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Prevalence and persistence of *Listeria monocytogenes* in premises and products of small food business operators in Northern Ireland

1

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18 **processing.**

19

20 **Running title:** ***Listeria monocytogenes* in SMEs in Northern Ireland**

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21 **Abstract**

22

23 Listeriosis is a foodborne disease, with a high mortality rate, that predominantly effects the elderly.
24 Under European Union legislation, EC 2073/2005, food business operators are encouraged to
25 undertake sampling to ensure that the food processing environment, and required to ensure that food
26 products, are free of *Listeria monocytogenes*. To determine the prevalence of *L. monocytogenes* in
27 smaller food processing facilities in Northern Ireland, 24 companies submitted six processing
28 environment swabs and two food samples every two months for eighteen months (July 2015 to
29 November 2016) for *L. monocytogenes* examination. The prevalence of *L. monocytogenes* was 4.6%
30 in food samples, and 6.3% in processing environment swabs. Over the duration of the study, 96
31 isolates of *L. monocytogenes* were obtained, one from each positive sample, except for two meat
32 samples that had >100 cfu/g, where two isolates were obtained from each sample. No seasonality in
33 occurrence of *L. monocytogenes* was seen for food isolates but significantly higher numbers of
34 positive processing environment swabs were found in the warmer months of May, July and
35 September ($p = 0.007$). Pulsed Field Gel Electrophoresis (PFGE) analysis revealed the presence of 27
36 pulsotypes; 9 pulsotypes were shared between different facilities and 9 were persistent. Based on a
37 Combase predictive growth model, 77.5% (n=130) of the foods tested were predicted to support the
38 growth of *L. monocytogenes*. All of the isolates carried the pathogenicity genes *inl*A and *act*A and
39 71.4% carried *qac*H, which confers resistance to quaternary ammonium compounds which are
40 frequently used in sanitizers. Whole genome sequencing of the isolates allowed multi-locus sequence
41 typing to be undertaken. The data indicated that the sequence types identified included those with
42 disease-causing ability, highlighting the disease-causing potential of the isolates.

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45 **1. Introduction**

46

47 Clinical invasive infection by *Listeria monocytogenes*, listeriosis, is rare in healthy humans.
48 However, there are subsections of the population that are vulnerable to invasive infection, including
49 the immuno-compromised, the elderly and pregnant women (CDC, 2017). From a public health
50 perspective, finding ways to reduce exposure of vulnerable consumers to *L. monocytogenes* in ready-
51 to-eat (RTE) foods is important. Despite this, the number of cases of listeriosis reported in the EU
52 has increased. In 2015, the number of confirmed human cases of listeriosis reported in the European
53 Union was 2,206 (0.46 cases per 100,000 population), which was similar to 2014. In 2015, nineteen
54 member states reported 270 deaths due to listeriosis, which was the highest annual number of deaths
55 reported since 2008 (EFSA & ECDC, 2016). In a systematic review of the literature, De Noordhout
56 et al. (2014) estimated the case fatality rate was 23.5%. The susceptibility of older people is of
57 special concern in the UK due to its ageing population (Harper, 2016).

58 Since *L. monocytogenes* is a ubiquitous environmental bacterium (Farber & Peterkin, 1991;
59 Hellberg & Chu, 2016; Montero et al., 2015), food processing environments are at a continuous risk
60 of colonisation by *L. monocytogenes*. For many RTE products the main, but not sole contamination
61 mechanism, is by transfer of *L. monocytogenes* strains from raw materials into niches in the plant
62 environment and subsequent transfer from these niches into final products (Tompkin, 2002). Such
63 cross-contamination has been shown in many studies (Bolocan et al., 2015; Leong et al., 2017;
64 Muhterem-Uyar et al., 2015; Stessl et al., 2014), although cross-contamination from the food to the
65 processing environment cannot be ruled out. Effective cleaning (the removal of soil) followed by
66 sanitising (the destruction of microorganisms), or the application of heat can remove
67 *L. monocytogenes* from processing environments (Murugesan, Kucerova, Knabel, & Laborde, 2015;
68 Zottola, 1994). However, any failings in these procedures can result in contamination of products that
69 have been rendered *Listeria*-free by the critical control point of cooking (Currie et al., 2015; Leong et

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70 al., 2017; Swaminathan & Gerner-Smidt, 2007). Accordingly, the European Union has legislated to
71 ensure the safety of RTE products that support the growth of *L. monocytogenes* (EU, 2005). For
72 foods supporting growth of *L. monocytogenes* (apart from foods for infants or special dietary
73 purposes, where complete absence is required), absence is required when the product leaves the
74 manufacturer, unless the manufacturer can demonstrate that the numbers will be <100cfu/g at the end
75 of the shelf life. Foods not supporting growth must have <100cfu/g at the end of the shelf life.

76 Since RTE food processing environments are recognised as a significant source of *L.*
77 *monocytogenes* contamination (Beno et al., 2016; Tompkin, 2002), it is important for food business
78 operators (FBOs) to have an appropriate surveillance programme to monitor and control the risk of *L.*
79 *monocytogenes* contamination of the final product. Environmental monitoring programmes are
80 recommended in the EU (EU, 2005) and required in some food sectors in the United States of
81 America (FDA, 2011) and contribute to the identification and tracking of *L. monocytogenes* along the
82 food chain, and within food processing facilities. Such programmes can have an impact on avoiding
83 cross-contamination to food (Ho, Lappi & Wiedman, 2007). Applying genetic fingerprinting, such as
84 pulsed field gel electrophoresis (PFGE) or whole genome sequencing (WGS) can assist studies on
85 isolate characterization and tracking (Dalmasso & Jordan, 2015; Schmid et al., 2014; Stasiewicz,
86 Oliver, Wiedmann, & den Bakker, 2015), especially with regard to determining persistence of *L.*
87 *monocytogenes* in food processing facilities.

