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

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A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University, for the award of Doctor of Philosophy

April 2020

Declaration

I, Alva Smith declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. This work has not been submitted for any other degree or personal qualification. Where other sources of information have been used, they have been duly acknowledged.

Alva Jay Smith

“The role of the infinitely small in nature is infinitely large”

- Louis Pasteur

Abstract

Listeria monocytogenes is an intracellular bacterial pathogen that has been responsible for many severe disease outbreaks resulting in multiple fatalities. *L. monocytogenes* enters the body through contaminated food and is known to contaminate fresh leafy produce such as spinach and lettuce. In the UK there are many stakeholders in the fresh leafy produce supply chain (FLPSC) including growers, processors and retailers, making the FLPSC complex and the management of bacterial contamination in the supply chain difficult. *L. monocytogenes* is sporadically detected in the FLPSC by routine testing, but currently, little is known about the strains of *L. monocytogenes* present in the UK FLPSC, their potential pathogenicity and phenotypic characteristics. Furthermore, data on *L. monocytogenes* survival in environments associated with the FLPSC, like horticultural soils and the leaf surface, is scarce and requires further investigation. Due to the potential risk of the 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.

Given the importance of these factors, 15 *L. monocytogenes* strains isolated from the UK FLPSC were characterised using a range of genotypic (MLST and WGS) and phenotypic (including biofilm formation and rates of swarming motility) methods. WGS revealed a genetically diverse population spanning two lineages but showed some indistinguishable pairs of isolates, suggesting cross contamination may have occurred in the supply chain. Following characterisation, selected isolates (from lineages I and II) were examined for their ability to survive in environments associated with the FLPSC including horticultural soils and spinach leaves. Results showed no differences in soil survival between strains, but strains were detected at a higher level (in sterilised soil) for longer in a clay loam soil compared to other types of soil in the short term (over 70 days). In contrast, *L. monocytogenes* was detected at higher levels in sandy soil using an illumina based NGS method in the long term. Washing contaminated spinach in chlorine water at operational levels (60ppm) was found to reduce *L. monocytogenes* number by 1log but electrolysed water (a chlorine alternative) showed lower efficacy at operational free levels of free chlorine, suggesting electrolysed water is not a viable alternative to chlorine wash at these levels. Survival on spinach leaves was influenced

by temperature and whether the leaves were subjected to a chlorine wash, or not. *L. monocytogenes* populations grew to a higher level on contaminated spinach at room temperature vs. when contaminated leaves were refrigerated. This demonstrated the importance of maintaining refrigeration temperatures throughout the supply chain. At both room and refrigeration temperatures, washing spinach leaves in a chlorine wash meant that *L. monocytogenes* populations grew to a higher level over the shelf life of the spinach. In addition, the endogenous microflora present on ready to eat (washed) spinach was less diverse and abundant than spinach which hadn't been washed.

Overall, the results of this work suggest that WGS technology should be phased into *L. monocytogenes* surveillance programmes in the FLPSC for purposes of source tracking and risk assessment so incidences of *L. monocytogenes* contamination can be controlled or reduced e.g. by cleaning and sanitation of affected areas. Furthermore, implementing this technology may give customers (retail) and consumers added confidence that growers and processors are informed of the *L. monocytogenes* risk in their supply chain and demonstrates a precautionary, rather than reactionary approach to consumer safety. Results from soil survival experiments suggest that *L. monocytogenes* found in the FLPSC can survive for extended periods in horticultural soils and thus, it was concluded that soil is a potential source of contamination in the FLPSC. However, the level of *L. monocytogenes* in horticultural soil declines quickly and transfer of the bacteria to soil on a large scale (in the field for example) is unlikely, suggesting that this risk of contamination from this source is low. Furthermore, sporadic contamination from this source is hard to prevent due to the intimate nature of the growing environment (soil) with fresh produce products. Results from the final chapter of this work show the chlorine wash at operation levels for the UK FLPSC is an effective sanitiser with regards to *L. monocytogenes* in a pre-wash contamination scenario. On the other hand, it was shown that chlorine wash may enable *L. monocytogenes* populations to reach a higher level on spinach leaves when the leaves have been contaminated post-wash by reducing the abundance and diversity of endogenous leaf microflora.

Dedication

I would like to dedicate this work to my Gramps, Paul Shadforth, who was a constant source of love and inspiration for me. He sadly passed away during the PhD process and will be missed dearly, by everyone he knew. Rest in Peace, Pops.

Acknowledgments

Firstly, I would like to express my gratitude to my supervisors at Edinburgh Napier University, Prof. Ian Singleton, Dr. Clare Taylor, Dr. Nick Wheelhouse for their continuous support during the entirety of this project. I am grateful for the discussions, advice, company and patience they have shown me during my studies. Their encouragement has been a huge help to me over the past 3 years and I'm incredibly grateful and proud to have been able to carry out the project under their mentorship.

I would also like to extend my gratitude to the faculty staff at Edinburgh Napier University, without whom research at the institution wouldn't be possible, especially Allen Flockhart who was a great help during long hours in the lab. I'm incredibly thankful for the discussions, and the laughs. Furthermore, I'd like to thank the post graduate community at Edinburgh Napier University for all their support.

Thanks to Dr. Jim Monaghan from Harper Adams University for being so welcoming during my time there and who's advice and direction have been invaluable. Thanks too, to Dr. Ed Moorhouse and Richard Barrett, my supervisors from the fresh produce industry who provided great support with their insightful knowledge of the industry.

