Persistent *Listeria monocytogenes* strains isolated from mussel production facilities form more biofilm but are not linked to specific genetic markers

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Abstract

Contamination of mussels with the human pathogen *Listeria monocytogenes* occurs during processing in the factory, possibly from bacteria persisting in the factory's indoor and outdoor areas. In this study, a selection of persistent (n=8) and sporadic (n=8) *Listeria monocytogenes* isolates associated with mussel-processing premises in New Zealand were investigated for their phenotypic and genomic characteristics. To identify traits that favour or contribute to bacterial persistence, biofilm formation, heat resistance, motility and recovery from dry surfaces were compared between persistent and sporadic isolates. All isolates exhibited low biofilm formation at 20°C, however, at 30°C persistent isolates showed significantly higher biofilm formation after 48 h using cell enumeration and near significant difference using the crystal violet assay. All 16 isolates were motile at 20°C and 30°C and motility was fractionally higher for sporadic isolates, but no significant difference was observed. We found persistent isolates tend to exhibit greater recovery after incubation on dry surfaces compared to sporadic isolates. Two of the three most heat-resistant isolates were persistent, while four of five isolates lacking heat resistance were sporadic isolates. Comparison of genome sequences of persistent and sporadic isolates showed that there was no overall clustering of persistent or sporadic isolates, and that differences in prophages and plasmids were not associated with persistence. Our results suggest a link between persistence and biofilm formation, which is most likely multifactorial, combining subtle phenotypic and genotypic differences between isolates.

Keywords: *L. monocytogenes*, persistence, biofilm, heat resistance

42 ## 1. Introduction

43 *Listeria monocytogenes* is a Gram-positive motile rod-shaped bacterium that is commonly
44 encountered in the environment. It is capable of surviving and even growing in harsh environmental
45 conditions, over a wide temperature range, in acidic environments and it also tolerates high
46 osmolarity (Yousef 1999).

47 *L. monocytogenes* is capable of infecting animals as well as humans, causing the bacterial infection
48 listeriosis, which leads to death in up to 30% of the cases (Rocourt et al. 2000). High-risk groups
49 include pregnant women, immunocompromised people and the elderly. *L. monocytogenes* can cross
50 the placental barrier, thereby leading to the abortion of the foetus. It can also cross the blood-brain
51 barrier and/or the intestinal barrier, leading to life-threatening bacterial infections. Common sources
52 of infection are processed food, including raw milk products and ready-to-eat (RTE) chilled products
53 (Allerberger and Wagner 2010). However, unprocessed foods such as fruit and vegetables have also
54 been identified as vectors (Garner and Kathariou 2016, Martinez et al. 2016).

55 Studies showed that contamination of seafood with *L. monocytogenes* usually occurs during
56 processing rather than from raw products in the seafood-processing environment (Autio et al. 1999,
57 Fletcher et al. 1994). The source of contamination therefore consists of either persistent strains
58 harboured within a processing facility or from transient sporadic strains passing through the facility.
59 Bacterial persistence is generally defined as the long-term occurrence of genetically indistinguishable
60 strains isolated from the same environment (Schmitz-Esser et al. 2015).

61 The ability of strains to persist in a food-processing environment has been the topic of two reviews
62 (Carpentier and Cerf 2011, Ferreira et al. 2014), however the reason for persistence remains poorly
63 understood. Persistence of strains in food-processing plants can either be due to genotypic and
64 phenotypic features that facilitate the expression of specific genes and lead to enhanced tolerance
65 towards environmental threats, or to the attachment of bacteria to niches and harbourage sites by

66 chance and subsequent survival due to insufficient cleaning and sanitation procedures (Abee et al.
67 2016).

68 Bacteria persisting in a food-processing plant not only pose a health concern/threat but also present
69 a risk factor for the economy (Ferreira et al. 2014, Ryser and Marth 2007). The ongoing sampling and
70 testing for *L. monocytogenes* in food-processing plants is a huge financial burden. To reduce the risk
71 for consumers and the financial stress for the producer, it is important to identify genetic traits
72 and/or phenotypic behaviour that favours persistence of *L. monocytogenes* within or around a
73 processing facility. Interventions that reduce attachment to surfaces and persistence of bacteria can
74 assist in preventing contamination.

75 During a two-year weekly sampling programme in the New Zealand mussel industry, sporadic and
76 persistent *L. monocytogenes* isolates were collected and further characterised (Cruz and Fletcher
77 2011). Strains were considered persistent if they had been isolated over a time period of 6 months or
78 more from the same processing facility and were genetically indistinguishable by pulsed field gel
79 electrophoresis (PFGE) (Cruz and Fletcher 2011). The current study was designed to compare the
80 phenotypes of such persistent isolates with those of presumed non-persistent isolates isolated from
81 the mussel processing environment.

82 During processing in a typical New Zealand mussel-processing plant, bacterial strains encounter
83 different environmental conditions (Figure 1). After arrival of mussels in the processing factory from
84 aquaculture sites, they are kept in large bags under chilled storage in the bale store (7–8°C). The
85 mussels are transferred to the cook room where they undergo heat treatment (which differs
86 between different plants) to facilitate opening and to inactivate microorganisms. A debyssing process
87 removes the mussel beards before the mussels are shucked manually or by an automated system.
88 The product is then cooled, individually quick frozen and packed for export. There are several steps in
89 the processing chain that may induce persistence of bacteria.

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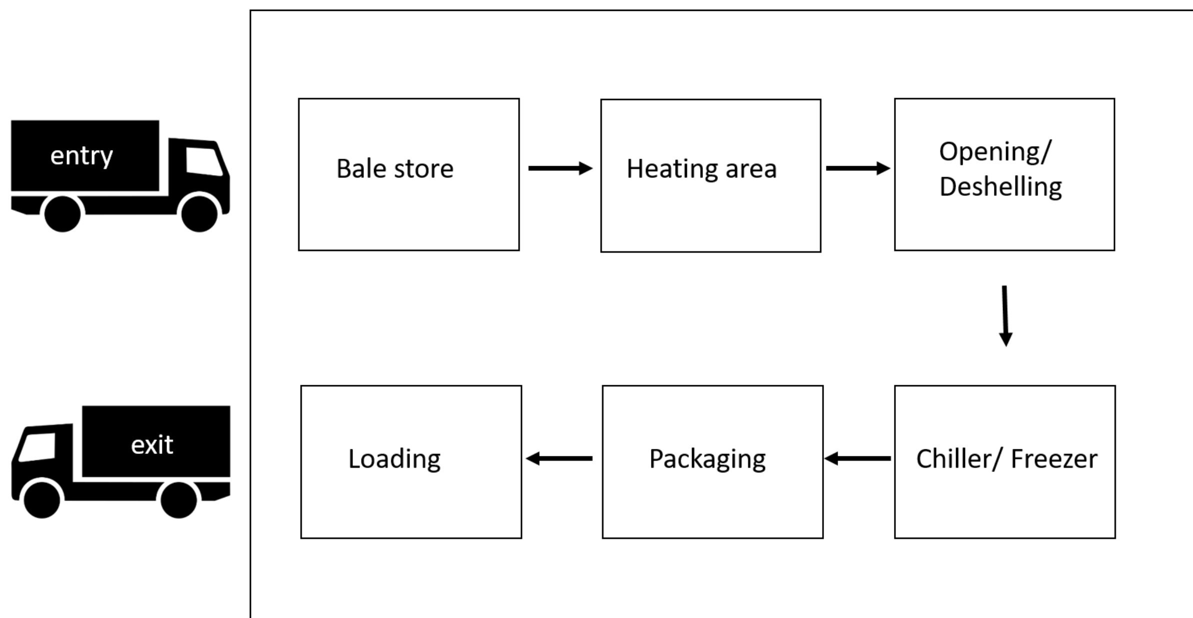

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92 **Figure 1**. Schematic process line for mussel processing. Mussels are stored in bales for up to 6 days, but ideally are
93 processed after 3–4 days. The processing from leaving the bale store (7–8˚C) to final packaging takes about two hours. The
94 heat treatment varies in different plants: One stage heat treatments with temperatures of 76–78˚C are applied for around
95 150 s or a 2-stage heat-shock process with higher temperatures at first (93–96˚C for 22 s) and a subsequent second step
96 with lower temperatures. The packaged product is stored at -18°C after being individually quick frozen ready for export.