88 Whilst large scale food processors will be aware of strategies and interventions to exclude
89 *L. monocytogenes,* small to medium sized enterprises (SMEs) may require assistance. These are
90 defined in European Union document 2003/361/EC as, inter alia, having fewer than 250 employees,
91 but in the current project most of the SMEs involved had <20 employees. In the Republic of Ireland
92 (RoI), a research project on assessment of *L. monocytogenes* was considered to have contributed to a
93 reduction of *L. monocytogenes* in food and food processing environments, leading to a decreased risk
94 to public health (Leong et al., 2017).

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95 The aim of this study was to assess the occurrence and persistence of *L. monocytogenes* in 24
96 RTE food processing facilities in Northern Ireland over an eighteen-month period (July 2015 to
97 November 2016). Regular monitoring of the processing environments and products of the RTE food
98 manufacturers, with molecular characterisation of the *L. monocytogenes* strains isolated was
99 undertaken.
100
101 **2 Materials and Methods**
102
103 *2.1* L. monocytogenes *monitoring program*
104 Staff of the College of Food, Agriculture & Rural Enterprise (CAFRE), Cookstown, led the
105 recruitment of FBOs to be involved in the programme. In total, 24 companies participated in this
106 study with staff attending a half day workshop for training in sampling procedures. All participating
107 FBOs provided sketch plans of their premises and marked the environmental sites to be sampled
108 during the programme. At the workshop the FBOs received detailed instructions which included
109 information on how to take swab samples, which areas to sample, and on the packaging and shipment
110 of the samples to the laboratory. This was designed to ensure consistent sampling by all participants.
111 In addition a video of the appropriate sampling procedures was made and placed on YouTube for
112 subsequent access by participants to ensure uniform sampling. For swab samples, all FBOs were
113 asked to take samples from three specific areas: a drain in the main processing hall, an area of floor
114 (1 m^2) and a storage shelf. Because of the variation in layout and type of the facilities, the area to
115 swab for the remaining samples was to be chosen by the FBO from anywhere in the food processing
116 environment, and the location noted. Cutting areas, walls, other drains and pooled water were
117 suggested as optimum locations. For food samples, FBOs were instructed to send two food samples
118 which were at the stage of being ready to be sent from the processing facility. All sampling took

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119 place during normal production conditions. Management practices were assessed by means of a
120 detailed questionnaire submitted to all participants.
121 From July 2015 to November 2016, a total of 24 food processing facilities from seven food
122 sectors (cooked meat, horticultural products, sandwich, baked goods, salads, seafood and dairy
123 [Table 1]) were analysed bimonthly for the presence of *L. monocytogenes*. All of these food
124 processing facilities produced RTE food products, and were distributed throughout Northern Ireland
125 (NI). Sampling packs, which consisted of a polystyrene box (DS Smith, UK) containing six pre-
126 moistened 3M sponge-stick swabs (Technopath, Ireland), a sterile liquid container (VWR, Ireland),
127 two sterile bags (VWR, Ireland), two cable ties, and two ice packs, were sent to all participating food
128 processing facilities two weeks prior to the assigned sampling date.
129

130 *2.2 Microbiological analyses*

131 All microbiological media were supplied by Oxoid, (Basingstoke, UK), unless otherwise
132 stated. Sampling kits were dispatched to FBOs two weeks before the target sampling date, and
133 samples were sent from FBOs by courier on the day of sampling, to arrive at AFBI the next morning,
134 where they were analysed immediately (less than 24 h after sampling). The methods used were
135 detection of *Listeria* spp. (including *L. monocytogenes*), BS EN ISO 11290-1:1996/Amd 1:2004, and
136 enumeration of *Listeria* spp. (including *L. monocytogenes*) as described in BS EN ISO 11290-
137 2:1998/Amd 1 2004, except that only agar *Listeria* according to Ottavani and Agosti (ALOA) was
138 used. Briefly, for food samples, for the enumeration of *Listeria* spp. 25 g of sample were added to
139 225 ml of Fraser broth base (CM0895 without selective supplements), blended for 2 min (Colworth
140 400, Seward Limited, Worthing, UK) then allowed to stand for 1 h. The samples were then plated
141 (0.1 ml) onto plates of ALOA agar and incubated (37ºC for 48 h), with examination after 24 h and 48
142 h. Plates with less than 150 typical colonies were counted. Where plates yielded presumptive *L.*
143 *monocytogenes,* five colonies were purified and confirmed, using API *Listeria* (bioMérieux UK

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144 Limited, Basingstoke, RG22 6HY), and the final count obtained by multiplying the presumptive
145 count by the percentage of confirmed *L. monocytogenes* colonies. For swabs, 90 ml of Fraser broth
146 base with half strength supplements was added to the swab in the bag in which it was transported to
147 the laboratory, followed by incubation and sub-culture as described above.
148
149 *2.3 Pulsed field gel electrophoresis*
150 The PFGE analysis was carried out using the International Standard PulseNet protocol
151 (PulseNetUSA, 2013). *Listeria monocytogenes* isolates were grown overnight in Brain Hearth
152 Infusion (BHI) agar at 37°C, subsequently a suspension in 10 mM Tris:1 mM EDTA buffer, pH 8.0
153 (TE) was prepared with an approximate OD_{610} of 1.0. To 400 µL of cell suspension was added 20
154 mg/ml of Lysozyme which was incubated at 55°C for 20 min. After incubation, 20 µl of Proteinase K
155 stock (20mg/ml) was added, followed by 400 µl of SeaKem Gold agarose 1%. Plugs were prepared
156 by dispensing the mix into plug moulds. Solid plugs were then lysed for 2 h in 5 ml of cell lysis
157 buffer (50mM Tris, 50mMEDTA, pH8.0 + 1% Sarcosyl) supplemented with 25 µl of 20 mg/ml
158 proteinase K solution. The plugs were then washed twice in distilled water and four times in TE
159 buffer.
160 DNA was digested with 10 U/µl of the restriction enzyme *AscI* FastDigest (Fisher Scientific,
161 Ireland) and 50 U/µl of the restriction enzyme *ApaI* FastDigest (Fisher Scientific, Ireland); the
162 restricted DNA was run in a 1% SeaKem Gold agarose gel for 21 h as described in the PulseNet
163 protocol, on a CHEF-DR III (Bio-Rad). After staining with 1 µg/ml ethidium bromide solution, the
164 gels were observed with the Alpha Imager (Alpha Innotech, DE). Analysis of the gels was performed
165 with BioNumerics v7.0 software (Applied Maths) using and UPGMA (unweighted pair group
166 method with averages) and the Pearson coefficient with 1% tolerance.
167
168 *2.4 Whole genome sequencing*

