monocytogenes and Listeria spp. in a small-scale
456   mushroom production facility. *J. Food Prot.* 76, 608–15. doi:10.4315/0362-028X.JFP-12-
457   292.

458   Yanagida, F., Chen, Y.-S., and Shinohara, T. (2006). Searching for bacteriocin-producing lactic
459   acid bacteria in soil. *J. Gen. Appl. Microbiol.* 52, 21–28. doi:10.2323/jgam.52.21.

460   Yang, H., Kendall, P. a, Medeiros, L. C., and Sofos, J. N. (2009). Efficacy of sanitizing agents
461   against Listeria monocytogenes biofilms on high-density polyethylene cutting board
462   surfaces. *J. Food Prot.* 72, 990–998.

463   Yun, H. S., Kim, Y., Oh, S., Jeon, W. M., Frank, J. F., and Kim, S. H. (2012). Susceptibility of
464   Listeria monocytogenes biofilms and planktonic cultures to hydrogen peroxide in food
465   processing environments. *Biosci. Biotechnol. Biochem.* 76, 2008–2013.
466   doi:10.1271/bbb.120238.

467   Zhao, T., Doyle, M. P., and Zhao, P. (2004). Control of Listeria monocytogenes in a Biofilm by
468   Competitive-Exclusion Microorganisms. *Appl. Environ. Microbiol.* 70, 3996–4003.
469   doi:10.1128/AEM.70.7.3996.

470   Zhao, T., Podtburg, T. C., Zhao, P., Chen, D., Baker, D. a, Cords, B., et al. (2013). Reduction by
471   Competitive Bacteria of Listeria monocytogenes in Biofilms and Listeria Bacteria in
472   Floor Drains in a Ready-to-Eat Poultry Processing Plant. *J. Food Prot.* 76, 601–7.
473   doi:10.4315/0362-028X.JFP-12-323.

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Table 1: Spectrum of activity of CFS from anti-listerial bacteria (ALB)

Organisms[a]	Isolated from[b]:	Serotype/ Serogroup[c]	Cell-free supernatants of ALB isolates[d]							
			Ca27	Ca29	Ca30	Ca46	Ca55	Sp32	Sp34	Sp41
L. monocytogenes strains:										
63/21	Milk sock filter	3a*	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +
105	Hay in cow barn	3b*	+	+ +	+ +	+	+	-	+	+ +
1628	Mushroom industry	4a*	+ +	+	+ +	+	+	-	+ +	+
1634	Mushroom industry	7*	+ +	+ + +	+ +	+	+	+	+ +	+ + +
6179	Cheese isolate	1/2a*	+	+	+	+	+	+	+	+ +
ATCC 15313	Rabbit	1/2a*	+	+ +	+ +	+	+ + +	-	+ +	+ +
ATCC 19112	Clinical isolate	1/2c*	+	+	+	+	+	-	+	+
ATCC 19116	Chicken	4c*	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
ATCC 19117	Sheep	4d*	+	+ +	+	+ +	+	+	+	+ +
ATCC 19118	Chicken	4e*	+	+	+	+	+	-	+	+ + +
DPC 4605	Unknown	3c*	+	+ + +	+ +	+	+ + +	-	+ +	+
EGD-e	Rabbit	1/2a*	+ +	+ + +	+ +	+ +	+ + +	+	+ +	+ +
NCTC 11994	Soft cheese	4b*	+	+ + +	+ +	+ +	+ + +	+	+	+ +
Scott A	Clinical isolate	4b*	+ +	+ +	+ +	+	+	+	+ +	+ +
1698	Mushroom industry	1/2a-3a**	+	+	+	+	+	-	+	+
1702	Mushroom industry	1/2b-3b-7**	+ +	+ +	+ +	+	+ + +	+	+ +	+
1703	Mushroom industry	1/2a-3a**	+ +	+ + +	+ + +	+	+ + +	-	+ + +	+
1721	Mushroom industry	1/2a-3a**	++	++	++	+	++	+	+	++
1722	Mushroom industry	4b-4d-4e**	+ +	+ + +	+ +	+	+ +	+ +	+ +	+ + +
2081	Mushroom industry	1/2a-3a**	++	++	+++	+	+	-	++	+
Other organisms:			-	-	-	-	-	-	-	-
Escherichia coli NCTC 9001	Clinical isolate		-	-	-	-	-	-	-	-
Salmonella Typhimurium SARB 67			-	-	-	-	-	-	-	-
Staphylococcus aureus NCTC 6571	Lake water		-	-	+	-	-	-	+	+

[a] ATCC: American Type Culture Collection; DPC: Teagasc Food Research Centre Moorepark Culture Collection; NCTC: National Collection of Type Cultures; SARB: Salmonella Reference Collection B.