97 The relationship between biofilm formation and persistence has been investigated previously
98 (Abdallah et al. 2014, Bonsaglia et al. 2014, Møretrø and Langsrud 2004). The sessile living form has
99 been shown to be the preferred form of existence for many bacteria and it is known that microbial
100 biofilms exhibit higher resistance to cleaning and sanitising (Pan et al. 2006), which may result in
101 bacterial persistence in food processing plants. Biofilm formation is a response to nutrient
102 deprivation and is seen as a stress response (Blaschek et al. 2015). Motility has been linked to biofilm
103 formation: some researchers found that flagella function as an adhesin (Vatanyoopaisarn et al.
104 2000), whereas others suggest flagella are used as a motility factor (Lemon et al. 2007). Regardless of
105 the different findings, flagella seem to play an important role in attachment to surfaces, which is the
106 first step of biofilm formation. Lemon et al. (2007) found that non-motile mutants including flagella-
107 minus mutants and with non-functional (paralyzed) flagella, exhibited impaired biofilm formation at

108 30˚C. Most of the *L. monocytogenes* strains do not express flagella above 30°C (Peel et al. 1988), but
109 are still able to form biofilms, suggesting that there are different mechanisms of attachment for
110 biofilm formation. As most of the food-processing plants and much of a mussel-processing facility
111 operates at temperatures below 30°C, bacterial flagella and motility may influence attachment.

112 Previous research focused on the influence of pH, salinity and resistance to sanitisers to identify
113 features that may lead to bacterial persistence in food-processing plants (Nilsson et al. 2011). The
114 focus of the current study was to identify links between persistent isolates and biofilm formation,
115 motility, survival under dry conditions and heat sensitivity and to identify genetic factors contributing
116 to persistence.

117 2. Materials and Methods

118 2.1 Strains and growth conditions

119 To assess the phenotype of the persistent and sporadic isolates, we selected two representative
120 isolates for four persistent pulsotypes (three identified in a previous study (Cruz and Fletcher 2011)
121 and a fourth pulsotype identified from a fourth factory) , seven sporadic isolates with different
122 pulsotypes (isolated only once over the sampling period of 2 years) and a strain isolated from a food-
123 poisoning outbreak in New Zealand due to contaminated smoked mussels (Brett et al. 1998) (Table
124 1). These strains were chosen from different locations at five different factories at different times in
125 an attempt to capture diversity from those sourced. The selected strains had all been isolated by
126 environmental swabbing either within processing plants or on their factory grounds except for the
127 outbreak strain that had been isolated from the final product. The outbreak strain could be linked to
128 isolates from the environment of the mussel-processing facility by several typing methods (Brett et
129 al. 1998), but is considered sporadic/non-persistent for the present study as the environmental
130 swabs were collected with a big time gap (November 1990 and July 1993). All 16 isolates belonging to
131 the 12 pulsotypes were recovered from -80°C stock and grown on Tryptic Soy Agar enriched with
132 0.6% Yeast Extract (TSAYE) (Difco, NZ) plates. A colony was freshly picked for each experiment.

133 Overnight cultures were usually prepared in Tryptic Soy Broth enriched with 0.6% Yeast Extract

134 (TSBYE) (Difco, NZ) and then used for subsequent experiments.

135 **Table 1**. Strains of *Listeria monocytogenes* used in the experiments

strain	pulsotype	persistent	Plant[1]	isolation	date
15A04	3814	+	A	heating	29/08/07
27A05	3814	+	B	heating	07/09/10
15G01	5132	+	C	external area[2]	07/02/08
16J10	5132	+	B	opening	01/05/08
32C06	5588	+	D	opening	03/06/11
33H04	5588	+	D	heating	01/12/11
15A07	6502	+	A	bale store	06/09/07
31H06	6502	+	A	heating	07/02/11
16A01	3860		outbreak	smoked mussels	??/12/92
15B09	8779		B	opening	07/12/07
15D07	5176		C	heating	19/12/07
15G10	3832		A	packaging	08/02/08
16H02	0101		E	opening	23/04/08
16J08	8981		C	bale store	30/04/08

17A02	7002	A	external area[2]	28/04/08
19B07	3880	C	heating	07/04/09

136 [1] Plants A, B and C are coded as published in Cruz and Fletcher (2011).

137 [2] Strains obtained from areas surrounding the factory (shell, truck park, waste belt roof, entrance
138 doors and floors)

139

140 **2.2 Biofilm formation**

141 An overnight culture (2 µL) grown in TSBYE (Difco) at 37°C was used to inoculate 96-well plates
142 (polystyrene, Greiner, F-Bottom, 655161) filled with 198 µL fresh Brain-Heart-Infusion broth (BHI)
143 (Difco) and incubated statically at 20°C and 30°C for 24 and 48 h, respectively. After incubation,
144 media was removed by inverting plates and wells were washed three times with double-distilled
145 water (ddH_2O). To determine biofilm production the biofilm mass was stained with 200 µL of an
146 aqueous 0.1% crystal violet (BDH) solution and incubated for 45 min at 30°C. After incubation the
147 wells were washed three times with ddH_2O to remove excess dye and then air dried. Bound crystal
148 violet was then destained using 200 µL 96% ethanol (EtOH) per well. The optical density was
149 measured at 595nm with a microplate reader (VersaMax, Molecular Devices). Obtained values were
150 blank corrected and averaged for each isolate. The experiment was performed twice with two
151 replicates.

152 For cell enumeration performed in parallel, the bacteria were grown as above and the wells washed
153 three times with ddH_20 to remove planktonic cells. The sessile cells were detached and resuspended
154 by rigorous pipetting using 500 µL phosphate buffered saline (PBS – NaCl 8.0 g/L, KCl 0.2 g/L,
155 Na_2HPO_4 1.42 g/L, KH_2PO_4 0.24 g/L, pH 7.4). Tenfold dilutions were prepared in PBS and plated on
156 Brain Heart Infusion (BHI) agar plates using the drop plate method (Chen et al. 2003). Colony forming

157 units (CFU) were enumerated after 24 h of incubation at 37°C. The experiment was performed twice
158 with five replicates.

159 **2.3 Motility assay**

160 Motility was tested on semi-solid agar plates composed of BHI broth and 0.3% agar. The plates were
161 inoculated from single colonies using a sterile pick and incubated at 20, 30 and 37°C for 24 h
162 according to a method described previously (Knudsen et al. 2004). Motility was measured using the
163 visible halo around the inoculum. The experiment was performed once with three technical
164 replicates. Results were analysed using analysis of variance (ANOVA) and the variation between the
165 three replicates was used as the residual error.