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169 DNA was extracted from all isolates using a PureLink Genomic DNA Kit (Thermofisher
170 Scientific, Paisley, UK) as per the manufacturer's instructions. The whole genome sequencing of all
171 *L. monocytogenes* isolates was performed as previously described (Ugarte-Ruiz et al., 2015) using
172 Illumina MiSeq 2 × 250 bp paired-end sequencing. To analyse the data quality, FastQC was used
173 (Andrews, 2016). To trim and crop the sequencing reads, Trimmomatic was used with the following
174 parameters: (v0.32) 'leading' and 'trailing' setting of 3, a 'slidingwindow' setting of 4:20 and a
175 'minlength' of 36 nucleotides (Bolger, Lohse, & Usadel, 2014). BWA-MEM (v0.7.7-r441) was used
176 to map the reads using the genome sequence of *L. monocytogenes* EGD (HG421741) as described by
177 Li & Durbin, 2010. VelvetOptimiser (v2.2.5) using n50 optimization was used to perform assembly
178 (Gladman & Seeman, 2012; Zerbino & Birney, 2008). The reference strain *L. monocytogenes* EGD
179 (HG421741) was used to complete contigs using ABACAS (v1.3.1) (Assefa, Keane, Otto, Newbold,
180 & Berriman, 2009). Multi locus sequence type (MLST) was determined using PubMLST
181 (https://github.com/tseemann/mlst). Genome annotation was provided by using Prokka (Seemann,
182 2014). To read the genomes, and assess them for presence of internalin A, *act*A,
183 *Listeria* pathogenicity islands 3 and 4, the stress survival islet SSI-1, *bcr*ABC and *qac*H, Artemis and
184 ACT software were used (Carver, Harris, Berriman, Parkhill, & McQuillan, 2012).
185
186 *2.5 Measurement of pH and a_w.*
187 A subset of food samples (n=130) was analysed to determine the pH, using the methodology
188 of BS 4401-9-1975, ISO 2917-1974, and water activity (a_w) determined according to British Standard
189 method BS ISI 21807:2004, using a Rotronic HygroLab 3 (Rotronic Instruments [UK] Ltd, Crawley,
190 RH10 9EE, UK). These represented two samples of each of the products submitted by FBOs.
191
192 *2.6 Statistical analyses*

193 All statistical analyses were undertaken by the Biometrics & Information Systems Branch,
194 AFBI, using Genstat Release 18.1 for Windows (VSN International Ltd, Hemel Hempstead, HP2
195 4TP, UK). One-way analysis of variance was used to study temporal differences with $P < 0.05$
196 indicating significance.

197

198 **3 Results**

199

200 3.1 *Occurrence of* L. monocytogenes

201 Overall, the 24 FBOs submitted 1,598 samples for analysis. These consisted of 1,203 swabs
202 and 395 food samples. Seventy-six swabs (6.3%) and eighteen food samples (4.6%) yielded
203 *L. monocytogenes*, with 5.9% of samples being positive overall. All the food samples had <100cfu/g,
204 except for two samples of cooked meat, one that had >2000 cfu/g and one that had 140 cfu/g. *L.*
205 *monocytogenes* were not isolated from twelve of the premises tested; four produced dairy products
206 and the eight others a variety of products, including hot smoked salmon, pasta sauces, pâtés and
207 ready meals, baked goods, cooked chicken, and salad and vegetable based products. The overall
208 prevalence of *L. monocytogenes* at the different facilities is shown in Table 1, while the type of
209 positive sample, is shown in Table 2. Overall, 96 *L. monocytogenes* isolates were obtained during an
210 eighteen-month sampling schedule, one from each positive sample, except for two meat samples that
211 had >100cfu/g, where two isolates were obtained from each sample. Study of the number of positive
212 samples with time (data not shown) suggested that there were differences in the prevalence of
213 *L. monocytogenes* between the seasons of summer and winter (summer = May, July, September;
214 winter = November, January, March). No statistically significant difference was found between the
215 numbers of positive food samples found in summer and winter however, for processing environment
216 swabs, significantly more positive samples were found during the summer months, $p = 0.007$.