I wish to express my appreciation for Stefan Smith, Adam Croy, Dr. Lee Curley, Nikki Peddie and the rest of my Edinburgh family for their support. I'm incredibly grateful that they made me feel so welcome north of the border. I've made friends for life in Edinburgh and will remember my time there fondly, thanks to them.

To my girlfriend, Ruth Watson, thank you for your love and support. Having you by my side served as a source of constant strength through many difficulties. Thank you for listening and understanding. We did it.

Finally, I wish to express my deepest gratitude for my family, especially my Grandparents Mary Smith and Shirley Shadforth, my big Brother Joe and my Mam and Dad, Peter and Paula Smith, who inspire me every day with their kindness, generosity and love. Thank you for everything.

Publications

1. Smith, A., Hearn, J., Taylor, C., et al. (2019) Listeria monocytogenes isolates from ready to eat plant produce are diverse and have virulence potential. Int J Food Microbiol 299:23–32. doi: 10.1016/j.ijfoodmicro.2019.03.013
2. Smith, A., Moorhouse, E., Monaghan, J., et al. (2018) Sources and survival of Listeria monocytogenes on fresh, leafy produce. J Appl Microbiol 1–13. doi: 10.1111/jam.14025

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Abbreviations

°C	Degrees Celsius
µl	Microlitres
µmol	Micromolar
ANOVA	Analysis of variance
BHI	Brain heart infusion
bp	Base pairs
CFU	Colony forming units
cgSNPs	Core genome single nucleotide polymorphisms
CNS	Central nervous system
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dw	dry weight
EtOH	Ethanol
EU	European Union
FLPSC	Fresh leafy produce supply chain
g	gram
h	hours
ISO	International organisation for standardization
l	litre
LIPI	Listeria pathogenicity island
LLO	Listeriolysin O
min	minutes
ml	Millilitres
MPN	Most probable number
MLST	Multi locus sequence typing
NGS	Next generation sequencing

nm	Nanometres
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed gel-field electrophoresis
PLCs	phospholipases C
pmol	picomolar
PMSCs	Premature stop codons
RBCs	Red blood cells
rpm	revolutions per minute
RTE	Ready to eat
s	seconds
SDW	Sterilised distilled water
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SSI	Stress survival islet
ST	Sequence type
TAE	Tris base, acetic acid and EDTA
TSB	Tryptone soy broth
UK	United Kingdom
V	volts
VBNC	Viable but non-culturable
VDFB	Virulence factors of bacterial pathogens
WGS	Whole genome sequencing

Chapter 1 General Introduction

1.1. General introduction

Listeria monocytogenes is a Gram-positive, facultative anaerobic, opportunistic bacterial pathogen. The bacterium is actively mobile via peritrichous flagella expressed throughout the bacterial body at environmental temperatures but not at human body temperature (Carvalho et al. 2014). It is the causative agent of listeriosis, a disease which predominantly affects immunocompromised people including the elderly, immunosuppressed (e.g. those undergoing cancer treatment or HIV/AIDS patients) and pregnant women together with their unborn or new-born babies. Advanced listeriosis manifests as encephalitis, septicaemia and meningitis and has a 20-30% mortality rate (Ramaswamy et al. 2007). *L. monocytogenes* mediates the switch between environmental saprophyte to intracellular pathogen through complex regulatory pathways that modulate the expression of virulence factors in response to environmental cues (Gray and Boor 2006). Contaminated foodstuffs are the main cause of infection by this bacteria and there have been several well-documented, high-profile outbreaks from this source over recent years (Salamina et al. 1996; Makino et al. 2005; Pichler et al. 2011; McCollum et al. 2013; Garner and Kathariou 2016). Foodborne *L. monocytogenes* infections accounted for 99% of all illnesses from *L. monocytogenes* in the USA in 2006 (Scallan et al. 2011).

Because of the risk of infection from food, national safety authorities impose stringent limits on the number of *L. monocytogenes* cells that can contaminate food products. Guidelines in the United States advise that *L. monocytogenes* should not be present (<1 CFU 25 g^{-1}) in ready to eat (RTE) foods that support the growth of *L. monocytogenes* and should not be equal to or above 100 CFU per g 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 per g 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). These low limits of acceptable contamination are due to the risks of *L.*

monocytogenes infection in highly susceptible individuals (Pouillot et al. 2016) coupled with the bacterium's ability to grow in a range of food substrates (Berrang, Brackett and Beuchat, 1989; Leong *et al.*, 2013; Jami *et al.*, 2014). Uncooked foods are the most likely source of infection because the time-temperature combination of the cooking process is normally sufficient to destroy *L. monocytogenes* (Nightingale et al. 2004a). Foods which have previously been identified as sources of *L. monocytogenes* 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. In the UK, the potential risk from this bacterium has been increased by the recent trend for 'healthy convenience' in the food industry that has resulted in a rise in consumption of fresh produce lines and minimally processed products, which include fresh produce components (FSA 2007). These products are consumed raw, preventing destruction of the bacteria through cooking.