166 **2.4 Survival of planktonic and biofilm cells on dry surfaces**

167 Survival on dry surfaces was tested as described previously with minor modifications (Castelijn et al.
168 2013). To grow the biofilms, colonies were picked from TSAYE agar plates and used to inoculate 10
169 mL TSBYE and then incubated at 37°C overnight. An overnight culture (2 μL) was used to inoculate
170 96-well plates containing 198 μL BHI per well. The 96-well plates were incubated at 30°C for 48 h and
171 then washed three times with ddH_2O to remove planktonic and loosely attached cells. The plates
172 were air dried under a laminar air flow until fully dried. For the planktonic cultures, 10 mL of TSBYE
173 was inoculated with colonies picked from TSAYE plates and incubated overnight at 37°C. This
174 overnight culture (100 μL) was then used to inoculate 10 mL of BHI and incubated for 24 h at 25°C.
175 Each culture (10 μL, ~10^7CFU) was then pipetted onto 96-well plates and left to air dry under the
176 laminar airflow. Dilutions of planktonic cultures were prepared and plated to determine the cell
177 concentration (Day 0). The 96-well plates were stored at 25°C and viable cells were determined on
178 days 0, 1, 2, 5, 7 and 14. PBS (500 μL, pH 7.4) was used to detach and resuspend the biofilms and
179 planktonic cells from the 96-well plates by rigorous pipetting. Tenfold dilutions were prepared and
180 cell concentration was determined using the drop plate method. Cultures were plated on BHI agar
181 plates and incubated at 37°C for 24 h. The experiment was performed twice with five replicates.

182 Repeat measures analysis of variance was performed, looking for differences between the means for
183 days, persistent versus sporadic isolates and biofilm vs planktonic cultures, allowing for the
184 difference between isolates within those groups. An antedependence model (which allows the
185 variability of the data to be different at different time-points, and estimates the correlation between
186 each time point and the preceding one) was used because observations on a culture/well may have
187 been correlated over time.

188 **2.5 Heat resistance**

189 Overnight culture grown in TSBYE at 37°C (100µL) was used to inoculate 9.9 mL BHI and grown for 24
190 h at 30°C. Heat resistance was determined after treatment of the cultures at 58°C for 5 min.
191 Overnight culture (4 x 1.5 mL) was pipetted in 4 x 2 mL Eppendorf tubes and heat treated. After 5
192 min cultures were allowed to cool down at room temperature for 5 min or 2 h, respectively. Decimal
193 dilutions of untreated (t0) and treated cultures (t5min and t2h) in PBS were plated on BHI agar
194 plates. Agar plates were incubated at 37°C and colonies were enumerated after 24 h of incubation.
195 CFU counts at each time were compared using analysis of variance, with experiment as a block term
196 (random effect) and strain as the treatment factor; the change from time 0 to 5 min, time 0 to 2 h
197 and 5 min to 2 h was also analysed using ANOVA. Furthermore, the results before and 5 min after
198 heat treatment were analysed using a one-tailed t-test.

199 Furthermore, 1.5 mL culture of untreated and heat-treated cells (cooled down for 5 min or 2 h) was
200 centrifuged and resuspended in the same volume of PBS containing the red fluorescent stain
201 propidium iodide (PI – 20 µM). Non-fluorescent PI is excluded from cells with intact membranes and
202 can only enter and bind to DNA in cells with damaged (permeabilised) membranes. The fractions of
203 unstained cells with intact membranes and that of PI-stained cells with (transiently) permeabilised
204 membranes were determined using a BD FACS Aria III flow cytometer (50000 events per
205 measurement).

206 Combining FCM analysis and plate count methods allows analysis of short-term membrane-damaging
207 effects (after 5 min and 2 h recovery) and impact on culturability of cells assayed after 24 h
208 incubation on BHI agar medium.

209 CFU/mL at each time were compared using analysis of variance, with experiment as a block term
210 (random effect) and strain as the treatment factor; the change from time 0 to 5 min, time 0 to 2 h
211 and 5 min to 2 h was also analysed using ANOVA. CFU values were $\log_{10}$ transformed before analysis.
212 Furthermore, the results before and 5 min after heat treatment were analysed using a one-tailed t-
213 test.

214 **2.6 Genome sequencing and analysis**

215 Genomic DNA was isolated from *L. monocytogenes* isolates using the DNeasy Blood & Tissue Kit
216 (Qiagen), and used for genome sequencing at the Earlham Centre, Norwich, UK. Libraries had an
217 average insert size of ~600 bp and were generated using the Illumina NexteraXT kit, according to the
218 manufacturer's instructions (Illumina UK, Cambridge). The libraries were used for high-throughput
219 DNA sequencing using the Illumina MiSeq with 250 nt paired end reads. Reads were QC-ed, trimmed
220 and adaptors removed using standard protocols. Genome sequences were assembled using SPAdes
221 3.6.2 (Bankevich et al. 2012) with assembly statistics generated by Quast v. 2.0 (Gurevich et al. 2013).
222 Genomes were annotated using Prokka v. 1.11b (Seemann 2014). Core genome single nucleotide
223 polymorphisms (SNPs) were identified using the parSNP program v. 1.2 from the Harvest suite
224 (Treangen et al. 2014) with the "-a 13 -c -x" switches, and used for the generation of phylogenetic
225 trees using the default settings of parSNP. Multilocus sequence typing (MLST) was performed *in silico*
226 using the scheme provided by the Institut Pasteur (http://bigsdb.pasteur.fr/listeria/listeria.html),
227 with Figtree (http://tree.bio.ed.ac.uk/software/figtree/) used for annotation of phylogenetic trees.
228 After clustering, genome sequences were compared using Roary (Page et al. 2015) for pangenome
229 analysis with Scoary (Brynildsrud et al. 2016) for statistical evaluation, and Mauve (Darling et al.
230 2004) for direct comparison. PHASTER (Arndt et al. 2016) was used for assessment on whether

231 prophages in the *L. monocytogenes* genomes were intact, questionable or incomplete, using the
232 default settings. The FASTQ reads, isolate information and genome assemblies have been uploaded
233 to the European Nucleotide Archive (ENA) at the EMBL-EBI (http://www.ebi.ac.uk/ena), as project
234 PRJEB19211. Accession numbers for ENA (reads and assemblies) are provided in appendices A3.