217 Of the positive samples, 47.8% were in drains and on floors, while 19.1% were from food
218 samples. The remainder were from other processing environment sites, for example trolleys, tables
219 and walls.
220
221 *3.2 Analysis of the isolates by pulsed field gel electrophoresis*
222 The isolates were analysed by pulsed field gel electrophoresis (PFGE); two isolates that were
223 not recoverable were not included in the PFGE analysis, leaving PFGE analysis on 94 isolates. A
224 total of 27 distinguishable pulsotypes were identified, based on > 90% similarity. Figure 1 shows a
225 minimum spanning tree, representing the PFGE profiles of all the isolates. The different colors
226 represent different food categories. Within a circle, each segment represents an isolate, while the
227 circle represents a pulsotype where the isolates show > 90% similarity. The length of the line
228 between the circles represents the distance of the relationship between the pulsotypes/isolates.
229 Pulsotypes with only one isolate are represented by a circle with no segments. Of the 27 pulsotypes,
230 there were 10 with a single isolate and 8 with more than 4 isolates. Nine of the pulsotypes had
231 isolates from more than one food sector, indicating possible cross contamination between food
232 sectors (Table 3 gives more detail on pulsotypes shared across food sectors).
233 For each of the companies which were positive for *L. monocytogenes*, Table 3 shows the
234 number of pulsotypes, number of persistent pulsotypes and the number of pulsotypes shared between
235 different food sectors (widespread pulsotype). A persistent pulsotype was defined as repeated
236 identification of an isolate of the same pulsotype over a period longer than 6 months. Of the 27
237 pulsotypes identified, 21 of these were identified in 3 of the companies tested. This shows the
238 diversity of the isolates obtained, but also shows a degree of similarity in the isolates from the
239 different companies (Figure 2).

240 Persistent pulsotypes were identified at 6 of the 12 facilities. Of the 27 pulsotypes, 9 were
241 persistent. Five pulsotypes showed a cross-contamination scenario in 3 of the companies, where
242 indistinguishable pulsotypes were found on food and in the processing environment.
243 The PFGE profiles obtained in this study were compared with a database of strain PFGE
244 profiles obtained in a similar study in the RoI. There were no similarities at the level of >90%
245 between the isolates from this study and isolates obtained previously.
246 Comparison between the pulsotypes obtained in this study and about 2,500 PFGE profiles in a
247 database of international isolates at Teagasc, Moorepark, Ireland, showed that of the 27 pulsotypes
248 obtained in this study, 10 were comparable with international isolates. These included similarities
249 with strains from Ireland, Austria, Romania, Czech Republic, Turkey and Australia (data not shown).
250 Seven of the pulsotypes from the current project were >90% similar to mushroom production chain
251 isolates from a project in the RoI . Figure 3 shows an example of one of these.
252 A comparison between the pulsotypes from this study and those of clinical isolates from the
253 ROI was made. Seven of the pulsotypes identified in this project were similar to pulsotypes from
254 clinical isolates at >90% (data not shown).
255
256 *3.3 Potential for growth of* L. monocytogenes *on the food products*
257 The pH and a_w data were used as the input into the Combase *L. monocytogenes* computer
258 growth prediction model (http://browser.combase.cc) to obtain an estimate of the mean generation
259 time at three storage temperatures: 4, 6, and 8°C. Combase predicted that 19 of the 130 samples
260 would not support the growth of *L. monocytogenes* due either to their pH being below 4.40 (14
261 samples), or their a_w being below 0.934 (5 samples). The pH and a_w for the remaining 111 samples
262 which were predicted to support growth is shown in Supplementary Figure 1. Over 80% of the 111
263 samples had a pH greater than 5.5 and over 86% had a a_w greater than 0.97. To assess, and compare,
264 the potential of each foodstuff to support the growth of *L. monocytogenes,* an arbitrary growth

265 parameter was chosen. This was the ability of the food to support ten generations of growth,
266 equivalent to approximately 1,000-fold growth, in seven days or less. This corresponded to growth
267 from 1 cell per 10 g of food to the limit of 100 cfu/g, and was intended to be a parameter
268 comprehensible to the FBOs (data not shown). At 4°C, growth predictions showed that none of the
269 foods supported 10 generations of growth in one week. However, at 6°C growth could exceed this
270 target in 55.0% of the foods, and at 8°C this rose to 77.5% of the foods.

271

272 *3.4 Whole genome sequencing of the isolates*

273 All of the *L. monocytogenes* isolates were subjected to WGS and 91 sequences were obtained;
274 the quality of the DNA did not allow WGS to be completed for 5 strains. The genome sequences
275 were then analysed for the presence of four virulence-associated genes: internalin A, *inl*A; actin
276 assembly protein, *act*A; *Listeria* pathogenicity island 3, LIPI-3 and *Listeria* pathogenicity island 4,
277 LIPI-4.Three genes indicative of stress tolerance were also studied; stress survival islet, SSI-1;
278 a resistance cassette, which contributes to resistance to quaternary ammonium compounds, *bcr*ABC;
279 and the quaternary ammonium compound-resistance gene, *qac*H (Fox, Allnutt, Bradbury, Fanning, &
280 Chandry, 2016). All 91 isolates carried functional genes for *inl*A and *act*A and 65 (71.4%) carried
281 *qac*H. None carried LIPI-3, LIPI-4, or *bcr*ABC.

282 These whole genome sequences were analysed to determine the MLST of the isolates, a
283 feature frequently used for clinical characterisation of isolates. Twelve MLSTs were
284 found(Supplementary Figure 1), and in nine FBOs some MLSTs were isolated on more than one
285 occasion (Table 4).

286

287 **4 Discussion**

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289 The results of this study show that 50% of the 24 food processing facilities where sampling
290 took place were free of *L. monocytogenes* in the food and the food processing environment for the
291 18-month duration of the sampling. It is significant that *L. monocytogenes* was not detected in any of
292 the 5 of the dairy processing companies where sampling took place (all the companies used
293 pasteurised milk, with the main products being ice cream, yoghurt and cheese). Sandwich production
294 companies and horticultural product production facilities were the food sectors where the highest
295 number of facilities were positive – 4 of 4 and 3 of 4, respectively. In a similar study in the RoI, 10 of
296 54 facilities were completely free of *L. monocytogenes* for the 3-year duration of that study (Leong et
297 al., 2017). Correlation of data on management practices at facilities that were positive and negative
298 for *L. monocytogenes* did not identify any practices that would lead to an increase or decrease in
299 occurrence of *L. monocytogenes* (data not shown). From a food safety perspective, a *L.*
300 *monocytogenes*-free processing environment will have a reduced risk of *L. monocytogenes*
301 contamination of the food produced.