Due to significant consumer risk, the fresh produce industry is subject to heavy internal and external regulatory pressure to minimise *L. monocytogenes* contamination. Therefore, any contamination of produce by this bacterium is of substantial concern to companies that operate in the fresh produce supply chain. 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). *L. monocytogenes* contamination of fresh leafy produce lines, such as salad ingredients (lettuce, wild rocket etc.) and leafy brassicas (kale, spinach etc.) is important in terms of public health because these foods account for a significant proportion of the UK market in both volume and value (Keynote 2015). Furthermore, these food items are more "high-risk" in terms of bacterial contamination because of their leaf structures and proximity to the ground. During the UK growing season (late spring to late summer) when temperatures are most favourable and consumer demand is high, these leafy salad vegetables are typically grown on a large scale in a field as opposed to glasshouses, which may be used to extend the growing season or provide additional protection. When the crop is harvested, it may be further processed in a dedicated facility, which washes, cuts and

packages the crop depending on customer (retail outlets) requirements. Transport to and from the farm, the processing facility and retail units is undertaken by a fleet of refrigerated trucks. Thus, the fresh leafy produce supply chain (FLPSC), from farm to fork, is complex and contains a diverse range of environments. *L. monocytogenes* can be detected during routine sampling of fresh leafy produce throughout the supply chain from soil, recently harvested crops, the processing environment and in the final the product itself, although detection tends to be sporadic.

For companies which operate in the supply chain, determining the source of *L. monocytogenes* on product remains difficult due to its ubiquitous nature in the environment. To source track *L. monocytogenes*, an increasing array of tools are becoming available with the gold standard being whole genome sequencing (WGS). These tools can characterise strains of the bacterium into various subtypes based on phenotypic (e.g. serotyping based on cell surface antigens) and genotypic (e.g. single nucleotide polymorphisms in the whole genome) methods. However, use of these tools on a day-to-day basis in the food industry is not currently 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 (Makino et al. 2005; Pichler et al. 2011; Gaul et al. 2013; McCollum 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 the avoidance of contaminated area). Subtyping can also indicate the potential risk of *L. monocytogenes* isolates. However, routine sampling often only characterises isolates down to the species level and ignores such benefits.

Due to the potential risk 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. Given the importance of source tracking, risk assessment and understanding the ability of *L. monocytogenes* to survive in the FLPSC, this thesis concentrates on characterising the *L. monocytogenes* isolates found in the FLPSC using physiological and molecular methods and determines the

bacterium's survival in a range of environments associated with the FLPSC including soil and the leaf surface.

1.2. 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 reviewed in depth previously (see Monaghan and Beacham (2017)), but it is useful to provide a brief overview here. The chain starts in the field where a crop typically takes between 3-24 weeks to grow before harvest, either 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 1.1).

Industry wide 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 protocols are specified by the retail customers (i.e. supermarkets, etc.) of companies that grow, and process produce to reduce microbial contamination of crops. Minimum standards do exist with regards to food for human consumption (UK/EU legislation) but these are usually trumped (in terms of being scrupulous) by standards set by retail customers. Common protocols include preventing farmers from growing crops on land that has been treated 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 and presence of *L. monocytogenes* or indicator species (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 et al. 2009). Despite these measures, *L. monocytogenes* continues to sporadically contaminate fresh leafy produce.

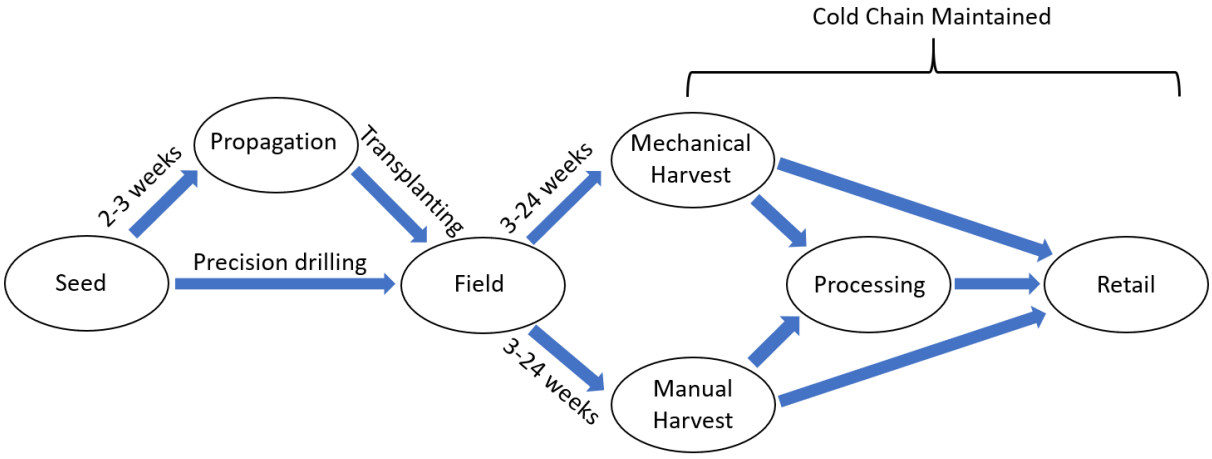

Figure 1.1. **Schematic diagram of a summary of the fresh produce supply chain**

1.3. 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 1.1/Figure 1.2). 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). Overall, *L. monocytogenes* has many potential contamination routes to fresh produce.