[b] ALB: anti-listeria bacteria; All strains were obtained from the Teagasc Food Research Centre culture collection

[c] *, serotype; **, serogroup

[d] -, no zone of inhibition; +, inhibition zone <3 mm; ++, inhibition zone 3–6 mm; +++, inhibition zone >6 mm

Table 2: Competitive exclusion assay on 120h *L. monocytogenes* biofilms formed on stainless steel by *Lc. lactis*.

| Tests | Biofilm after 120h (Log_{10} CFU/cm^2 ± SD)[a] | |
	L. monocytogenes	*Lc. lactis*
L. monocytogenes only	5.1 ± 1.0[A]	
L. monocytogenes and *Lc. lactis* co-culture biofilm	1.1 ± 1.2[B]	6.3 ± 0.6
L. monocytogenes biofilm and *Lc. lactis* supernatant	5.0 ± 0.4[A]	

[a] Values with different letters (A-B) are significantly different ($P \leq 0.05$)

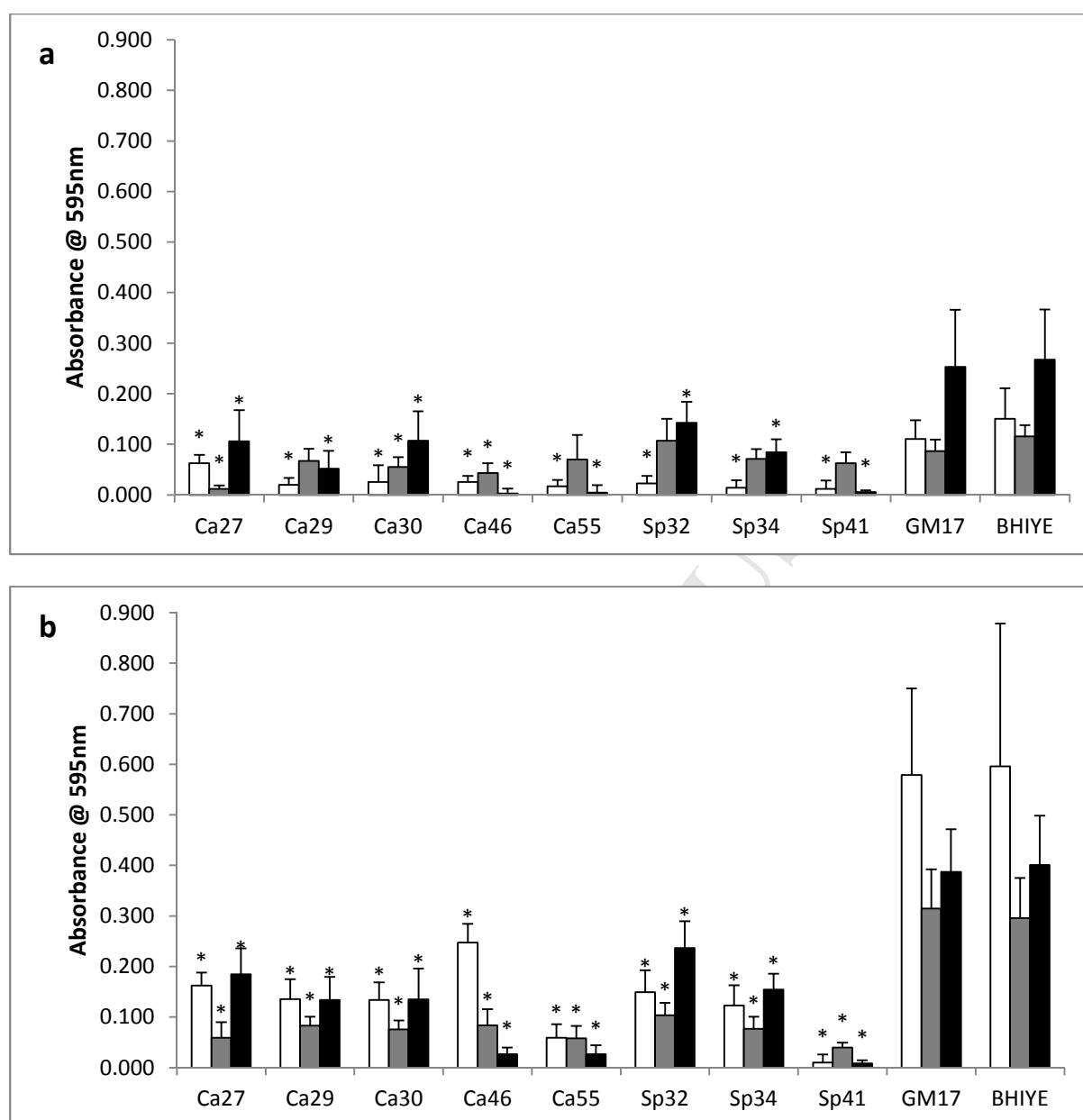CFU: colony forming units; SD: standard deviation

Figure 1: Absorbance values for crystal violet assay for determination of biofilm reduction by LAB CFS on 72h biofilms of three *L. monocytogenes* strains (☐LM 1698, ◼LM 1721 and ◼ LM 2081) formed at 18°C (**a**) and 25°C (**b**). For each graph, columns with an asterisk (*) on top indicate a significant difference (*P*<0.05) between the value from the CFS treatment and its respective GM17 control, for each *L. monocytogenes* strain. A reduction of biofilm is determined as an average value from LAB CFS treated biofilm which is significantly less (*P*<0.05) than the GM17 and BHIYE values.

Highlights

- Lactic acid bacteria are naturally present in the mushroom production environment.
- *Lactococcus lactis* subsp. *lactis* strains isolated from growth substrates had anti-listerial activity.
- Co-culture biofilm experiments with *Lc. lactis* resulted in a four log reduction of *L. monocytogenes*.