Sources and Survival of *Listeria monocytogenes* on Fresh, Leafy Produce

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Summary

Listeria monocytogenes in an intracellular human pathogen which enters the body through contaminated food stuffs and is known to contaminate fresh leafy produce such as spinach, lettuce and rocket. Routinely, fresh leafy produce is grown and processed on a large scale before reaching the consumer through various products such as sandwiches and prepared salads. From farm to fork, the fresh leafy produce supply chain is complex and contains a diverse range of environments where *L. monocytogenes* is sporadically detected during routine sampling of produce and processing areas. This review describes sources of the bacteria in the fresh leafy produce supply chain and outlines the physiological and molecular mechanisms behind its survival in the different environments associated with growing and processing fresh produce. Finally, current methods of source tracking the bacteria in the context of the food supply chain are discussed with emphasis on how these methods can provide additional, valuable information on the risk that *L. monocytogenes* isolates pose to the consumer.

Keywords: Listeria, monocytogenes, Food Safety, Food Processing, Microbial Contamination, Soil,

Introduction

Listeria monocytogenes is a Gram-positive, facultative anaerobic, opportunistic bacterial pathogen. It is the causative agent of listeriosis, a disease which predominantly affects immunocompromised people including the elderly, immunosuppressed and pregnant women together with their unborn or new-born babies. Contaminated foodstuffs are the main cause of infection and there have been several well-documented, high-profile outbreaks from this source over recent years (Garner and Kathariou 2016). Because of the risk of infection from food, safety authorities impose limits on the number of *L. monocytogenes* cells that can contaminate food products. Guidelines in the USA advise that *L. monocytogenes* should not be present (<1 CFU $25g^{-1}$) in ready to eat (RTE) foods that support the growth of *L. monocytogenes* and should not be equal to or above 100 CFU g^{-1} for foods that do not support the growth of *L. monocytogenes* (Center for Food Safety and Applied Nutrition 2017). Legislation on *L. monocytogenes* contamination of RTE foods in the EU requires that *L. monocytogenes* number remains less than 100 CFU g^{-1} for the shelf life of the product unless it has been demonstrated that *L. monocytogenes* has the potential to exceed this number (European Commission 2005). In such cases the food producer must demonstrate *L. monocytogenes* absence in raw materials and the production environment (i.e. there is no potential for contamination of the final product). Limits are set on the number of *L. monocytogenes* allowed in RTE food due to the risk of *L. monocytogenes* infection in highly susceptible individuals coupled with the bacterium's ability to grow in a range of food substrates (Leong *et al.* 2013; Jami *et al.* 2014). Although incidence of listeriosis is relatively low compared to other foodborne bacteria, the disease outcome if often more serious, making it a priority pathogen for many countries. Furthermore, *L. monocytogenes* can grow at refrigeration temperatures (Chan and Wiedmann 2009), meaning it presents an added danger to consumers over other food pathogens such as *Salmonella* and *E. coli*.

Foods which have been previously implicated in *L. monocytogenes* infections include milk, soft cheeses, deli or sandwich meats and fresh produce, which encompasses both fresh fruit and vegetables (Cartwright *et al.* 2013). Several reports have demonstrated *L. monocytogenes* presence in

a wide variety of fresh produce samples (Zhu *et al.* 2017) and other minimally processed foods. Other than a potentially tragic loss of life, the economic consequences of a *L. monocytogenes* outbreak are significant due to a loss of consumer confidence and subsequent drop in product sales and related value (McCollum *et al.* 2013). This review focuses on *L. monocytogenes* contamination of fresh leafy produce lines, such as salad ingredients (lettuces, wild rocket etc.) and leafy brassicas (kale, spinach etc.), which account for a significant proportion of the UK market and are "high-risk" in terms of bacterial contamination because of their leaf structures and proximity to the ground. The fresh leafy produce supply chain (FLPSC), from farm to fork, is complex and contains a diverse range of environments where *L. monocytogenes* can be detected during routine sampling of fresh leafy produce throughout the supply chain. For example, in soil, recently harvested crops, the processing environment and in the final the product itself, although detection tends to be sporadic.

L. monocytogenes is more likely to be detected in environments where soil contamination is present due to its ubiquity in the environment and presence in soil. Owing to this ubiquitous nature, companies that operate in the supply chain have difficulty determining the source of contaminating *L. monocytogenes* on fresh leafy produce. For source tracking, an increasing array of tools are becoming available with the gold-standard being whole genome sequencing (WGS). However, use of these tools on a day to day basis in the FLPSC is not yet feasible due to their cost, complexity of analysis, and expertise required to interpret data. In contrast, during outbreaks of disease, the advent of subtyping techniques has enabled source tracking of *L. monocytogenes* after an outbreak has been identified (Pichler *et al.* 2011; Gaul *et al.* 2013). Once a contamination source is located or indicated, regulatory bodies and companies that operate in the supply chain take appropriate precautionary measures to avoid further contamination (e.g. increased sanitation regimes or avoidance of the contaminated area). Subtyping can also indicate the potential risk of *L. monocytogenes* isolates. The species can be split into four evolutionary lineages (I, II, III, IV), where most of human clinical cases are caused by lineages I & II (Orsi *et al.* 2011). Despite the added benefits that subtyping provides in terms