Table 1.1.**Possible sources of *L. monocytogenes* on fresh leafy produce from the growing and 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)

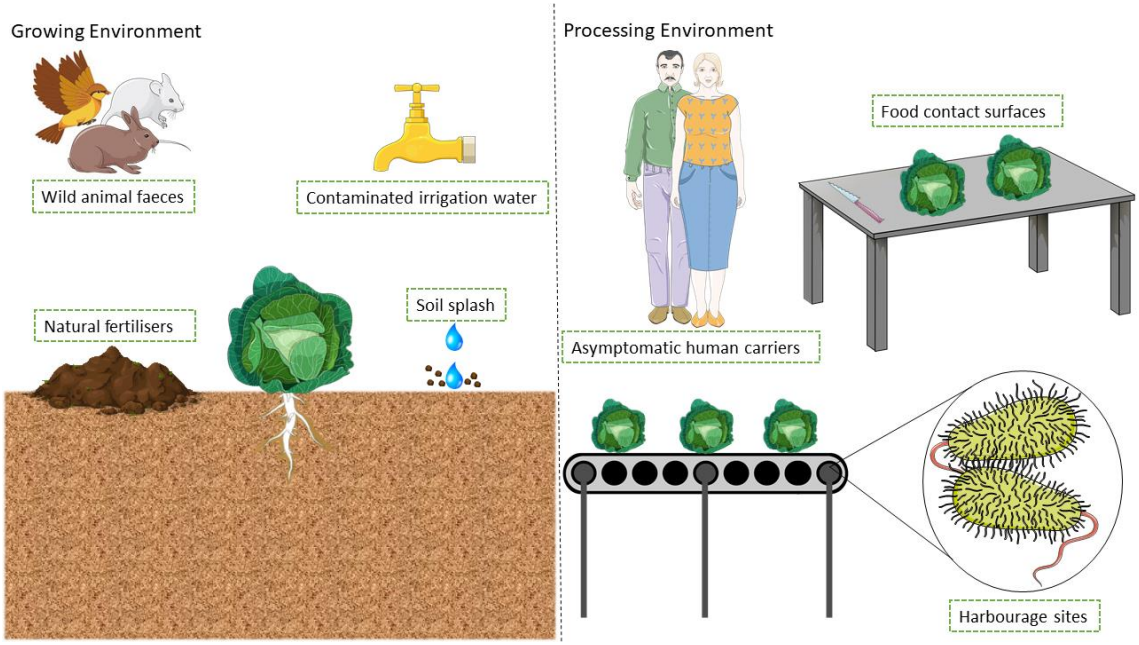

Figure 1.2. **Schematic diagram of possible sources of *L. monocytogenes* in the fresh produce supply chain.**

1.4. Outbreaks of *L. monocytogenes* associated with fresh produce

Sporadic and epidemic cases of listeriosis are exclusively caused by the consumption of contaminated foods, and while the incidence of listeriosis is relatively low compared to other bacterial pathogens (Allerberger and Wagner 2010), the disease outcome is often more serious, making it a priority pathogen for diagnosis and subsequent reporting in 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 enterica* and *Escherichia coli*.

Outbreaks caused by RTE food products such as deli meats, dairy products and fresh produce are more likely because a bacterial kill step (i.e. the cooking process) is not undertaken before eating. The potential of these products to be significant routes of infection has been demonstrated in two of the largest recent outbreaks of listeriosis. Between 01 Jan 2017 – 05 Jun 2018 in South Africa, there were 1049 laboratory confirmed cases of listeriosis, with 209 reported deaths as a result of consumption of contaminated Polony (National Listeria Incident Management Team 2018). Cantaloupe melon, another RTE product, was identified as the source of a large outbreak in the USA, where 147 cases of listeriosis were reported, with 33 deaths (McCollum et al. 2013).

As outlined above (section 1.2), fresh produce may have an increased risk of *L. monocytogenes* contamination because there are many potential sources of *L. monocytogenes* in both the growing and processing environments for these products. Fresh produce outbreaks such as those outlined in Table 1.2. lead to a loss of consumer confidence and related product value, hence the importance of preventing such outbreaks for the stakeholders in the FLPSC that produce and process this type of food product.

Table 1.2. **List of *L. monocytogenes* outbreaks associated with fresh produce**

Location	Year	Cases/Deaths	Food Vehicle	Reference
San Giorgio di Piano, Italy	1993	39/0	Rice salad	(Salamina et al. 1996)
Texas, USA	2010	10/5	Chopped celery	(Gaul et al. 2013)
Colorado, USA	2011	147/33	Cantaloupe melons	(McCollum et al. 2013)
Illinois and Michigan, USA	2014	05/02	Mung bean sprouts	(Garner and Kathariou 2016)
California, USA	2014	32/1	Caramel apples	(CDC Centers for Disease Control and Prevention 2016)
Ohia, USA	2016	19/1	Packaged salads	(CDC Centers for Disease Control and Prevention 2016)
California, USA	2016	9/3	Frozen vegetables	(CDC Centers for Disease Control and Prevention 2016)
Multi-country, Europe	2018	47/9	Frozen vegetables	(European Food Safety Authority 2018)