235 **3. Results**

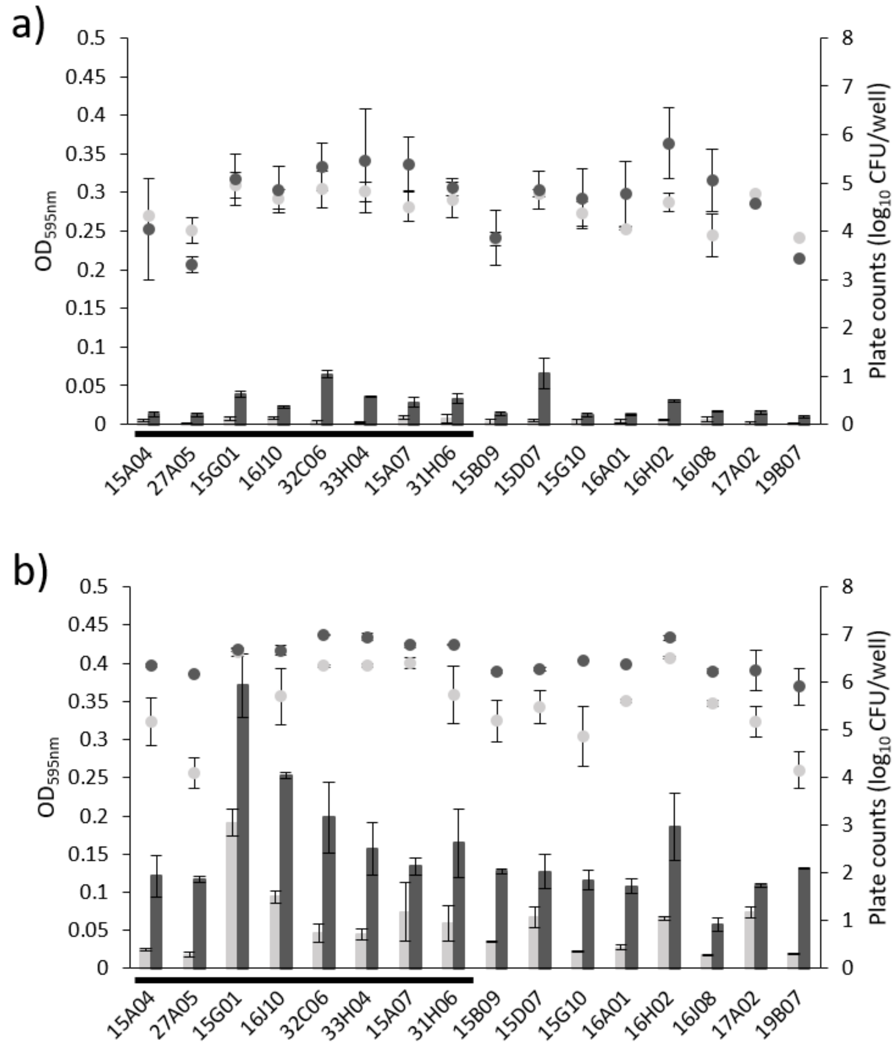
236 **3.1 Persistent isolates form more biofilm**

237 We evaluated the ability of the *L. monocytogenes* isolates to produce biofilm by the crystal violet
238 assay and determined the number of viable biofilm cells by plating. Generally, biofilms are composed
239 of living cells, dead cells and extracellular polymeric substance including carbohydrates, DNA and
240 lipids. Crystal violet attaches to negatively charged surface molecules and determines the whole
241 biofilm mass including all of the above mentioned. Viable biofilm cells are of special risk as they are
242 the ones capable of reproducing and contaminating new surfaces and foods. Combining both
243 methods gives a good indication of *L. monocytogenes* persistent and sporadic isolates biofilm
244 formation capacity and associated contamination risk.

245 Biofilm formation was studied at two different temperatures (20°C and 30°C) after 24h and 48h
246 incubation. At 20°C biofilm mass was higher after 48 h compared to 24h incubation for all isolates
247 (Figure 2a). However, biofilm mass was relatively low with all OD_{595nm} values ≤ 0.066 after 24 h.
248 Viable cell counts ranged between 3.83 and 4.94 log_{10} CFU/well after 24 h incubation and between
249 3.31 and 5.82 log CFU/well for 48 h incubation (Figure 2 a). Prolonged incubation times resulted in a
250 marginal increase of number of viable cells but greater increases in biomass. The average for all
251 measurements after 48 h was 0.28 log CFU/well higher than after 24 h incubation. Log_{10} counts were
252 higher after 24 h incubation compared to 48 h incubation for some isolates, which could be due to
253 increased cell death by 48 h.

254 At 30°C, biofilm mass and cell counts were higher than at 20°C for all isolates tested. The average
255 mean for the cell counts was 0.94 log_{10} CFU/well higher after 48 h incubation compared to 24 h
256 (Figure 2 b). All 16 isolates had more viable cells present in their biofilm after 48 h incubation.

257 Comparing the dataset for the persistent isolates with the sporadic isolates (Appendices A1), ANOVA
258 revealed two differences between persistent and non-persistent isolates; biofilm mass after 48 h
259 incubation at 30°C was higher for persistent isolates (average of 0.19 compared to 0.12; $p = 0.053$),
260 and the number of cells in the biofilm was higher at 30°C and 48 h for persistent isolates (average of
261 6.68 log_{10} CFU/well compared to 6.33; $p = 0.031$). Overall, biofilm formation increased for sporadic
262 and persistent isolates with increasing incubation time and temperature.



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264 **Figure 2.** Biofilm formation measured through crystal violet staining (bars) and plate count cell enumeration (dots) at 20°C
265 (a) and at 30°C (b) after 24 h (light grey bars and dots) and 48 h (dark grey bars and dots) incubation in Brain Heart Infusion
266 Broth. Error bars represent standard deviation of two independent experiments with n= 2 for the crystal violet staining and
267 n=5 for the cell enumeration. Underlined strains are persistent. Isolates of the same pulsotype are found next to each
268 other.

269 ### 3.2 Persistence is not correlated to motility

270 All tested isolates were motile and showed higher motility at 30°C compared to 20°C, which is quite
271 likely simply due to higher growth rate at 30˚C. Six out of 16 isolates were motile at 37°C, with two of
272 the six isolates being persistent. The involvement of flagella in *L. monocytogenes* attachment to
273 surfaces has been thoroughly investigated (Lemon et al. 2007, Todhanakasem and Young 2008,

274 Vatanyoopaisarn et al. 2000). The difference in motility between 20˚C and 30˚C and the interaction
275 (temperature/strain) was significant ($p<0.05$), however, the proportion of variability was
276 comparatively small, indicating the differences were not very large (the 20°C and 30°C results had a
277 correlation of 0.68). The mean motility was higher for sporadic isolates compared to persistent
278 isolates at 20°C and 30°C (Appendices A2). Testing whether there was a difference between
279 persistent and sporadic isolates, against the variation between isolates, indicated no significant main
280 effect ($F = 1.2$ on 1 and 14 df, $p = 0.294$) and no significant persistent x temperature interaction ($F =$
281 1.6 on 1 and 14 df, $p = 0.224$).

282 ### 3.3 Heat resistance might contribute to persistence

283 Heat resistance was examined by exposing all 16 isolates to 58°C heat for 5 min. The short-term
284 effect of heat exposure on the (non)persistent isolates was investigated by determining the fraction
285 of membrane-damaged cells with propidium iodide (PI) using flow cytometry and the long-term
286 effect was measured through cell plating.