302 All of the positive samples came from 12 facilities, 4 of 4 sandwich producing companies, 3
303 of 4 horticultural product production facilities, 2 of 3 meat companies, 1 of 2 baked goods companies
304 and 1 of 1 seafood company. It should be noted that two samples of meat products sampled prior to
305 distribution were found to exceed 100 cfu/g, and were therefore not fit for sale. In these cases, the
306 FBOs were informed and appropriate action was taken. All other food samples (n=393) had <100
307 cfu/g. In this study, sandwiches had significant *Listeria* contamination, as was shown by (Cossu et
308 al., 2016), and they have been implicated in cases of listeriosis (Silk, McCoy, Iwamoto, & Griffin,
309 2014). This show the importance of anti-listeria controls in sandwich producing facilities.

310 The overall occurrence in food and in the processing environment was 4.6% and 6.3%,
311 respectively. Comparing the results with other published surveys is not applicable as, 1) different
312 methodologies (for sampling and analysis) are frequently used, 2) in many surveys, one facility is
313 surveyed on several occasions or several facilities are surveyed on one occasion, 3) some surveys are

314 targeted at facilities that have been shown to be positive. In extensive surveys, using similar
315 methodologies, Leong et al, (2017) found that 3.8% of 4667 processing environment samples and
316 4.2% of 1202 food samples were positive, while Beno et al. (2016) found 1.4% of 4430 processing
317 environment samples positive. Muhterem-Uyar et al. (2015) reported a higher occurrence in meat
318 processing facilities than in dairy processing facilities (32.0% and 8.8%, respectively), attributing the
319 higher occurrence in meat processing facilities to cross-contamination from carcasses. In addition,
320 not all the meat processing facilities produced ready-to-eat food.

321 The locations most commonly associated with *L. monocytogenes* contamination were areas
322 such as floors and drains (Kells & Gilmour, 2004; Leong, Alvarez-Ordonez, & Jordan, 2014; Ruckerl
323 et al., 2014; Schoder, Rossmanith, Glaser, & Wagner, 2012). This prevalence was not consistent over
324 the 18-month period of the study as processing environment samples were more frequently positive
325 during the warmer months of May to September ($p = 0.007$). This is in contrast to other studies such
326 as that undertaken in the RoI (Leong et al., 2017), where no seasonality was found. Weather, such as
327 warm or wet conditions, can affect the prevalence of *L. monocytogenes* (Hellberg & Chu, 2016).
328 However, in this study, no obvious cause for the seasonality observed was determined. In contrast,
329 the statistical analyses showed no seasonal effects with regard to prevalence in the food samples.
330 Seasonality of contamination has been reported in dairy products (Meyer-Broseta, Diot, Bastian,
331 Rivière, & Cerf, 2003), but no dairy products were positive in this study.

332 The ninety-four isolates genetically characterised by PFGE resulted in 27 distinguishable
333 pulsotypes; 33% of these pulsotypes showed persistence at the processing facility. Nine of the
334 pulsotypes were found in different food sectors. If more extensive sampling and analysis were
335 undertaken, it is possible that other pulsotypes would be identified as persistent. The persistence of *L.*
336 *monocytogenes* indicates either repeated contamination events or the resistance of *L. monocytogenes*
337 to the hygiene procedures in the facility (for review see Carpentier & Cerf, 2011). Persistence of *L.*
338 *monocytogenes* in the food processing environment presents a risk of cross contamination to the food

339 being produced. In order to address such persistence, more stringent hygiene measures, or hygiene
340 barriers on access to the facility (including for raw materials) are necessary. Such measures can
341 reduce cross contamination to the final product and contribute to the prevention of public health
342 issues (Awofisayo-Okuyelu et al., 2016; McCollum et al., 2013; Montero et al., 2015).

343 The results of the project show the diversity of the isolates obtained (Figure 1), but also
344 shows a degree of similarity in the isolates from the different companies (Figure 2). For example, the
345 PFGE profile of an isolate from the floor in front of a sink at one facility was >90% similar to a food
346 isolate from the same facility and isolates from processing environment and food samples from other
347 facilities (Figure 2). Further studies analysing the WGS data in more detail may help to resolve this
348 issue and determine how similar the isolates are.

349 The presence of the same *L. monocytogenes* pulsotype in different companies (widespread
350 occurrence) shows the possibility of cross contamination between different production units and
351 sometimes different food sectors. This can happen when services are shared, like transporters, raw
352 materials, couriers, etc. In the current study, there were no known links between the companies where
353 the same pulsotype was found in different facilities, except for one case where the same company
354 operates two facilities. Widespread contamination was detected in the mushroom industry. In Ireland,
355 the mushroom industry is a very 'cross border' industry, where companies have premises in both NI
356 and RoI. This could explain the occurrence of seven common pulsotypes that were commonly found
357 in both NI and the RoI. Alternatively, there are global clones of *L. monocytogenes* (Chenal-
358 Francisque et al., 2013), indicating that it is possible for the same pulsotype to be found in different
359 facilities that have no apparent epidemiological link.

360 Cross contamination was shown at four facilities, where *L. monocytogenes* isolates from
361 processing environment swabs had the same pulsotype as food samples in the same facility. PFGE
362 does not discriminate between the two possible scenarios, namely if the contamination went from the
363 food to the environment or vice-versa. Further analyses would be required to differentiate these

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364 scenarios. Persistence of *L. monocytogenes* in the food processing environment presents a risk of
365 cross contamination to the food being produced. Cross contamination from the processing
366 environment has been previously reported (Ivanek, Grohn, Wiedmann, & Wells, 2004; Leong et al.,
367 2017; McCollum et al., 2013) and has been implicated in outbreaks of listeriosis (Currie et al., 2015).