1.5. Summary of the *L. monocytogenes* infection cycle

The *L. monocytogenes* infection cycle starts when it enters the gut of a host from contaminated food where it proceeds with the intracellular infection cycle through entry into intestinal epithelial cells. Here the bacteria replicates and, after translocation via lymph and blood, the liver is the first organ where *L. monocytogenes* arrives before the infection is controlled by host cell-mediated immune response (Vazquez-Boland et al. 2001). In healthy individuals ingesting a large inoculum (> 10^6 - ~10^9 CFU g^{-1}), clinical symptoms are restricted to a self-limited febrile gastroenteritis (Drevets and Bronze 2008). However, if replication is not controlled by host innate immune response, the bacteria escape from immune clearance and continue to divide and replicate. This can ultimately lead to the development of severe central nervous system (CNS) infection with or without bacteraemia i.e. listeriosis. As such, in immunocompromised people, a reduced immune response means that *L. monocytogenes* has the potential to invade the CNS causing severe disease and even death. Symptoms include fever, myalgia, septicaemia and meningitis. *L. monocytogenes* has the highest case fatality rate amongst foodborne diseases at around 20-30% which makes it high priority and concern for both food production and processing companies and the regulatory bodies that oversee them.

As a model intracellular pathogen, the infection cycle of *L. monocytogenes* has been studied extensively. Briefly, *L. monocytogenes* binds to E-cadherin, a host cell invasion molecule, and enters the cell using a family of invasion proteins, the internalins which include InlA and InlB. Following internalisation, the bacterium mediates its escape from the membrane bound vacuole by secreting a pore forming cytolysin known as Listeriolysin O (LLO) (Freitag et al. 2009). When in the host cell cytosol, *L. monocytogenes* replicates using nutrients from the host and moves through the cell and into adjacent cells using the actin polymerisation force through the surface protein ActA (Liu 2008). Once inside adjacent cells, the bacteria secrete LLO and two phospholipases C (PLCs) to escape the double membrane vacuole formed as a result of cell to cell spread (Figure 1.3.) (Portnoy et al. 2002).

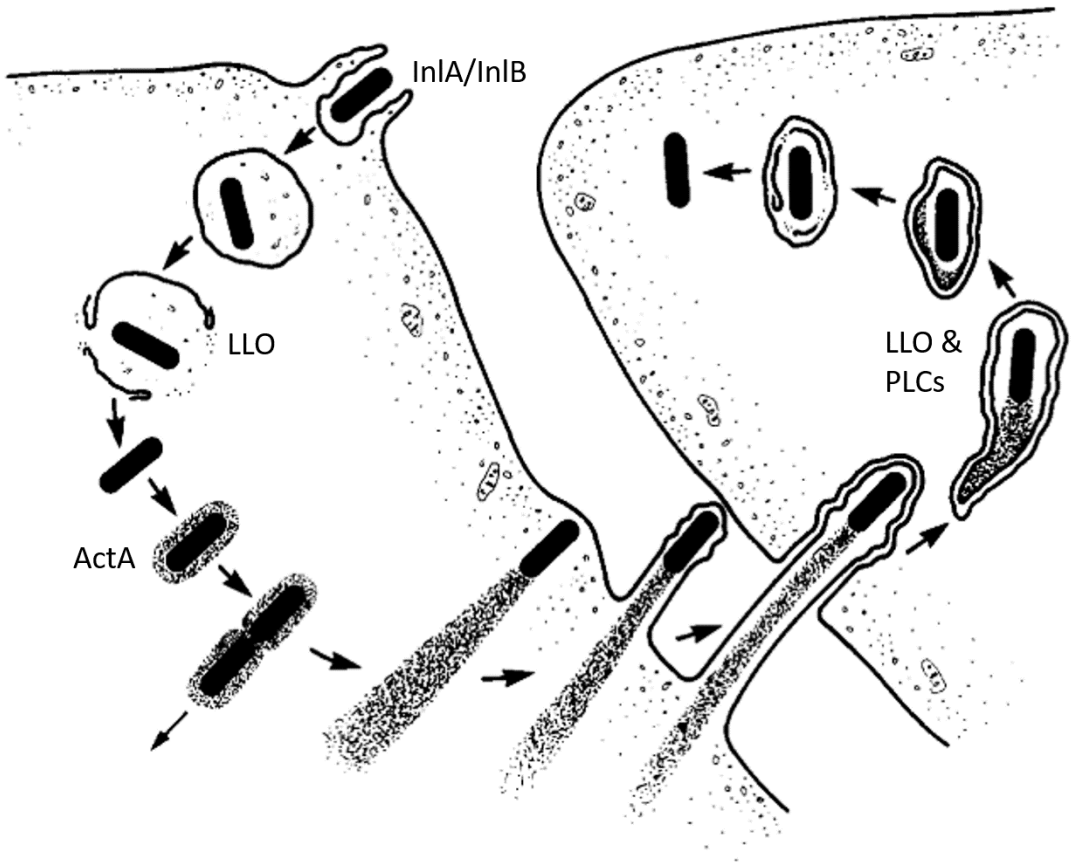

Figure 1.3. **The *L. monocytogenes* intracellular infection cycle.** Cartoon depicting; entry to host cell via InlA & InlB, escape from vacuole mediated by LLO, actin polymerisation using ActA, transport to adjacent cell and escape from double vacuole mediated by LLO & PLCs. All proteins are mentioned in the text. Adapted from Tilney and Portnoy (1989).

1.6. *Listeria monocytogenes*: an organism adapted to survive in the fresh leafy produce supply chain

Outside the host, 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. This ability to withstand physiochemical stresses is a major factor in *L. monocytogenes* ability to contaminate chilled and minimally processed foods.