of potential risk assessment, routine sampling in the FLPSC often only characterises isolates down to the species level as currently, all *L. monocytogenes* are treated equally for regulatory purposes. Owing to the potential risk of foodborne illness from this bacterium, source tracking, risk assessment and understanding the ability of *L. monocytogenes* to survive in the FLPSC should be considered key factors in tackling *L. monocytogenes* contamination of fresh leafy produce and reducing risk to the consumer. In this review, possible sources of *L. monocytogenes* contamination in the FLPSC and the mechanisms behind *L. monocytogenes* survival in this environment are discussed and the benefits of subtyping *L. monocytogenes* isolates found in the FLPSC in the context of source tracking and risk evaluation are outlined.

The Structure of the Fresh Leafy Produce Supply Chain

Fresh leafy produce types include but are not limited to, baby spinach (*Spinacia oleracea*), lettuce (*Lactuca sativa*), rocket (*Eruca sativa*), kale (*Brassica oleracea*) and herbs such as coriander (*Coriandrum sativum*). These crops are distributed to the consumer through a variety of end products such as whole head crops, mixed bagged salads and sandwich ingredients. The FLPSC has been summarised (see Monaghan and Beacham, 2017), but it is useful to provide a brief overview. The chain starts in the field where a crop typically takes between 3-24 weeks to grow before being harvested mechanically or by hand. After harvest, a crop may be packaged in field (as is the case with whole head lettuce) where the product is cooled and transported to the retailer, or subjected to further processing such as washing, cutting and packaging in a dedicated facility (Figure 1).

Protocols have been developed and applied to the growing process to reduce the risks of microbial contamination of fresh produce supplied to retail outlets. These include preventing farmers from growing crops on land that has been amended with raw manure and not irrigating crops in the immediate period before harvest to reduce the risk of contamination from irrigation water. Microbiological testing for *L. monocytogenes* throughout the FLPSC is obligatory through regulation and/or customer specifications. The presence of *L. monocytogenes* or those of the *Listeria* genus in

sampled product or surrounding areas results in 1) an increase in the scope and frequency of testing and 2) a review of the risk assessment with emphasis on possible sources of the bacteria (Monaghan and Hutchinson 2015). Despite these measures, *L. monocytogenes* continues to sporadically contaminate fresh leafy produce. A detailed review of *L. monocytogenes* outbreaks and prevalence associated with fresh produce is provided by Zhu, Gooneratne and Hussain, (2017).

Potential Sources of *L. monocytogenes* Contamination in the Fresh Leafy Produce Supply Chain

L. monocytogenes has been isolated from soil, waterways and vegetation where it exists as a saprophyte (Welshimer 1968; Locatelli *et al.* 2013a) from domestic and wild animals where it is harboured in the intestine and shed in faeces (Hellström *et al.* 2008; Hellström 2011) and from food contact surfaces in processing facilities (Leong *et al.* 2014). Such environments are significantly associated with production and processing of leafy produce and the pathogen can potentially be transferred to the product surface through several transmission routes (Table 1). Survival of *L. monocytogenes* in these environments is key to its transmission to foodstuffs. For example, *L. monocytogenes* can persist in a food processing facility for months and re-contaminate product passing through that facility (Leong *et al.* 2017).

L. monocytogenes: An Organism Adapted to Survive in the Fresh Leafy Produce Supply Chain

To survive in the FLPSC *L. monocytogenes* must withstand various environmental pressures such as, competition with other microbes, cleaning, desiccation, nutrient starvation and fluctuation in temperatures. *L. monocytogenes* can grow between temperatures of 0 - 45°C and a pH of 4.1 to 9.6 (Liu 2008; Shabala *et al.* 2008). Liu *et al.* (2005) also showed that *L. monocytogenes* recovers well after treatment with a pH 12 solution and was resistant to saturated (40% v/v) NaCl for at least 20h.

123 This ability to withstand physiochemical stresses is a major factor in *L. monocytogenes* ability to
124 contaminate chilled and minimally processed foods.

125 Exposure to environmental stresses induces the *L. monocytogenes* stress response, mediated by the
126 alternative sigma factor σ^B which regulates several stress, virulence and transporter associated
127 genes (e.g. *lmo2230, ltrC, ctc, inlA-E & opuC operon*) and related proteins (Kazmierczak *et al.* 2003).
128 Phenotypic investigations with strains lacking *sigB* demonstrate the important role that σ^B plays in
129 protecting against osmotic, oxidative, acid and detergent stresses (Ferreira *et al.* 2001, 2003). PrfA,
130 another important *L. monocytogenes* regulatory protein, plays a central role in the bacterium's
131 transition from soil to gut environments by activating and deactivating key virulence factors from a
132 set of environmental cues (Heras *et al.* 2011). Cold-adaptation is especially important for *L.*
133 *monocytogenes* survival in the FLPSC as low temperatures are readily encountered in the growing
134 and processing environments and during storage of products. *L. monocytogenes* has an innate ability
135 for cold adaptation, partly regulated by σ^B using a variety of mechanisms including the uptake of
136 cryoprotective osmolytes and peptides and the maintenance of cell surface fluidity (Tasara and
137 Stephan 2006). Biofilm production (Ferreira *et al.* 2014) and the ability to enter a protective, viable
138 but non-culturable (VBNC) state (Oliver 2010; Ayrapetyan and Oliver 2016) may also facilitate *L.*
139 *monocytogenes* survival in environments associated with the FLPSC.