368 In a comparison between the pulsotypes identified in this study and those obtained in other
369 countries, 10 common pulsotypes were identified. Again, this shows the existence of global clones of
370 *L. monocytogenes*, not necessarily connected to epidemiological data (Chenal-Francisque et al.,
371 2013). The relationship between these clones is unclear, and further studies would be required to
372 clarify this. For example, a comparison of whole genome sequences can highlight the presence of
373 single nucleotide polymorphisms (SNPs) between strains with indistinguishable PFGE profiles. This
374 is especially important where PFGE similarities are shown in the comparison with clinical isolates,
375 but in the absence of epidemiological data no link can be made between isolates from food and
376 disease-causing isolates.

377 Since *L. monocytogenes* was present in half of the food processing facilities participating in
378 this study, there is potential for cross-contamination to food products. Thus, it is relevant to
379 determine if these foods support the growth of *L. monocytogenes*. Although not foolproof, predictive
380 microbiology can give an indication of growth potential. Nineteen of the 130 food products tested
381 (using Combase predictions based on pH and water activity) were predicted not to support the growth
382 of *L. monocytogenes*; these were mainly low pH products such as yoghurts, cheese, fruit products and
383 coleslaw, which all had a pH below 4.4. While the pH of cheese can increase post-production due to
384 the actions of the endogenous microflora (Schoder, Skandamis, & Wagner, 2013), this was unlikely
385 to occur with the cheese products studied, hence they would be not support growth during their
386 normal shelf life.

387 The remaining 111 food samples, i.e. 85%, all had pH and a_w values which would support the
388 growth of *L. monocytogenes* at 4°C, 6°C and 8°C, according to the ComBase model. While 4°C is the

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389 intended temperature of a domestic refrigerator, a UK-wide microbiological study found that the
390 mean temperature of pre-packed meats on retail display was 6.8°C, with 71.3% of samples above the
391 industry guideline of 5°C, and 32.7% being stored above 8°C (Madden, 2014). Hence, the
392 temperatures of 4 and 8°C, and the intermediate temperature of 6°C, were chosen for the growth
393 studies. Growth in the foodstuffs over a seven-day period was determined using the arbitrary
394 criterion of calculating the time for 10 generations of microbial growth (approximately a 1,000-fold
395 increase in numbers), using the generation time predicted by ComBase. This allowed a simple metric
396 to be communicated to FBOs. At 4°C none of the 111 foodstuffs were predicted to support such
397 growth. However, increasing the temperature to 6°C resulted in 55% of the samples failing the test,
398 while 77.5% failed at 8°C. This shows the importance of temperature in controlling the growth of *L.*
399 *monocytogenes*, and the importance of proper control over the temperature of domestic refrigerators.
400 In order to help them comply with Commission Regulation (*EC*) No 2073/2005, this information was
401 communicated to participants. Additionally, guidance for FBOs undertaking challenge studies to
402 determine the ability of foods to support growth of *L. monocytogenes* in the food matrix has been
403 published (Beaufort, Bergis, Lardeux, Polet, & Botteldoorn, 2014). Thus, for most of the food
404 products analysed in this study, an initial contamination of one *L. monocytogenes* per 10 g could
405 exceed the regulatory level of *L. monocytogenes* cited in Commission Regulation (EC) No 2073/2005
406 (100 cfu/g) within a week, if they were subjected to abuse temperatures found in retail premises.
407 The Gastrointestinal Bacteria Reference Unit, National Infection Service, Public Health
408 England, supplied data on clinical MLSTs from the UK (April 2016-March 2017), where 10 MLSTs
409 were found (STs 1, 5, 6, 8, 20, 21, 121, 155, 204 and 394). ST204 was the most common sequence
410 type identified in this study, comprising 30 isolates. This MLST was isolated from a number of
411 different food processing facilities, and was found for the duration of the project. All of the clinical-
412 associated MLSTs were found in the isolates from this study, where they comprised 98% of the
413 isolates. Furthermore, the National *Salmonella, Shigella & Listeria* Reference Laboratory in the RoI

414 reported that eight of the nine most common MLSTs isolated during this study were also found as
415 clinical isolates. However, based on a study of fifteen ST204 isolates, this MLST was reported as
416 being mainly an environmental isolate (Fox et al., 2016). It should be noted that not all isolates with a
417 given MLST are genetically identical. For example, 86% of the ST204 isolates carried the *qac*H
418 gene, and were therefore different from the 14% which did not carry it.

419 Overall, most of the isolates for which WGS data was obtained (71%) carried the *qac*H gene,
420 which confers resistance to quaternary ammonium salts, the basis of many sanitizers used in the food
421 industry (Sidhu, Sørum, & Holck, 2004). The high rate of carriage of *qac*H by ST204 isolates found
422 in this study contrasts with its absence in the 15 isolates described in the report of Fox et al. (2016),
423 and may reflect selection for this property in food processing environments. Resistance to quaternary
424 ammonium salts may also be a contributory factor to the high prevalence of this MLST found in this
425 study.

426 Study of the WGS data showed that all of the isolates obtained carried the virulence genes
427 *inl*A and *act*A (Fox et al., 2016). However, determining the true pathogenic abilities of the isolates
428 was beyond the scope of this study; further work on the WGS data and on invasion assays would be
429 required to further investigate pathogenic properties of the isolates (Chen et al., 2016).

430 Overall, the results obtained showed that most participating FBOs can produce RTE food free
431 from *L. monocytogenes*, and many were able to keep their production facility free from this pathogen.
432 Hence, most FBOs appear to have determined effective control strategies and practical interventions.
433 However, WGS data suggests some FBOs have persistent strains, and that many of the isolates could
434 be potential pathogens. Therefore, there is a need for FBOs to continually assess control strategies
435 and practical interventions for the exclusion of *L. monocytogenes*.