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

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

1.7. Characterising *L. monocytogenes* by molecular methods reveals diversity within species

L. monocytogenes can be classified into at least 14 different serotypes– these serotypes are defined based on the reactions of somatic (O) and flagellar (H) antigens with a series of polyvalent and monovalent antisera (Palumbo et al. 2003). Furthermore, the species is divided into at least 4 distinct genetic lineages (I, II, III &

IV). These lineages occupy different ecological niches, but most isolates fall into lineages I & II, where isolates from these lineages are more commonly associated with food and human clinical cases of disease. In general, most outbreaks are caused by isolates from lineage I (Orsi et al. 2011). To further classify isolates, researchers rely on molecular techniques such as multi locus sequence typing (MLST), pulsed gel field electrophoresis (PFGE) and whole genome sequencing (WGS) which will all be described in greater detail later in this section. Characterising the bacteria in these ways has traditionally been a way of identifying a commonality between related cases of listeriosis and more virulent types of the bacteria. More recently, the power of WGS in terms of differentiating between closely related strains has allowed outbreak investigators to determine the source of *L. monocytogenes* on contaminated foodstuffs. Differentiating between closely related strains is key to source tracking in outbreak investigations. The remainder of this section describes the molecular methods mentioned above which have been used historically to characterise *L. monocytogenes* and discusses their advantages and disadvantages in the context of isolates relevant to food and clinical sources.

1.7.1. Diversity in the species based on serotyping

Serotyping *L. monocytogenes* is based on the presence of O & H antigens on the surface of the bacterial body and is has traditionally been used as a first line typing method over other, more laborious typing methods such as PFGE. Since 95% of all human illness is caused by just 4 serotypes out of the 14 serotypes (1/2a, 1/2b, 1/2c, and 4b), previously, serotyping was used to outline potential outbreak strains compared to those which might sporadically be isolated (Doumith et al. 2004). The established, serological method for serotyping which involves analysis of the patterns of somatic (O) and flagella (H) antigens in *L. monocytogenes* can be seen in Seeliger and Hohne (1979). However, this technique is labour intensive and is often limited by cost, availability of reagents and technical expertise to perform such an analysis. It has also been noted that the reproducibility of serotyping is not always satisfactory (Doumith et al. 2004). Due to these difficulties in the original method, numerous researchers have developed a PCR based serotype identification method for *L. monocytogenes* (Borucki and Call 2003; Doumith et al. 2004; Wei and Knabel 2005). Whilst this new method is more cost effective, less labour intensive and cheaper,

serotyping still fails to characterise *L. monocytogenes* to the precision required for reliable source tracking during outbreak investigations.

1.7.2. Multilocus sequencing typing (MLST) can be used as an important surveillance tool for *L. monocytogenes*

MLST is a technique, which uses the typing (sequencing) of various loci to characterise isolates in a bacterial species. The MLST scheme used to characterise *L. monocytogenes* isolates is determined by the allelic profile of 7 housekeeping genes, these are; ABC transporter (*abcZ*), beta-glucosidase (*blgA*), catalase (*cat*), succinyl diaminopimelate (*dapE*), D-amino acid aminotransferase (*dat*), L-lactate dehydrogenase (*ldh*) and histidine kinase (*lhkA*). Briefly, the method involves extracting DNA from an isolate then conducting PCR amplification of each of genes outlined above, using specific primer pairs. Products from PCR reactions are then sequenced and the sequences are submitted to an international database (https://bigsdb.pasteur.fr/listeria/listeria.html) to allow comparison to previous strains and generate a sequence type. The PCR primers are often generated with sequencing tails so that PCR products can be sequenced using just one set of sequencing primers. A more detailed method for MLST of *L. monocytogenes* isolates can be seen in Salcado et al. (2003). MLST of isolates is generally now coordinated by the Institut Pasteur in France, where researchers can find instructions, online tools and guidance for submission of data so that isolates can be compared internationally.

MLST has been used previously as a surveillance and observation tool to characterise the subtype of *L. monocytogenes* (Wu et al. 2016). Whilst other subtyping tools such as PFGE offer more discriminatory power, MLST defines genetically coherent groups based on a common ancestor (Nemoy et al. 2005) which allows phylogenic analysis and determination of the relatedness of strains. Furthermore, MLST can be used in conjunction with whole genome sequencing to corroborate historical MLST data (Ruppitsch et al. 2015) whilst PFGE cannot. Variants on the MLST methods such as multi-virulence-locus sequence typing (MVLST) aim to analyse the genetic diversity, evolution and ecology of *L. monocytogenes* based on genes which evolve more quickly over time than housekeeping genes (Kimura 2006). In the case of MVLST these are virulence genes. Whilst MLST and associated methods are appropriate and powerful tools for investigating the evolutionary patterns and genetic diversity of *L.*

monocytogenes isolates from the environment (Linke et al. 2014b), they lack the discriminatory power to appropriately differentiate between closely related isolates which is key to outbreak investigations.