140 These mechanisms ensure that *L. monocytogenes* has a more robust cross-stress tolerance
141 compared to other food-borne pathogens such as *E. coli* or *S. enterica* allowing it to survive in food
142 and food associated environments. For this reason, *L. monocytogenes* should not be considered in
143 the same way as other food-borne pathogens and comparatively stronger measures relating to
144 contamination of food and food associated environments are employed to control its presence.

145 *L. monocytogenes* Prevalence in Soil

146 Fresh produce begins its journey through the FLPSC as a seed or transplant in the soil. Soil is a
147 complex, nutritionally rich, heterogeneous environment which is in a state of 'dynamic equilibrium'

148 and contains an abundance of endogenous microbiota, mesofauna and macrofauna (Vivant *et al.*
149 2013). Soil is an environmental niche for *L. monocytogenes* and the bacterium has been readily
150 isolated from soil samples from different locations including meadows, mountainous regions and
151 forests (Linke *et al.* 2014a).

152 Whilst *L. monocytogenes* is nearly always found in low numbers, needing selective enrichment to be
153 detected (i.e. ISO 11290-1 for the presence/absence of *L. monocytogenes* in samples), the bacterium
154 can be found in around 17% of soil samples (Locatelli *et al.* 2013a). Data on the occurrence of *L.*
155 *monocytogenes* in soil from fresh leafy produce production fields indicate between 4% and 11% of
156 soil samples harbour the bacterium (Weller *et al.* 2015). To survive in soil, *L. monocytogenes* must
157 endure physiological stresses and competition from other soil dwelling microorganisms.

158 ## Factors Affecting *L. monocytogenes* Survival in Soil

159 Biotic factors have an important role in determining the size and growth characteristics of the *L.*
160 *monocytogenes* population in soil. McLaughlin *et al.*, (2011) showed an increase in the *L.*
161 *monocytogenes* population of over one log in 4 days from an initial inoculum of 10^7 CFU g^{-1} soil in
162 sterilised soil whilst the population decreased nearly two logs in the same time in unsterilised soil.
163 The suppressive effect of endogenous soil microbiota on *L. monocytogenes* survival in soil has been
164 demonstrated by many authors and reviewed expertly by Vivant, Garmyn and Piveteau, (2013). For
165 example, using a pathogen death rate model, Moynihan *et al.*, (2015) showed that the suppressive
166 effect on *L. monocytogenes* survival by the native soil microbiota increases with an increasingly
167 diverse population. Additionally, when a partial reconstruction of the soil microbiota is re-inoculated
168 into soil after sterilisation, it has a significant suppressive effect on *L. monocytogenes* survival
169 (McLaughlin et al. 2011).

170 *L. monocytogenes* survival in soil is variable by soil type, ranging from rapid decline to long-term
171 persistence, but generally, removing the bacterial population (sterilisation by autoclaving or other)
172 enables *L. monocytogenes* to survive for longer compared to the identical unsterilised soils (Locatelli

et al. 2013b). This effect could be due to competition for nutrients and space combined with inhibitory bacteriocins which are produced by soil bacteria to kill or inhibit the growth of competitors (Bruce *et al.* 2017), meaning that a large inoculum is not sustainable in the soil. Survival has been shown to be dependent on soil type and abiotic factors such as soil texture (especially clay content), pH and basic cation saturation ratio (BCSR) appear to be significant drivers of *L. monocytogenes* survival in soil (Locatelli *et al.* 2013b). Owing to this variation in soil survival (dependant on soil type), there is a need to determine how *L. monocytogenes* survives in soils typically used in the intensive production of fresh leafy produce. This information will infer the risk these commercially important soils pose to fresh leafy produce in terms of *L. monocytogenes* contamination and may allow growers to consider alternative soils to reduce the likelihood of *L. monocytogenes* survival.

