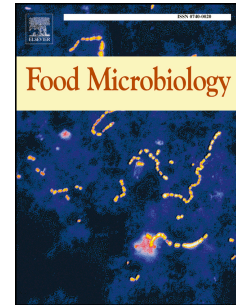


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Lactococcus lactis subsp. *lactis* as a natural anti-listerial agent in the mushroom industry

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

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25

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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52 1 Introduction

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

71 The primary concern in food production and processing environments, including the
72 mushroom industry, is that *L. monocytogenes* biofilms can form on approved materials (e.g.
73 stainless steel, rubber and polymers), including food contact surfaces (Beresford et al.,
74 2001; Bridier et al., 2014; Di Bonaventura et al., 2008). Studies of disinfectants or sanitizing
75 agents that are currently used in the food industry (i.e. quaternary ammonium compounds,
76 chlorine based compounds and oxidising agents) have found that they can reduce biofilm on
77 different surfaces, but they are not consistently effective in dealing with *L. monocytogenes*
78 biofilms, as the reduction levels were dependent on biofilm age, type of surface, and
79 substrate on which the biofilms were formed (Amalaradjou et al., 2009; O'Neill et al., 2015;
80 Pan et al., 2006; Yang et al., 2009). In addition, several studies suggest that listerial biofilms
81 that have been exposed to certain antimicrobials, which did not result in complete removal
82 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 μ l
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>).

174 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.

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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 α s2-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* adheres 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-Vizueté, 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-](http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2012-09-27/eng/1357586653536/1357586653551)
348 [warnings/complete-listing/2012-09-27/eng/1357586653536/1357586653551](http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-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-](http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2014-08-06c/eng/1407377686071/1407377691861)
353 [warnings/complete-listing/2014-08-06c/eng/1407377686071/1407377691861](http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-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). Progressivemaue: 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-](https://www.teagasc.ie/media/website/publications/2015/Use-of-chemical-disinfectants.pdf)
433 [chemical-disinfectants.pdf](https://www.teagasc.ie/media/website/publications/2015/Use-of-chemical-disinfectants.pdf) [Accessed January 1, 2017].
- 434 Olasupo, N. A., Schillinger, U., Narbad, A., Dodd, H., and Holzappel, 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
<i>L. monocytogenes</i> 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:										
<i>Escherichia coli</i> NCTC 9001	Clinical isolate		-	-	-	-	-	-	-	-
<i>Salmonella</i> Typhimurium SARB 67			-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> 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	
	<i>L. monocytogenes</i>	<i>Lc. lactis</i>
<i>L. monocytogenes</i> only	5.1 ± 1.0 ^A	
<i>L. monocytogenes</i> and <i>Lc. lactis</i> co-culture biofilm	1.1 ± 1.2 ^B	6.3 ± 0.6
<i>L. monocytogenes</i> biofilm and <i>Lc. lactis</i> 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

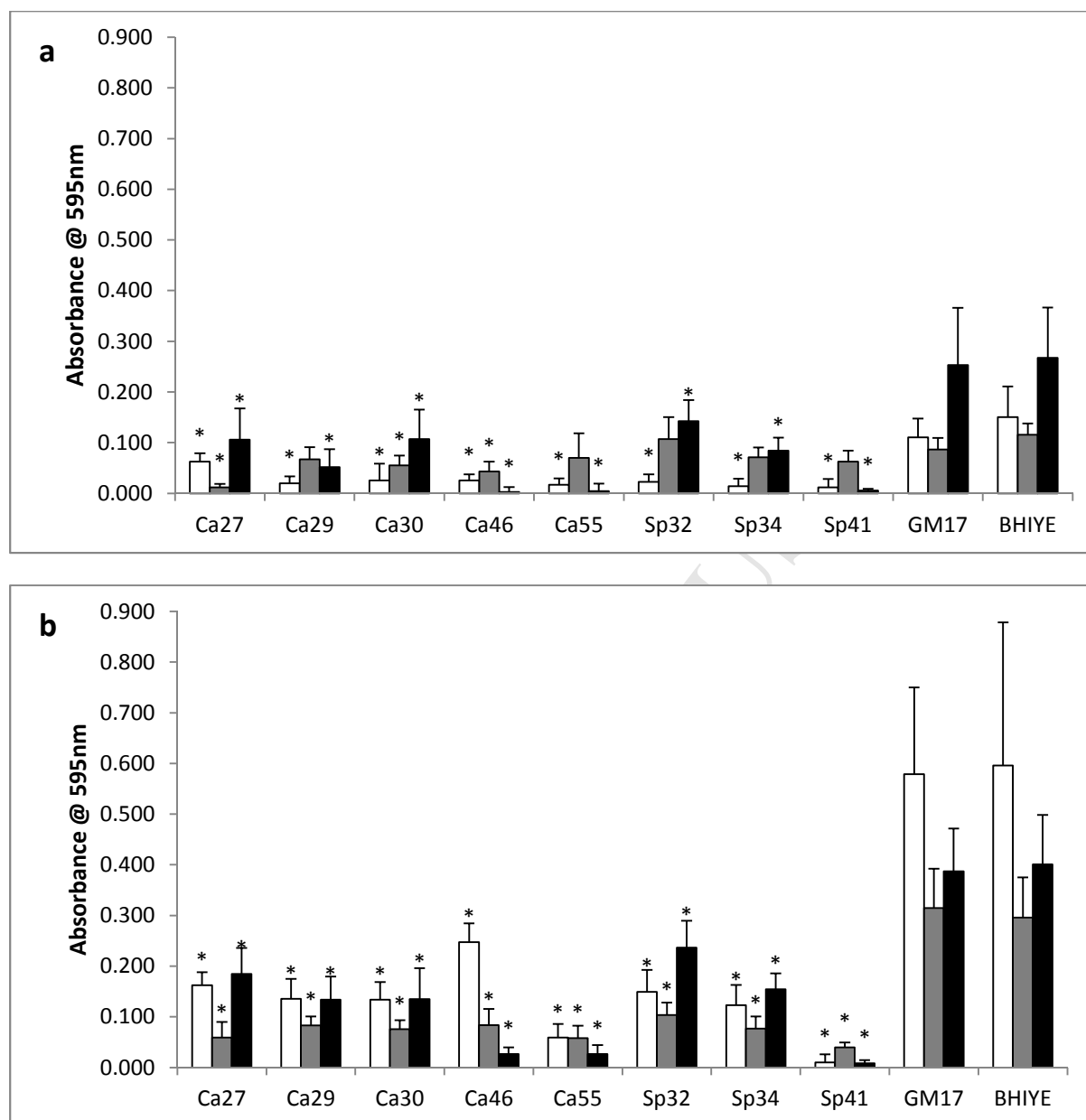


Figure 1: Absorbance values for crystal violet assay for determination of biofilm reduction by LAB CFS on 72h biofilms of three *L. monocytogenes* strains (□ LM 1698, ■ LM 1721 and ■ LM 2081) formed at 18°C (a) and 25°C (b). For each graph, columns with an asterisk (*) on top indicate a significant difference ($P<0.05$) 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*.