1.7.3. Pulsed field gel-electrophoresis (PFGE) – the traditional outbreak investigation method

PFGE is a technique used for the separation of large DNA molecules, which involves a variation in the standard electrophoresis protocol by alternating a voltage gradient to improve the resolution of larger DNA molecules. A standard protocol for PFGE with *L. monocytogenes* isolates is provided by Pulsenet (2013) who also curate a platform for cooperating groups to share experimental data across laboratories meaning that large scale outbreaks can be more easily identified (https://www.cdc.gov/pulsenet/index.html). This method is traditionally thought of as 'fingerprinting' and many studies and outbreak investigations have outlined the effectiveness of the method in distinguishing between closely related strains and identifying outbreak strains (Fugett et al. 2007; Laksanalamai et al. 2012; Dahl et al. 2017). A high congruence between PFGE and MLST results has been recognised (Henri et al. 2016) but PFGE is more discriminatory. Having previously been the 'gold-standard' in molecular characterisation of *L. monocytogenes* strains, technological advances in bioinformatics and computer processing power have yielded a new technology - entirely sequence based which requires little work in the laboratory and generates a plethora of data. This technology is whole genome sequencing (WGS) and is discussed in detail in the context of microbial sampling and source tracking in the fresh produce supply chain, later in this chapter (Section 1.12.)

1.8. *L. monocytogenes* survival in soil

1.8.1. *L. monocytogenes* prevalence in soil

Fresh produce begins its journey through the FLPSC as a seed or transplant in the soil. Soil is a complex, nutritionally rich, heterogeneous environment which is in a state of 'dynamic equilibrium' and contains an abundance of endogenous microbiota, mesofauna and macrofauna (Vivant *et al.* 2013). Soil is considered to be an environmental niche for *L. monocytogenes* and the bacterium has been readily isolated

from soil samples from different locations including meadows, mountainous regions and forests (Linke et al. 2014a) (Table 1.3).

Table 1.3. **Soil and related environments associated with the recovery of *L. monocytogenes* from samples.** ND = not done.

References	Sample Type	Samples positive for *L. monocytogenes* (%)	Dominant serotypes
(Sauders et al. 2012)	Soil & vegetation	4.32	ND
(Linke et al. 2014a)	Soil & water	6	1/2a, 3a, 4b, 4d, 4e
(Weller et al. 2015)	Soil, faecal, leaf & water from spinach field	9	ND
(Locatelli et al. 2013a)	Soil	17	1/2a, 3a, 4b, 4d, 4e
(Nightingale et al. 2004b)	Faecal, feed, soil & water samples from ruminant farms	24.4	ND

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

1.8.2. Factors affecting *L. monocytogenes* survival in soil

Biotic factors have an important role in determining the size and growth characteristics of the *L. monocytogenes* population in soil. McLaughlin *et al.*, (2011) showed an increase in the *L. monocytogenes* population of over one log in 4 days from an initial inoculum of 10^7 CFU g^{-1} soil in sterilised soil whilst the population decreased nearly

two logs in the same time in fresh soil. The suppressive effect of endogenous soil microbiota on *L. monocytogenes* survival in soil has been demonstrated by many authors and reviewed expertly by Vivant *et al.,* (2013). For example, using a pathogen death rate model, Moynihan *et al.*, (2015) showed that the suppressive effect on *L. monocytogenes* survival by the native soil microbiota increases with an increasingly diverse population. Additionally, when a partial reconstruction of the soil microbiota is re-inoculated into soil after sterilisation, it has a significant suppressive effect on *L. monocytogenes* survival (McLaughlin et al. 2011).

L. monocytogenes survival in soil is variable by soil type, ranging from rapid decline to long-term persistence, but generally, removing the bacterial population (sterilisation by autoclaving or other) enables *L. monocytogenes* to survive for longer compared to the identical fresh 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). Overall, evidence suggests that there are many factors affecting *L. monocytogenes* survival in soil (Figure 1.4). 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 and subsequent contamination.

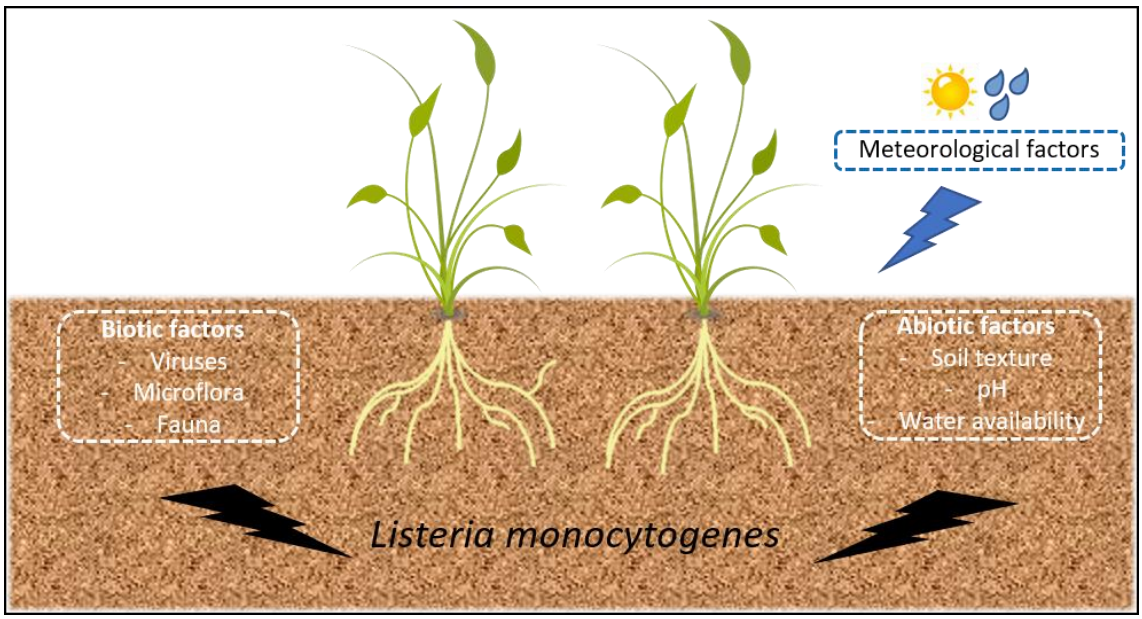

Figure 1.4. **Schematic diagram of some factors affecting *L. monocytogenes* survival in soil.** Adapted from Vivant et al. (2013).