Mechanisms of *L. monocytogenes* Survival in Soil

L. monocytogenes survival in the soil has been shown to be significantly affected by the response regulator AgrA and corresponding genes; this regulator controls genes responsible for the transport and metabolism of amino acids and related molecules, genes responsible for motility & chemotaxis and genes that code for other regulators (Vivant *et al.* 2015). Emphasis has also been placed on the role of transporters, which are upregulated by AgrA and allow *L. monocytogenes* to recruit an extensive range of substrates for energy production in the soil (Piveteau *et al.* 2011). Interestingly, *agrA* and *agrD* deletion mutants have altered ability to adhere to surfaces, suggesting the *agr* system's involvement in the early stages of biofilm formation (Rieu *et al.* 2007). Biofilm production and the ability of *L. monocytogenes* to survive in soil appear to be intimately linked as mutants which lack *Lmo0753* (a *prfA* like transcription factor gene) form poor biofilms and show poor survival in soil compared to wild-type strains (Salazar *et al.* 2013). Furthermore, *Lmo0753* is highly conserved in lineage I & II strains, which are more commonly isolated from the soil than lineage III and IV strains (Locatelli *et al.* 2013a; Linke *et al.* 2014b). *SigB* too plays an important role in soil survival – it

198 regulates the stress response after *L. monocytogenes* entry to the soil allowing the bacteria to stop
199 multiplying as a response to nutrient limitation, similar to entry to the stationary phase (Piveteau *et*
200 *al.* 2011). Entry to the soil also causes prfA to be down-regulated, subsequently de-activating key
201 virulence factors whilst genes involved with mobility, chemotaxis and the transport of carbohydrates
202 are up-regulated (Vivant *et al.* 2017).

203 ## Is the Viable but Non Culturable (VBNC) State as a Potential *L. monocytogenes* Strategy for Soil Survival?

204 VBNC cells are metabolically active bacteria that have lost the ability to develop colonies on rich
205 laboratory media and cannot therefore, be detected by conventional methods (i.e. direct plate
206 count). This state is believed to be a survival strategy to minimise energy requirements (Li *et al.*
207 2014). A variety of pathogenic bacteria including *L. monocytogenes* enter a protective VBNC state in
208 response to nutrient starvation, incubation outside the normal temperature, increased or reduced
209 osmotic concentrations and heavy metal exposure (Oliver 2010). Indeed, research has shown that a
210 large fraction of the *L. monocytogenes* population becomes VBNC in microcosms containing pig
211 manure and digestates from agricultural biogas plants (Desneux *et al.* 2016; Maynaud *et al.* 2016).
212 Given that the soil environment may result in nutrient deprivation and other stresses known to
213 induce VBNC, this may also cause *L. monocytogenes* to turn VBNC, but data on this characteristic of
214 the bacterium in the soil environment is missing. Overall, there is evidence to suggest that the VBNC
215 state of *L. monocytogenes* may be important for soil survival, but this whole area requires further
216 study.

217 ## The Risk Posed from Soil Contaminated with *L. monocytogenes*

218 *L. monocytogenes* may be transferred from the soil to fresh produce through soil splash from
219 rainfall/irrigation or general soil contamination from mechanical or human activity. In an experiment
220 assessing the survival and transfer of the *L. monocytogenes* surrogate *L. innocua*, Girardin *et al.*,
221 (2005) demonstrated that transfer of this bacterium to the surface of parsley leaves occurred mostly
222 through soil splash from rain and irrigation after the bacterium was inoculated into the soil. The

223 authors also showed rapid decline of *L. innocua* numbers in soil and noted that when leaf surfaces
224 were contaminated with soil containing bacteria, the number of *L. innocua* was low.

225 Whilst only 1 *L. monocytogenes* cell per 25g $^{-1}$ of sample is required for detection of the bacteria on
226 fresh leafy produce (based on ISO 11290-1 methodology), illness caused by *L. monocytogenes* is
227 usually linked to consumption of food contaminated with a high number of the bacteria (European
228 Commission 1999). Using a dose response model Farber, Ross and Harwig, (1996) determined that
229 inoculum sizes of 10^5 and 10^7 *L. monocytogenes* cells would be required to cause listeriosis infection
230 in 10% and 90% of a 'high-risk' population respectively. These inoculum sizes contrast with the low
231 number of *L. monocytogenes* cells that survive in soil for extended periods and may suggest that
232 contamination of fresh leafy produce by soil borne bacteria is not likely to be a high risk to
233 consumers. However, recent evidence has shown that susceptible individuals can become ill after
234 consuming low levels of the bacteria (Pouillot *et al.* 2016) and infection with *L. monocytogenes* is
235 made more complicated due to the risk of repeated exposure and variation in susceptibility among
236 immunocompromised individuals (Buchanan *et al.* 2017). Therefore, whilst infection from low levels
237 of soil borne *L. monocytogenes* on leafy produce may not be high risk to consumers based on the
238 level of bacteria transferred, it is not possible to rule out infection of susceptible individuals from
239 this type of contamination.

240 Soil spoilage of product is common when growing leafy fresh produce, yet *L. monocytogenes*
241 outbreaks from this food type are rare, implying that soil is not a significant source of *L.*
242 *monocytogenes* in the FLPSC. When contamination does occur, the amount of *L. monocytogenes*
243 transferred to product is likely to be small/minimal based on previous data on the number of *L.*
244 *monocytogenes* present in soil (Locatelli *et al.* 2013a). Conversely, *L. monocytogenes* can proliferate
245 when in contact with a substrate such as cut produce (Salazar *et al.* 2017), but more research is
246 needed to determine its growth behaviours specifically for fresh leafy produce. Additionally, more

investigation is required to determine the effect that this change of environments has on the culturability and infectiveness of this pathogen.