436

437 **5 Conflict of Interest**

438 The authors declare that the research was conducted in the absence of any commercial or financial
439 relationships that could be construed as a potential conflict of interest.
440

441 ## 6 Author Contributions

442 Substantial contributions to the conception or design of the work; or the acquisition, analysis, or
443 interpretation of data for the work; RM, MH, KJ, VP, OG, NC
444 Drafting the work or revising it critically for important intellectual content; RM, MH, KJ, OG, NC
445 Final approval of the version to be published; RM, MH, KJ, VP, OG, NC
446 Agreement to be accountable for all aspects of the work in ensuring that questions related to the
447 accuracy or integrity of any part of the work are appropriately investigated and resolved. RM, MH,
448 KJ, VP, OG, NC
449

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452

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460 2014.
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Table 1. Occurrence of *L. monocytogenes* by category of food product produced by the food business.

Food category	Total samples	*L. monocytogenes* positive	% Samples Positive
Cooked meat	225	27	12.0
Horticultural productse[1]	398	26	9.1
Sandwich	286	24	8.4
Baked goods	128	10	7.8
Salads	136	5	3.7
Seafood	144	2	1.4
Dairy	281	0	0.0
Total	1,598	94	5.8

[1]Includes mushrooms, vegetables and fruits

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Table 2. Food premises yielding *Listeria monocytogenes* from the processing environment and/or products. To maintain anonymity, the premises listed have been designated in terms of the type of principal products.

Products from food premises	Number of isolates from each food premises	
	Processing environment swabs	Food product
Cooked Meats A[1]: pulled chicken, turkey and beef	17	2
Cooked Meats B: pork from fresh and cured meat	5	5[2]
Sandwiches A: sandwiches, rolls and wraps	10	2
Sandwiches B: sandwiches, salads, wraps and snacks	7	0
Sandwiches C: sandwiches, pasta and salad bowls, coleslaw, potato salad	0	4
Sandwiches D: sandwiches	1	0
Processed mushrooms	12	7
Baked goods: cakes, pies, sausage rolls	10	0
Salads: green and pasta salads, chicken tuna and egg mixes	5	0
RTE raw fruit pieces in consumer packs	5	0
RTE processed fish/shellfish	2	0
RTE vegetable products	2	0

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[1]Where more than one company manufactured a product type they have been designated successively, as A, B etc.

[2]Three food samples were positive and two of these samples yielded *L. monocytogenes* isolates from both enrichment cultures and enumeration plates.

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Table 3. Pulsotypes obtained from different food premises. To maintain anonymity premises listed have been designated in terms of their principal products

Products of Food Premises	Number of pulsotypes	Number of persistent pulsotypes	Number of pulsotypes shared with other companies (persistent isolates shared)
Cooked Meats A[1]	10	2	4 (1)
Sandwiches A	5	3	5 (3)
Salads	4	0	4
Cooked Meats B	2	1	0
Baked goods	4	3	1
Sandwiches B	3	0	1
RTE raw fruit	2	0	1
Sandwiches C	1	1	1
RTE vegetable products	2	0	2
RTE processed fish/shellfish	1	0	1
Processed Mushrooms	6	1	4 (1)
Sandwiches D	1	0	1

[1]Where more than one company manufactured a product type they have been designated successively, as A, B etc.

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Table 4. Sequence types (ST) isolated from individual premises on more than one occasion. To maintain anonymity, the premises listed have been designated in terms of the type of principal products.

Products from food premises	Total number of sequence types (ST)	Number of recurrent ST	Recurrent ST
Cooked Meats A[1]: pulled chicken, turkey and beef	8	4	ST204
Cooked Meats B: pork from fresh and cured meat	7	1	ST204
Sandwiches A: sandwiches, rolls and wraps	4	1	ST204
Sandwiches B: sandwiches, salads, wraps and snacks	4	1	ST6
Sandwiches C: sandwiches, pasta and salad bowls, coleslaw, potato salad	3	1	ST6
Processed mushrooms	8	2	ST8, ST204
Baked goods: cakes, pies, sausage rolls	6	2	ST6, ST20, ST121, ST21, ST121
Salads: green and pasta salads, chicken tuna and egg mixes	4	0	
RTE[3] raw fruit pieces in consumer packs	2	1	ST155

[1]Where more than one company manufactured a product type, they have been designated successively, as A, B etc.

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[2] RTE- Ready to Eat

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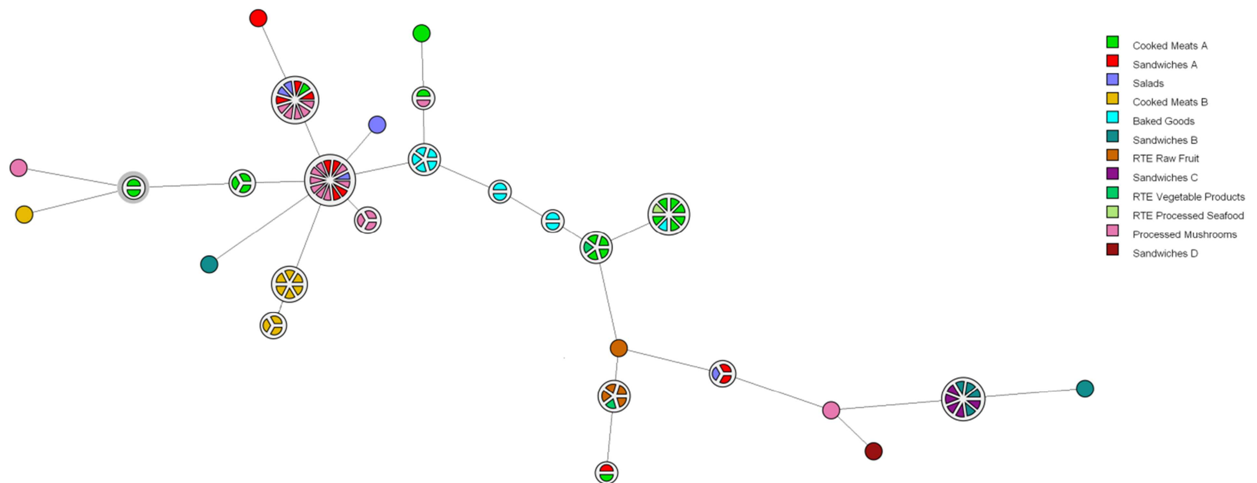