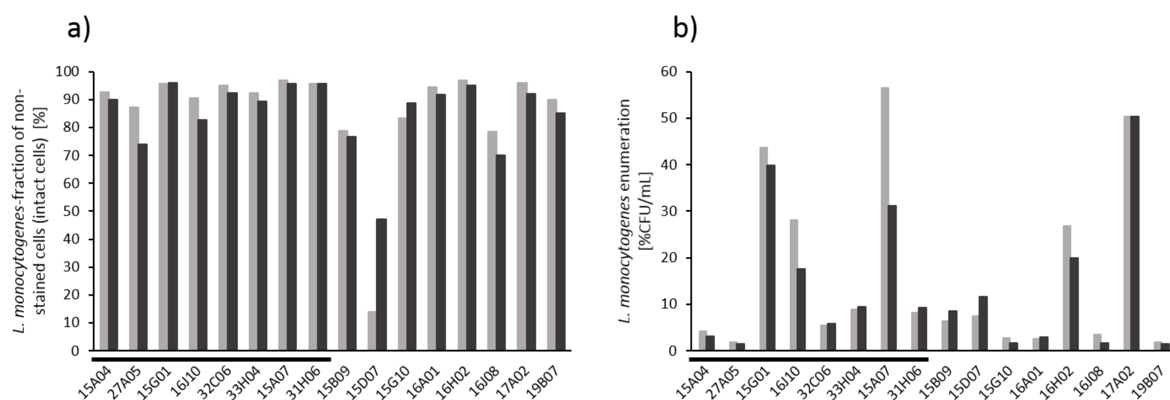
287 Notably, for seven out of eight persistent isolates (all except 27A05), the fraction of non-stained cells
288 indicating intact (non-damaged) membranes assayed 5 min after heat treatment, was higher than 90%,
289 whereas only three out of eight sporadic isolates showed the same effect (Figure 3a). After 2 h only
290 four out of eight persistent isolates and the same three out of eight sporadic isolates showed the same
291 effect.

292 Plate counts were determined at 0, 5 min and 2 h after heat treatment, to determine whether
293 recovery occurs. Heat treatment for 5 min at 58°C led to a decrease in cell numbers for all isolates.
294 Three isolates (15G01, 15A07, 17A02) showed highest heat resistance with the surviving fraction
295 after 5 min being approximately 43.7 %, 56.5 %, and 50.3 %, respectively (Figure 3). The first two of
296 the three belong to the persistent group. The sporadic isolate 16H02 and the persistent isolate 16J10
297 showed a heat resistance of 26.78% and 28.02%, respectively. All the remaining isolates exhibited
298 survival below 10% of the original population after heat treatment (Figure 3).

299 The results showed that there was no significant difference (Figure 3) (p=0.232, ANOVA), confirming
300 that none of the isolates showed ability to recover from heat stress 2 h post treatment. The
301 difference between 0 and 5 min was significant for 12 of the 16 examined isolates (p=0.005, one-
302 tailed t-test). Four isolates showed no significant decrease in cell numbers 5 min after heat
303 treatment, hence are more heat resistant. Three (15G01, 16J10, 15A07) of the four isolates were
304 persistent.

305 The three most heat-resistant isolates (15A07, 15G01 and 17A02) were isolated from either samples
306 within the bale storage or from the external area, so had not been exposed to any heat prior.
307 However, they could represent contamination of these areas from strains present within the plant.
308 The other two isolates that exhibited higher heat resistance were isolated in the opening area just
309 after the heating step so may have gone through heat treatment if introduced before the opening
310 step, but they also might have been introduced from outside sources.

311 Flow cytometry assesses recovery based on staining damaged cells, whereas cell plating measures
312 viable culturable cells. The high percentage of intact cells after heat treatment measured through
313 flow cytometry and the lower levels of recovered cells measured through plating suggest that
314 significant irreversible damage occurs such as cell replication stalling.



315

316 **Figure 3.** *Listeria monocytogenes* enumerated 5 min and 2 h after being heat treated at 58°C for 5 min expressed as fraction
317 of non-damaged cells for flow cytometry (a) (grey bars showing results for 5 min and black bars for 2 h after heat treatment)

318 and expressed as percentage of those present before the heat treatment for cell plating (b) (CFU/mL, grey bars 5 min after
319 heat treatment and black bars 2 h after heat treatment). Persistent strains are underlined.

320

321 **3.4 Survival under dry conditions**

322 Two experiments were run, testing 16 isolates comparing numbers of biofilm cells versus planktonic
323 cells on dry surfaces over a period of 14 days. Colony forming units (CFU) were counted at days 0, 1,
324 2, 5, 7 and 14.

325 Cell numbers decreased sharply on Day 1 compared to the initial concentration (Table 2). The mean
326 reduction for planktonic cells was 1.93 log_{10} CFU/well and 1.83 log_{10} CFU/well for biofilm cells after 1
327 day. Cell numbers decreased continuously over subsequent time periods.

328 CFU/mL were higher for planktonic cultures (Table 2) due to methodology so the changes from Day 0
329 were analysed for all groups. There was a significant change over time ($p<0.001$); the persistent
330 isolates tended to survive better than the sporadic isolates ($p=0.005$); and the time pattern was
331 different from planktonic and biofilm samples (the biofilm cells showed a steady decline; the
332 planktonic cells had a steep drop at day 5, but beyond that declined faster than the biofilms;
333 $p=0.067$) (Table 2). Planktonic cultures of all isolates tested showed reductions between 3.01–5.29
334 log_{10} CFU/well after 14 d incubation on dry surfaces, whereas biofilm cells exhibited reductions
335 ranging between 2.57–5.05 log_{10} CFU/well after 14 d. Comparing persistent versus sporadic isolates,
336 the planktonic cultures of the sporadic isolates showed highest reductions (3.76–5.29 log_{10} CFU/well
337 after 14 d) and biofilm cells of the persistent isolates showed lowest reductions (2.57–4.12 log_{10}
338 CFU/well) (Table 4).

339

340 **Table 2.** Enumeration of planktonic and biofilm cells of persistent and sporadic *L. monocytogenes* isolates on dry surfaces

341 after 1, 2, 5, 7 and 14 d incubation at 25°C

Days on dry surface	**persistent**				**sporadic**			
	planktonic		biofilm		planktonic		biofilm	
	Log_{10} CFU/well	SD	Log_{10} CFU/well	SD	Log_{10} CFU/well	SD	Log_{10} CFU/well	SD
0	7.364	0.142	5.996	0.838	7.263	0.148	6.140	0.640
1	5.587	0.703	4.457	0.808	5.185	0.489	4.014	0.767
2	5.350	0.540	4.078	0.573	4.949	0.429	3.299	0.824
5	5.005	0.499	3.512	0.597	4.633	0.454	3.103	0.770
7	3.950	0.466	3.161	0.618	3.636	0.439	2.746	0.519
14	3.459	0.676	2.805	0.678	2.836	0.697	2.274	0.977

342

343 **3.5 The two persistent isolates of each pulsotype behaved similarly**

344 Two representatives for each persistent pulsotype (Table 1) were subjected to the phenotype
345 analyses. Most phenotypic tests performed showed similar outcomes for the representatives of each
346 pulsotype with the occasional slight difference (Figures 2 and 3). This supports the use of PFGE typing
347 as a method of selecting persistent strains. The exception was 15A07 which had higher heat
348 resistance by the plate count method than 31H06. This was surprising in that 31H06 was isolated
349 from the heating area whereas 15A07 was from the bale store.

350 The selected representatives of pulsotype 3814 were isolated from two different processing plants,
351 and this pulsotype was also isolated from a third factory (Cruz and Fletcher 2011), suggesting that the
352 strain has been introduced from a common source, e.g., raw material. Both selected strains had been
353 isolated from the heating areas (outlined in Figure 1) as had most (13) isolates of this pulsotype (Cruz

354 and Fletcher 2011). However, both tested isolates were rather less heat resistant compared to the
355 other persistent strains (Figure 3). This pulsotype was widely distributed in factories with three
356 isolated from an external area, two from bale stores, one from an opening area and two from a
357 packing area (Cruz and Fletcher 2011).