L. monocytogenes Association with Pre-harvest Fresh Leafy Produce

As discussed above, whilst growing in the field, fresh leafy produce may be subject to *L. monocytogenes* contamination through soil splash where the bacteria is transferred to the surface of the leaves. Opportunistic human pathogenic bacteria, including *L. monocytogenes,* can also interact with fresh leafy produce through the root portion of the plant. For example, *E. coli* O157:H7 internalises to the root of lettuce and spinach plants (Wright *et al.* 2013). *L. monocytogenes* has been shown to internalise both into lettuce seedlings and mature plants – the former after 5 days of watering with contaminated water (10^5 CFU ml^{-1}) and the latter when the plant is grown hydroponically with repeated exposure to the same level of *L. monocytogenes* contaminated water (Standing *et al.* 2013). These conditions are unlikely to be encountered in the normal growing environment and so the ability of *L. monocytogenes* to internalise into crop plants under field conditions remains an open question. Opportunistic human pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium have also been shown to be associated with the rhizosphere – the narrow zone of soil influenced by the plant root. *L. monocytogenes* has a supposed preference for the rhizosphere (Dowe *et al.* 1997), but research with *L. monocytogenes* in this area is scarce. Crop plants produce root exudates, improve aeration in the soil and serve as a source of nutrients to soil bacteria, thus improving soil microbial growth and activity. Based on previous evidence this increase in microbial activity could have an increased suppressive effect on *L. monocytogenes* survival. Overall, research is needed to determine how *L. monocytogenes* survives in the soil in the presence of crop plants and whether this bacterium associates with the plant rhizosphere like other opportunistic pathogens.

L. monocytogenes Presence in the Processing Environment

After harvest, fresh produce may be cut, washed and packaged in a dedicated processing facility depending on customer requirements. The processing environment is kept clean through regular sanitation and hygiene barrier systems, such as the segregation of pre- and post-wash product, aim to prevent cross contamination. Despite these measures, *L. monocytogenes* enters the processing facility, unintentionally, through contaminated product and personnel. Cross-contamination of food from the processing environment does occur and research has highlighted that *L. monocytogenes* can persist in the food processing environment and contaminate food products passing through a food processing facility over time (Ferreira *et al.* 2014; Leong *et al.* 2017).

The fresh leafy produce processing environment is in some respects, a stark contrast to the soil – nutritionally poor abiotic surfaces are abundant, detergent application is frequent and refrigeration temperatures are typical. In spite of these different stresses, *L. monocytogenes* can be found in difficult to clean harbourage sites, such as drains, cracks in surfaces and crevices in machinery where disinfectants and sanitisers cannot properly reach (Jordan *et al.* 2015) and nutrients may be available to the bacteria through product debris and factory run off (i.e. water containing leaf juices and soil organic matter etc.). Evidence from factories suggests that *L. monocytogenes* can be introduced into the food processing environment easily, grows at operational temperatures and is resistant to several stresses which results in contamination of the processing environment.

Detection rates for *L. monocytogenes* in food processing facilities changes depending on the type of food processing facility being sampled (Jordan et al. 2015). It is important to note that authors vary in their sampling approach in the food processing environment and so differences in sampling locations and detection methods may influence detection rates between studies. Interestingly, in the largest study of its kind which monitored *L. monocytogenes* prevalence in food and environmental samples across 54 small food businesses in Ireland, fish processing facilities returned the lowest incidence of *L. monocytogenes* positive environmental samples (1.6%), followed by dairy

295 and meat processing facilities (both 4.1%) and vegetable (including fresh leafy produce) processing
296 facilities had the highest incidence of *L. monocytogenes* (9.5%) (Leong *et al.* 2017). Despite the
297 obvious presence of *L. monocytogenes* in the fresh produce processing environment, data on the
298 incidence of *L. monocytogenes* in processing facilities of this food group is scarce.

299 Harbourage Sites and Persistent Strains in Food Processing Facilities

300 Harbourage sites, also known as niches, reservoirs and hard to reach places, are areas in a
301 processing facility which are difficult to clean and may harbour *L. monocytogenes*. Harbourage sites
302 can arise from badly designed or worn equipment (e.g. hollow parts, cracks or crevices), and organic
303 matter from soil and product can be transferred to these areas and persist if not cleaned properly.
304 This process creates a supportive environment for bacterial growth and *L. monocytogenes* can be
305 introduced to harbourage sites from product contaminated outside the processing facility, or from
306 human carriers (Jordan *et al.* 2015). Low temperatures in processing facilities may inhibit the growth
307 of competitors, essentially selecting for *L. monocytogenes* in these niches. Additionally, these
308 harbourage sites may enable the selection of detergent resistant *L. monocytogenes* mutants through
309 ineffective cleaning due to the diluted levels of detergent that the harbourage site is exposed to
310 (Carpentier and Cerf 2011).