Figure 1. Minimum spanning tree summarising the data from the PFGE profiles of the 94 isolates tested. The different colours represent different food companies. Within a circle, each segment represents an isolate, while the circle represents a pulsotype where the isolates show > 90% similarity. The length of the line between the circles represents the distance of the relationship between the pulsotypes/isolates. Pulsotypes with only one isolate are represented by a circle with no segments.

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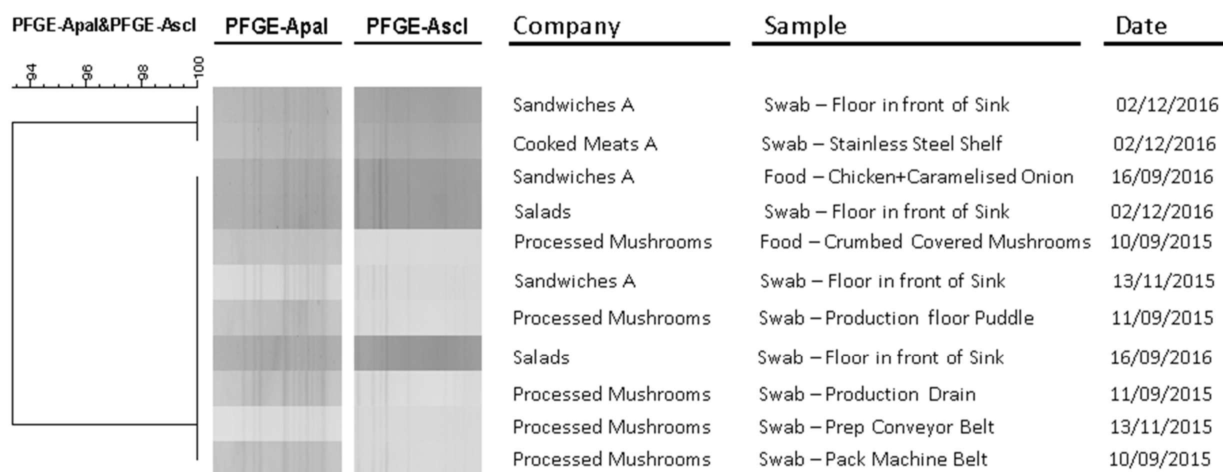

Figure 2. PFGE profiles showing similarity between *L. monocytogenes* isolates obtained from different food premises.

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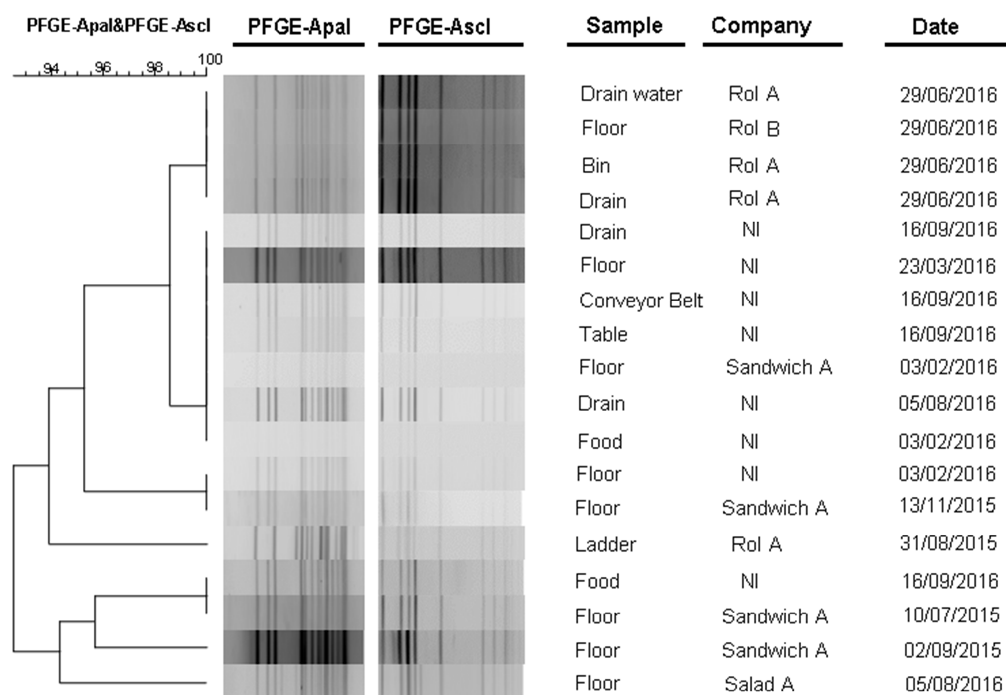

Figure 3. PFGE profiles of *L. monocytogenes* from mushroom producers in the Republic of Ireland (RoI) (A and B) compared with food producers in Northern Ireland (NI): a mushroom producer (NI), sandwich maker (Sandwich A) and a salad producer (salad A). Dates are those on which isolates were stored. The scale shows % similarity.

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- No *Listeria monocytogenes* were detected at 50% of the food processing facilities sampled
- At 4°C none of the foods tested were predicted to support growth of *Listeria monocytogenes*
- There were 27 distinguishable pulsotypes in the 96 isolates tested
- Sequence types detected can cause listeriosis

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