358 The selected representatives of pulsotype 5132 were isolated in the opening area and the external
359 area of the same plant. Strains of this pulsotype had been regularly isolated in these areas (four
360 isolates from the external area and 15 from the opening area) but was also found in the packing area
361 of the plant (five isolates) and the opening area (two isolates) and in product (one isolate) from
362 another nearby plant (Cruz and Fletcher 2011). This strain might have been introduced from the
363 outside environment possibly through the raw product or bags for mussel storage. It is likely that this
364 strain made it through the processing line into the opening and packing areas (Figure 1) surviving the
365 heat treatment, suggesting high heat resistance. Both isolates belonging to pulsotype 5132 exhibited
366 high viable cell numbers after heat treatment (two of the three highest heat-resistant strains
367 determined by plating).

368 The two selected isolates belonging to pulsotype 6502 were isolated from the bale storage and the
369 opening area of the same plant and eight other isolates with the same pulsotype had been obtained
370 in these locations but none from the other plants (Cruz and Fletcher 2011). This suggests
371 introduction from an outside source from the environment.

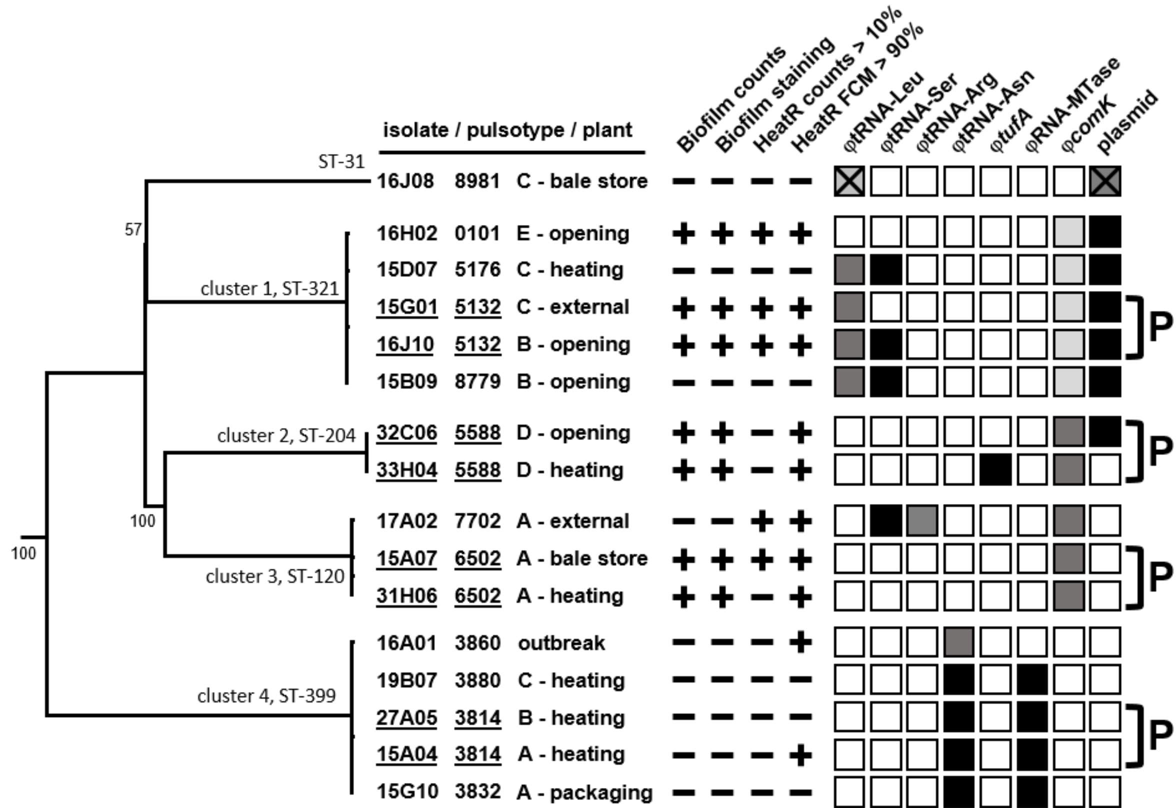
372 The two representatives of the pulsotype 5588 were isolated from the same factory in the opening
373 area and heating area and the pulsotype has been found in numerous other locations in that factory
374 including product, suggesting survival of the isolates through heat treatment and contaminating the
375 opening area.

376 **3.6 Comparative genomics of persistent and sporadic *L. monocytogenes* isolates**

377 The draft genome sequences of the eight persistent and eight sporadic isolates were determined
378 using Illumina sequencing technology with 250 nt paired end reads. The genome sizes varied
379 between 2.9–3.3 Mbp, consistent with previously released *L. monocytogenes* genomes. The
380 phylogenetic relationship between the 16 genomes was analysed using phylogenomic clustering
381 based on core genome single nucleotide polymorphisms (SNPs) (Figure 4). The 16 isolates were all
382 lineage II genomes, and clustered into four distinct clusters of five, two, three and five genomes,
383 respectively (labelled cluster 1–4). Isolate 16J08 branched separately from the other samples.
384 Clusters 1, 3 and 4 contained both persistent and sporadic isolates, with cluster 2 only containing
385 persistent isolates. The genomes within the clusters showed virtually no differences in core genome
386 SNPs, shown by the lack of further sub-branching within the clusters. The clusters each consisted of
387 genomes with a separate MLST sequence type, with cluster 1 consisting of ST-321, cluster 2 of ST-
388 204, cluster 3 of ST-120 and cluster 4 of ST-399, with 16J08 being ST-31.
389 All genomes were provisionally annotated using Prokka (Seemann 2014), and pangenome analysis
390 using Roary (Page et al. 2015) was used to search for genes or markers associated with persistence or
391 non-persistence within the 16 genomes, or markers associated with specific factories from which the
392 isolates were obtained. We were unable to detect such markers for either group, consistent with
393 previous reports where persistence and virulence of *L. monocytogenes* could not be linked to specific
394 genetic markers (Fagerlund et al. 2016, Fang et al. 2016, Stasiewicz et al. 2015). As there were
395 differences in pulsotypes within the four clusters, we used a combination of comparative genomics
396 utilities to identify the genomic changes that may account for the differences in pulsotypes between
397 the genomes within each cluster, using the multiple genome aligner Mauve (Darling et al. 2004). The
398 ST-321 genomes in cluster 1 were from two persistent isolates and three sporadic isolates, and
399 isolated from factories B, C and E.
400 The primary differences were in mobile genetic elements, with isolate 16H02 and 15G01 lacking a
401 ϕtRNA-Ser prophage, 16H02 lacking a ϕtRNA-Leu prophage, and 16H02 having a transposon with a
402 restriction-modification system lacking in the other four genomes (Fagerlund et al. 2016) (not shown

403 in Figure 4). The two ST-204 isolates from Cluster 2 were virtually identical, both persistent and came
404 from the same factory (D), but isolate 33H04 carries a ϕ*tufA* prophage, while isolate 32C06 contains
405 plasmid contigs also found in cluster 1. The ST-120 isolates from cluster 3 all came from factory A,
406 with two out of three isolates persistent (15A07 and 31H08), and differed from sporadic isolate
407 17A02 by lacking ϕtRNA-Ser and ϕtRNA-Arg prophages, as well as differences in the specificity
408 subunits of a *hsd* Type I restriction modification system (not shown), and having an inactivated gene
409 encoding a putative surface-located protein (not shown). Finally, the ST-399 genomes in cluster 4
410 included isolate 16A01 from an outbreak in 1992, and this genome lacked a ϕRNA-methyltransferase
411 prophage, and the ϕtRNA-Asn prophage in 16H02 was scored as questionable (i.e. possibly
412 incomplete) while it scored as intact in the four other isolates. When compared to the pulsotypes of
413 the isolates, the differences in these 30–50 kb prophages could in part explain differences in
414 pulsotypes, for instance in cluster 3, but not in clusters 1, 2 and 4.
415