311 *L. monocytogenes* is known to persist in the processing environment and harbourage sites are
312 thought to play an important role in persistence of the bacterium in processing facilities. In addition,
313 inappropriate cleaning and sanitation can add to the spreading *L. monocytogenes* in a processing
314 facility through the creation of aerosols. A persistent strain can be defined as repeated isolation of
315 an identical *L. monocytogenes* subtype (as determined by molecular subtyping) from a single
316 processing facility over 6 months. Persistent strains in the food processing environment have been
317 identified by several authors (Sauders *et al.* 2009; Stasiewicz *et al.* 2015; Fagerlund *et al.* 2016)
318 because identifying and subsequently eliminating persistent strains in the processing environment is
319 a key step in reducing consumer risk from *L. monocytogenes* contamination. Leong *et al.*, (2017)

determined that out of 4 food groups tested, vegetable processing facilities had the highest number of persistent strains and the highest diversity of pulsotypes which may reflect *L. monocytogenes* presence and distribution in the growing environment for fruit and vegetables.

Potential Survival Mechanisms of *L. monocytogenes* in the Food Processing Environment

Persistent strains have been shown to exist in the processing environment, but studies which have tried to explain the physiological characteristics which contribute to *L. monocytogenes* persistence vary in their findings. For example, it has been reported that persistent strains show enhanced adherence to food contact surfaces after short contact times (Lundén *et al.* 2000) with some studies suggesting that persistent strains form better biofilms than sporadic strains (Nowak *et al.* 2017) whereas others showing no difference in biofilm formation between persistent and sporadic strains (Magalhães *et al.* 2017). Persistent strains have also been shown to be more resistant to detergent stresses, although this attribute may be due to the characteristics of biofilms rather than intrinsic resistance of the bacterial cell (Pan *et al.* 2006). Cheng *et al.*, (2015) determined that persistent strains showed increased adherence and biofilm formation, but no difference was noted in sanitiser resistance between persistent and transient strains, demonstrating the lack of consensus in the literature. Whether persistent strains confer a physiological advantage compared to their non-persistent counterparts remains an open question as so far, research has generated mixed results which do not explain how persistent strains seem to be able to survive more readily in the processing environment.

In *L. monocytogenes,* σ^B, the major transcriptional regulator of stress response genes, plays an important role in resistance to detergent stresses at lethal levels (Ryan *et al.* 2008). In addition, *SigB* has been shown to be activated in biofilms and appears to be an essential gene for the formation of biofilms with increased resistance to disinfectants in *L. monocytogenes* (Van Der Veen and Abee 2010). *L. monocytogenes* biofilms contribute to persistence in the food processing environment as biofilms can be formed on many different surfaces and serve as a source of subsequent

345 contamination (Colagiorgi *et al.* 2017). Another aspect of *L. monocytogenes* physiology which may
346 contribute to persistence in the food processing environment is the ability of the bacteria to enter
347 the VBNC state. The VBNC state may be triggered in response to numerous physiological cues as
348 mentioned previously. Importantly, in the context of the food processing environment, the
349 sanitation procedure (cleaning and disinfection) leads to a loss in culturability of *L. monocytogenes*
350 and appearance of VBNC populations (Overney *et al.* 2017). By entering a protective, VBNC state, *L.*
351 *monocytogenes* may be able to further resist environmental stresses in the food processing
352 environment (Ayrapetyan and Oliver 2016). Upon entry into a suitable environment (e.g. a
353 harbourage site) VBNC *L. monocytogenes* can subsequently regain culturability and begin to
354 proliferate. Further evidence outlining the potential importance of VBNC *L. monocytogenes* in the
355 food processing environment is demonstrated by work indicating that chlorine stress induces the
356 VBNC state in *L. monocytogenes* and that these VBNC cells remain infectious in a *Caenorhabditis*
357 *elegans* model (Highmore *et al.* 2018).

358 Recent evidence has shown that *L. monocytogenes* ST121, a sequence type commonly associated
359 with food and food environments, carries a stress survival islet (SSI-2) that confers increased survival
360 under oxidative and alkaline stresses which are common in the food processing environment (Harter
361 *et al.* 2017). Overall, *L. monocytogenes* is well suited to surviving the various stresses presented by
362 the fresh produce processing environment and may have a competitive advantage over other
363 contaminating bacteria, facilitated through harbourage sites. Moreover, due to its ubiquitous nature
364 in the growing environment, recontamination of a processing environment in the FLPSC after
365 cleaning and disinfection is possible, meaning that regular sanitation regimes must be undertaken to
366 combat its continuing presence.

367 *L. monocytogenes* Survival on the Product Surface: Post-harvest

368 It has been shown that *L. monocytogenes* survives and grows on a range of fresh products including
369 lettuce (Beuchat and Brackett 1990), mixed vegetable salads (García-Gimeno *et al.* 1996), green and

red peppers and avocado pulp (Salazar *et al.* 2017). Studies such as these have outlined the importance of keeping produce at refrigeration temperatures to slow growth of *L. monocytogenes* populations, but have also demonstrated that post-harvest, *L. monocytogenes* can survive on the surface of fresh produce for extended periods. For example, *L. monocytogenes* can survive on the surface of an apple for up to 12 weeks from an initial inoculum of 3.5 log CFU ml^{-1} (Sheng *et al.* 2017).

Contamination events with relatively high levels of *L. monocytogenes* may be rare in the FLPSC, however, a small bacterial contamination on an injured leaf may lead to growth and colonisation similar to *Salmonella* and pathogenic *E. coli* (Koukkidis *et al.* 2016) increasing the risk to consumers. Of concern to the companies operating within the FLPSC is that any *L. monocytogenes* contamination (1 *L. monocytogenes* per 25g product as determined by ISO 11290-1) of the leaf surface can ultimately lead to a positive detection during routine sampling creating an expensive logistical issue and potential health threat.

Mechanisms of Survival on the Product Surface: Post-harvest

There is good awareness of the *L. monocytogenes* (plus other pathogens) contamination risk to fresh produce and fresh leafy produce is subject to a wash/decontamination step before packaging (ready to eat prepared products) or customers are advised to wash before use (non-prepared, whole head products). The specific requirements for product processing and consumer labelling are controlled by legislation with additional customer-specific demands. The wash step is intended to reduce foreign bodes, dirt and microbial load on the product surface and process wash water contains sanitisers to maintain the water quality during processing. The effectiveness of the wash step in reducing bacterial loads on lettuce leaves that have recently been contaminated with *L. monocytogenes* depends on the amount of time post contamination. Ölmez and Temur, (2010) showed a 99.9% reduction in *L. monocytogenes* when green leaf lettuce was subject to sanitiser treatments 6h after a contamination event. This efficacy was reduced to 90% after applying the

sanitiser treatments 48h post-contamination due to the formation of *L. monocytogenes* biofilms on the leaf surface. Biofilms also facilitate resistance to desiccation, an environmental stress readily encountered on the product surface. *L. monocytogenes* strains which are resistant to desiccation stress may present an increased contamination risk to the consumer due to their ability to survive on the leaf surface. Desiccation resistance has been shown to be influenced by serotype, origin, genotype and virulence with strains of serotype 1/2b being more resistant to desiccation stress than other serotypes (Zoz *et al.* 2017). A further contributing factor to *L. monocytogenes* contamination of post-harvest product is the bacterium's ability to adhere to and persist on abiotic surfaces in the processing environment such as stainless steel and polystyrene (Lee *et al.* 2017). *L. monocytogenes* forms biofilm on a range of abiotic surfaces and it is hypothesised that this characteristic of the bacterium aids in its persistence and subsequent recontamination of post-harvest produce.

Source Tracking *L. monocytogenes* in the Fresh Leafy Produce Supply Chain

Using Subtyping to Source Track *L. monocytogenes* Through the Supply Chain and Identify Persistent Strains

An important step in tackling *L. monocytogenes* contamination in the FLPSC is to identify the source of contaminating bacteria and persistent strains in environments where they may be subsequently eradicated. To do this in food associated environments, subtyping methods such as pulsed gel field electrophoresis (PGFE) and whole genome sequencing (WGS) must be employed. Once common subtypes have been identified, investigators can begin to link separate contamination events and search for commonality (source) between these events (e.g. a single processing facility, farm or deli counter). Subtyping of *L. monocytogenes* during outbreak investigations has successfully revealed sources of contamination including a celery processing environment (Gaul *et al.* 2013) and a cantaloupe processing environment (McCollum *et al.* 2013). Importantly, in the cantaloupe example, the authors did not find any evidence of *L. monocytogenes* in the raw material, establishing the

processing environment as the main source of contamination. These examples indicate the contamination risk from *L. monocytogenes* presence in 'bottle-neck' areas of food supply chains. The same rationale can be applied to contamination events in the FLPSC through regular sampling of fresh produce and surrounding environments. Leong *et al.*, (2017) used PGFE to subtype isolates from a variety of food processing facilities and were able to identify persistent strains in vegetable processing facilities which subsequently contaminated produce, but also recognise that elucidation of the specific source of contamination in a processing facility requires sampling over a longer time. Nastasijevic *et al.*, (2017) applied single nucleotide polymorphism (SNP) analysis to WGS data to determine the genetic relatedness of strains and trace contamination through a meat production facility to a single line (slaughter line) demonstrating that the use of subtyping techniques enables source tracking through a food processing environment. Identification of persistent strains and contamination sources would enable companies who operate in the FLPSC to employ a "seek and destroy" strategy (Stasiewicz *et al.* 2015) to eradicate the contaminating bacteria from contaminated environments. However, routine commercial sampling of fresh produce and surrounding environments is often infrequent and currently only identifies *L. monocytogenes* down to the species level. Even with the advent of subtyping techniques, source tracking in a processing environment remains difficult due to the risk of recontamination, i.e. if an indistinguishable strain is found in a processing environment and on a raw material it doesn't prove that the contamination came from the raw material or vice versa. To elucidate the specific source of *L. monocytogenes* in this context, companies in the FLPSC would have to embark on a regular sampling regime of both the processing environment and raw/processed product combined with molecular subtyping which may currently be beyond the scope (in terms of time and financial investment) of companies operating in this sector. Source tracking with WGS relies on well-designed sampling plans as the difficulties in distinguishing persistent and genetically similar, repeatedly reintroduced *L. monocytogenes* strains in a given environment have been noted (Stasiewicz *et al.* 2015).