416



417 **Figure 4.** Whole-genome comparison of persistent and non-persistent *L. monocytogenes* isolates demonstrates persistence
418 is not associated with a specific genetic lineage, and does not allow identification of genetic markers contributing to
419 persistence in the isolates investigated. The phylogenetic tree on the left is based on core genome single nucleotide
420 polymorphisms identified with the ParSNP program, with bootstrap values indicated at the branching points. The code of
421 the isolates, pulsotypes and the plant where they were isolated and the location are indicated, with underlined isolate
422 codes indicating persistent isolates. The clusters and the MLST sequence type are indicated in the tree, demonstrating that
423 persistence is not associated with a specific genetic lineage. The plus and minus symbols indicate level of biofilm formation
424 based on viable counts and crystal violet staining, whereas HeatR represents heat resistance, and FCM flow cytometry. Six
425 of the eight persistent isolates showed high results in at least three of the four shown tests whereas the sporadic isolates
426 exhibited lower biofilm formation and heat resistance. The sporadic isolate 16H02 was an outlier and might be in the
427 process of becoming persistent. The boxes on the right indicate differentially present mobile genetic elements (prophages
428 and putative plasmids) in these isolates. The φ code indicates the genomic locus where the prophage is inserted. The
429 shading of the prophage boxes indicates whether it is an intact (black), questionable (dark grey) or incomplete prophage
430 (light grey), based on the analysis performed using PHASTER. The crossed boxes with isolate 16J08 highlight that although
431 16J08 does have a prophage at the tRNA-Leu locus, it differs from the prophages of cluster 1 in the same location. Similarly,
432 the putative plasmid-contigs show significant sequence difference with those of clusters 1 and isolate 32C06 of cluster 2.

433 There were no prophages or plasmids specifically associated with persistence. Minor genetic changes were observed (not
434 shown), but again did not correlate with persistence, but only with genetic background/clusters.
435

436

437 **4. Discussion**

438 Persistence of pathogenic bacteria in a food-processing environment causes a health risk for the
439 consumer of the food products and a high financial risk for the producer, thus constituting both a
440 public health and economic concern. Identification of phenotypic and/or genotypic features of
441 pathogenic bacteria that favour persistence have been the focus of several research groups across
442 the world. In this study, we analysed phenotypic and genotypic features of *L. monocytogenes* to
443 identify differences between sporadic and persistent strains isolated from a mussel-processing
444 environment to determine key factors that might contribute to persistence in food-processing plants.

445 In this study eight persistent strains isolated from four different factories and eight sporadic strains
446 from four different sites (three factories identical with the persistent isolation) including one
447 outbreak isolate were subject to phenotypic assays and genomic analysis.

448 We observed that biofilm formation was significantly higher at 30°C compared to 20°C for persistent
449 isolates. In a previous study by Cruz and Fletcher (2011), where some of the same isolates used in
450 this study were investigated, persistence could not be linked to biofilm formation using TSBYE
451 medium (Cruz and Fletcher 2011). However,in the current study BHI medium was used, as it is a
452 commonly used medium for *L. monocytogenes* studies and a nutrient-rich medium that was
453 previously shown to promote *L. monocytogenes* biofilm formation (Nowak et al. 2015). Differences in
454 biofilm formation might be due to nutrient availability and composition as discussed in our previous
455 work (Nowak et al. 2015) and others (Kadam et al. 2013).

456 A recent study focused on growth behaviour of 31 persistent (repetitive isolation over 4–5 years for
457 small-scale industrial cheese producer and 8–15 months artisanal raw ewes' milk cheese producer)

458 and 10 sporadic *L. monocytogenes* strains at different temperatures (4°C, 22°C, 37°C) (Magalhaes et
459 al. 2016). Average growth rates for persistent strains were found to be higher at 22°C compared to
460 sporadic strains, which highlights the fact that certain temperatures (22°C) may favour bacterial
461 persistence.

462 In the present study persistent isolates were found to form significantly higher amounts of biofilm at
463 30˚C after 48 h incubation determined by plate counts. Nakamura et al. (2013) analysed biofilm
464 formation of persistent and transient strains at 32°C with the crystal violet assay and viable cell
465 counts using Modified Welshimer's Broth (MWB) as the medium (Nakamura et al. 2013). They found
466 that persistent strains formed significantly more biofilm than transient strains after 48 h incubation
467 and that there was no significant difference after 24h incubation. Viable cell counts were not
468 significantly different for persistent and transient strains in Nakamura's study, however, we noted
469 significant higher viable cell counts after 48h incubation for persistent strains. Furthermore, Borucki
470 et al. (2003) found that persistent strains (n=11) form more biofilm than sporadic strains (n=15)
471 (p=0.027) after 40 h incubation at 30°C determined by the crystal violet assay in MWB (Borucki et al.
472 2003). Ochiai et al. (2014) investigated biofilm forming ability of persistent strains at 30°C and 37°C
473 using the crystal violet assay and showed that persistent strains did not form more biofilm at 30°C,
474 but at 37°C (Ochiai et al. 2014).

475 However, some other research groups found no difference between biofilm-forming ability of
476 persistent and sporadic strains which might be due to differences in media and growth conditions as
477 discussed, next to strain variability (Djordjevic et al. 2002, Nilsson et al. 2011)

478 In the present study we evaluated the recovery of persistent and sporadic isolates from dry surfaces.
479 We observed reduction in cell numbers with increasing incubation for persistent and sporadic
480 isolates. Planktonic cultures showed a reduction of 3.01–5.29 $\log_{10}$ CFU/ well after 14 d. Survival of
481 planktonic cells of *L. monocytogenes* on dry surfaces (coated and uncoated stainless steel coupons)
482 has been tested previously (Møretrø et al. 2013, Takahashi et al. 2011). Møretrø et al. (2013) found

483 that there was a 1.5–2 $\log_{10}$ reduction per coupon for bacteria after 7 and 14 days, when incubated
484 at 12°C. Takahashi et al. (2011) coated coupons with a sterile food filtrate (minced tuna, cabbage or
485 ground pork) and tested survival of *L. monocytogenes* Scott A at 25°C compared to survival on
486 uncoated stainless steel coupons. After 15 d, cell numbers reduced from an initial concentration of
487 10^7 CFU/coupon to ~3 $\log_{10}$ CFU/coupon on uncoated coupons, which is consistent with our findings.
488 On coated coupons cell numbers reduced to ~3.5-5.5 $\log_{10}$ CFU/coupon after 15 d and after 30 d to
489 2.32-3.7 $\log_{10}$ CFU/coupon. Castelijn et al. (2013) examined survival of *Salmonella* spp. on dry
490 surfaces and observed that survival of biofilm cells is higher than survival of the planktonic
491 counterpart. We observed a difference in the decline pattern between planktonic and biofilm cells,
492 however, this difference was not significant. In our study we observed higher reduction of $\log_{10}$
493 CFU/well for planktonic cultures. Survival on dry surfaces has also been tested for *Acinetobacter*
494 *baumannii* where biofilm-forming and non-biofilm-forming strains were compared (Espinal et al.
495 2012). Biofilm-forming strains survived longer than the non-biofilm formers in dry conditions (36
496 days versus 15 days). We observed a reduction in cell numbers over time for all isolates tested with
497 the persistent strains showing better survival than the sporadic strains. Biofilm cells showed a steady
498 decline in cell numbers up until day 14 whereas planktonic cells had a sharp decline from day five to
499 seven. Bacterial cells tend to settle down in niches and harbourage sites that are hard to reach and
500 therefore hard to eradicate. Once attached, the bacteria can go unnoticed for an unknown
501 timeframe. It could be considered to test survival after longer incubation time (up to 30 d), however,
502 previous studies could not observe a further reduction from 15 d to 30 d (Takahashi et al. 2011).