As the cost of WGS reduces year on year however, this molecular subtyping method becomes more attractive. In terms of source tracking, WGS data gives a higher resolution (i.e. more distinction between genetically similar isolates) than PGFE, making it a more powerful and reliable tool (Moura *et al.* 2017). Implementation of WGS in the commercial microbiology laboratories which service the FLPSC by testing produce for pathogens produce is limited by expertise in the field, data interpretation and lack of infrastructure (Kwong *et al.* 2015). Implementation is also limited by cost, and whilst the cost per sample is reducing it still remains a significant cost which is prohibitive for such routine use in the FLPSC.

Subtyping *L. monocytogenes* by WGS Can Infer the Potential Risk of Isolates

In addition to being used as a source tracking tool, WGS can infer the risk posed by isolates found in the FLPSC by allowing genome-wide mapping and phylogenetic analysis. WGS can be used to group *L. monocytogenes* isolates based on their phylogenetic lineage. Other sequencing tools such as multi locus sequence typing (MLST) also provide this advantage but unlike MLST, WGS also provides data on the presence and intactness of specific and essential virulence associated genes in *L. monocytogenes* such as internalins (*InlA, InlB, InlC* & *InlJ*) essential for host cell internalisation, listeriolysin O (*hly*) essential for *L. monocytogenes* escape from phagosomes into the cytosol and listeriolysin S (*llsX*), essential for modifying host gut microbiota during infection (Wu *et al.* 2016; Quereda *et al.* 2017). Determining the presence and functioning of these genes could indicate the potential risk that *L. monocytogenes* isolates found in the FLPSC pose to the consumer, although it should be said that missing or non-functioning genes do not necessarily confer reduced virulence or avirulence in an isolate and more research is needed in this area.

Thus, when applied to *L. monocytogenes* isolates in the FLPSC, WGS is only able to give an indication of risk. However, implementation of this technique combined with a *L. monocytogenes* surveillance programme in the supply chain would give insight into the relatedness of the *L. monocytogenes* population that exists in the FLPSC, outlining the frequency with which strains are isolated (thus whether a strain is sporadic or persistent) and their source. WGS also provides phylogenetic

information on isolates and could therefore outline the potential risk they pose to the consumer. This information may be valuable to the companies that operate in the FLPSC by informing risk assessments associated with *L. monocytogenes* contamination, ultimately reducing the risk to the consumer.

Conclusion

Several molecular and physiological mechanisms contribute to *L. monocytogenes* survival in the FLPSC. There are many potential contamination routes in the growing environment of fresh leafy produce that may be difficult or impossible to prevent (e.g. transfer from wild animal faeces) and we suggest that whilst contamination from the soil is possible, it is of low risk to consumers due to the small number of bacteria transferred. *L. monocytogenes* can persist in a processing facility, facilitated by harbourage sites and recontaminate product passing through that facility, making this environment a high priority for the elimination of the bacteria. Although currently expensive, WGS should be used to identify persistent *L. monocytogenes* due to the additional valuable data it provides compared to other subtyping methods. As the cost of WGS reduces, *L. monocytogenes* isolates from the FLPSC should be characterised by this method to determine their source, relatedness and evaluate the risk they pose to the consumer. The authors recommend that future *L. monocytogenes* research should focus on; *L. monocytogenes* survival in soil, transfer to the product surface and subsequent survival on the product surface of fresh leafy produce, *L. monocytogenes* association with the product in the growing environment (i.e. in the soil), the VBNC state of *L. monocytogenes* in the context of survival in the food supply chain, how *L. monocytogenes* biofilms can be mitigated and removed and finally robust methods for determining sources of *L. monocytogenes* in the FLPSC.

Conflict of Interest Statement

The authors declare that no conflict of interest exists.

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730

731 Tables

732 Table 1. Possible sources of *L. monocytogenes* on fresh leafy produce from the growing and
733 processing environments

Environment	Source	Reference
Farm	Soil splash	(Monaghan and Hutchison 2012)
	Contaminated irrigation water	(Heaton and Jones 2008; Hellström 2011; Allende and Monaghan 2015; Weller *et al.* 2015)
	Application of natural fertilisers	(Girardin *et al.* 2005; Oliveira *et al.* 2011)
	Wild animal faecal contamination	(Weis and Seeliger 1975; Fenlon 1985; Inoue *et al.* 1992; Hellström *et al.* 2008; Haase *et al.* 2014)
Processing Environment	Cross contamination from human carriers	(Buchanan *et al.* 2017)
	Cross-contamination from food surfaces	(Khan *et al.* 2016; Buchanan *et al.* 2017; Overney *et al.* 2017)
	Cross contamination from harbourage sites	(Leong *et al.* 2017)

734

735 Figures

736 Figure 1. Summary of the fresh produce supply chain