503 In the present study we also investigated heat resistance but there was no consistent difference
504 between persistent and sporadic isolates observed after heat treatment at 58°C for 5 min although
505 two of the three most heat resistant isolates were classified as persistent. Lunden et al. (2008)
506 analysed 17 persistent and 23 non-persistent strains for heat resistance and found no significant
507 difference in survival of persistent and sporadic strains (55°C, 40 min), which is consistent with our
508 findings. In the present study heat resistance was investigated at 58˚C in a model setting to evaluate

509 overall robustness of the sporadic versus the persistent isolates. Majority of the isolates had been
510 sampled after heating stage of the processing line. Heat treatment at a sub-lethal level might
511 encourage cross-protection, which could result in higher resistance towards other environmental
512 stresses, including sanitisers and cleaning reagents. Cross-protection has been reported for several
513 bacterial species, where bacterial cells become more resistant to different environmental stresses
514 after being exposed to another stress, e.g. higher heat resistance after alkali stress exposure for
515 *Salmonella enteritidi*s (Inagaki et al. 2009) and *Enterococcus faecalis*. *L. monocytogenes* also showed
516 increased H_2O_2 resistance after exposure to salt stress (Bergholz et al. 2012) and exhibited higher
517 heat resistance after exposure to other environmental stresses (Lou and Yousef 1996).

518 We also used genome sequencing to assess whether there were any genetic markers that could
519 distinguish persistent and sporadic isolates used in this study. The 16 isolates used here were from
520 five different MLST sequence types, with four of the five sequence types containing both persistent
521 and sporadic isolates. While the isolates within each sequence type contained only a few core
522 genome SNPs which did not lead to major differences in genes predicted, there were differences in
523 the accessory genome, especially mobile genetic elements such as prophages and plasmids (Figure
524 4). As the prophages and plasmids are relatively large fragments (30–50 kb of sequence each), they
525 could be large enough to affect the PFGE banding pattern and hence result in a different pulsotype.
526 However, there was no clear association between the prophages/plasmids with persistence or
527 pulsotype observed (Figure 4). This is consistent with several recent studies on genomics of *L.*
528 *monocytogenes*, where significant variations in prophages have been observed, but were not linked
529 to specific phenotypes (Fagerlund et al. 2016, Fang et al. 2016, Stasiewicz et al. 2015). This strongly
530 suggests that the phenotypic differences are not caused by single mutations, phages or other genetic
531 differences, but could be unique to distinct *L. monocytogenes* lineages, or multifactorial combining
532 phenotypic stimuli and genetic background.

533 In food production plants such as mussel-processing facilities, products undergo several treatment
534 steps before being packed for export including washing and cooling as well as heating steps. As we

535 could isolate persistent strains from different processing facilities showing high biofilm formation
536 capacity and high numbers of viable biofilm cells, higher survival on drying surfaces and additional
537 higher heat resistance in some cases, it is crucial to critically evaluate and implement adequate
538 cleaning procedures to eliminate the risk for product contamination by persistent *L. monocytogenes*
539 strains.

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544 ## 6. Appendices

545 **A 1**. Means and standard deviations (SD) of biofilm formation of the persistent and sporadic *L. monocytogenes* strains
546 measured by the crystal-violet assay and cell enumeration at 20°C and 30°C after 24 and 48 h

			Crystal violet assay		**Cell enumeration**	
	Temperature (°C)	incubation (h)	OD_{595nm}	SD	Log_{10} CFU/well	SD
persistent n=8	20	24	0.005	0.003	4.597	0.311
		48	0.031	0.017	4.798	0.749
	30	24	0.069	0.055	5.809	0.839
		48	0.190	0.086	6.676	0.284
sporadic n=8	20	24	0.004	0.002	4.273	0.407
		48	0.022	0.019	4.628	0.727
	30	24	0.041	0.024	5.323	0.676
		48	0.120	0.036	6.332	0.288

547

548 **A 2**. Motility of persistent and sporadic *L. monocytogenes* strains after 24h incubation at 20°C and 30°C

Motility	**20°C**		**30°C**	
	halo [mm]	SD	halo [mm]	SD
persistent	4.05	0.63	7.70	3.07
sporadic	4.27	0.71	9.10	2.09

549

550 **A 3.** Accession numbers for genome assemblies and Illumina paired end sequencing reads. The sequencing reads and genome assemblies have been deposited in the European Nucleotide Archive

551 (ENA) as part of BioProject PRJEB19211.

BioProject	isolate	Sample code	Sequencing reads	Assembly	Contigs	Persistence
PRJEB19211	15A04	LDI10680	ERR1816990	GCA_900162275	FUIK01000001-FUIK01000018	Persistent
PRJEB19211	27A05	LDI10675	ERR1816985	GCA_900162135	FUJP01000001-FUJP01000018	Persistent
PRJEB19211	15G01	LDI10683	ERR1816993	GCA_900162555	FUIO01000001-FUIO01000023	Persistent
PRJEB19211	16J10	LDI14780	ERR1817086	GCA_900162285	FUJD01000001-FUJD01000027	Persistent
PRJEB19211	32C06	LDI10677	ERR1816987	GCA_900162495	FUKF01000001-FUKF01000018	Persistent
PRJEB19211	33H04	LDI10679	ERR1816989	GCA_900162345	FUKA01000001-FUKA01000023	Persistent
PRJEB19211	15A07	LDI14788	ERR1817093	GCA_900162245	FUIL01000001-FUIL01000028	Persistent
PRJEB19211	31H06	LDI14777	ERR1817095	GCA_900162415	FUKE01000001-FUKE01000028	Persistent
PRJEB19211	16A01	LDI10686	ERR1816996	GCA_900162565	FUIW01000001-FUIW01000017	Non-persistent

PRJEB19211	15B09	LDI10681	ERR1816991	GCA_900162295	FUIJ01000001-FUIJ01000027	Non-persistent
PRJEB19211	15D07	LDI10682	ERR1816992	GCA_900162155	FUIN01000001-FUIN01000026	Non-persistent
PRJEB19211	15G10	LDI10684	ERR1816994	GCA_900162115	FUIU01000001-FUIU01000018	Non-persistent
PRJEB19211	16H02	LDI10687	ERR1816997	GCA_900162165	FUIV01000001-FUIV01000024	Non-persistent
PRJEB19211	16J08	LDI14782	ERR1817088	GCA_900162445	FUIR01000001-FUIR01000048	Non-persistent
PRJEB19211	17A02	LDI10689	ERR1816999	GCA_900162215	FUJA01000001-FUJA01000040	Non-persistent
PRJEB19211	19B07	LDI14766	ERR1817005	GCA_900162375	FUJI01000001-FUJI01000016	Non-persistent

552

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