1.8.2. 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). Importantly *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 regulates the stress response after *L. monocytogenes* entry to the soil allowing the bacteria to stop multiplying as a response to nutrient limitation, similar to entry to the stationary phase (Piveteau *et al.* 2011). Entry to the soil also causes prfA

to be down-regulated, subsequently de-activating key virulence factors whilst genes involved with mobility, chemotaxis and the transport of carbohydrates are up-regulated (Vivant *et al.* 2017).

1.8.2.1. Is the viable but non-culturable (VBNC) state as a potential *L. monocytogenes* strategy for soil survival?

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

1.8.3. The risk posed from soil contaminated with *L. monocytogenes*

L. monocytogenes may be transferred from the soil to fresh produce through soil splash from rainfall/irrigation or general soil contamination from mechanical or human activity. In an experiment assessing the survival and transfer of the *L. monocytogenes* surrogate *L. innocua*, Girardin *et al.*, (2005) demonstrated that transfer of this bacterium to the surface of parsley leaves occurred mostly through soil splash from rain and irrigation after the bacterium was inoculated into the soil. The authors also showed rapid decline of *L. innocua* numbers in soil and noted that when leaf surfaces were contaminated with soil containing bacteria, the number of *L. innocua* was low.

Whilst only 1 *L. monocytogenes* cell per 25g $^{-1}$ of sample is required for detection of the bacteria on fresh leafy produce (based on ISO 11290-1 methodology), illness

caused by *L. monocytogenes* is usually linked to consumption of food contaminated with a high number of the bacteria (European Commission 1999). Using a dose response model Farber, Ross and Harwig, (1996) determined that doses of 10^5 and 10^7 *L. monocytogenes* cells would be required to cause listeriosis infection in 10% and 90% of a 'high-risk' population respectively. These inoculum sizes contrast with the low number of *L. monocytogenes* cells that survive in soil for extended periods and may suggest that contamination of fresh leafy produce by soil borne bacteria is not likely to be a high risk to consumers. However, recent evidence has shown that susceptible individuals can become ill after consuming low levels of the bacteria (Pouillot *et al.* 2016) and infection with *L. monocytogenes* is made more complicated due to the risk of repeated exposure and variation in susceptibility among immunocompromised individuals (Buchanan *et al.* 2017). Therefore, while infection from low levels of soil borne *L. monocytogenes* on leafy produce may not be high risk to consumers based on the level of bacteria transferred, it is not possible to rule out infection of susceptible individuals from this type of contamination.

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

1.9. *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 – in seedlings after 5 days of watering with contaminated water (10^5 CFU ml^{-1}) and in mature plants when 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 on *L. monocytogenes* in the rhizosphere 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, there is a need for improvement in our understanding of 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.

1.10. *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, which 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. Despite these different stresses, *L. monocytogenes* can be found in difficult to clean harbourage sites. Harbourage sites

are areas of food production facilities that are difficult to reach or to clean and disinfect properly. These sites include 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.). *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 change 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. 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%). This was followed by dairy and meat processing facilities (both 4.1%) and vegetable (including fresh leafy produce) processing facilities had the highest incidence of *L. monocytogenes* (9.5%) (Leong *et al.* 2017). Despite the obvious presence of *L. monocytogenes* in the fresh produce processing environment, data on the incidence of *L. monocytogenes* in processing facilities of this food group is scarce.

1.10.1. Harbourage sites and persistent strains in food processing facilities

Harbourage sites, also known as niches, reservoirs and hard to reach places, are areas in a processing facility, which are difficult to clean and may harbour *L. monocytogenes*. Harbourage sites can arise from badly designed or worn equipment (e.g. hollow parts, cracks or crevices), and organic matter from soil and product can be transferred to these areas and persist if not cleaned properly. This process creates a supportive environment for bacterial growth and *L. monocytogenes* can be introduced to harbourage sites from product contaminated outside the processing facility, or from human carriers (Jordan *et al.* 2015). Low temperatures in processing facilities may inhibit the growth of competitors, essentially selecting for *L. monocytogenes* in these niches. Additionally, these harbourage sites may enable the selection of detergent

resistant *L. monocytogenes* mutants through ineffective cleaning due to the diluted levels of detergent that the harbourage site is exposed to (Carpentier and Cerf 2011).

L. monocytogenes is known to persist in the processing environment, and harbourage sites are thought to play an important role in persistence of the bacterium in processing facilities. In addition, inappropriate cleaning and sanitation can add to the spread of *L. monocytogenes* in a processing facility through the creation of aerosols. A persistent strain can be defined as repeated isolation of an identical *L. monocytogenes* subtype (as determined by molecular subtyping) from a single processing facility over 6 months. Persistent strains in the food processing environment have been identified by several authors (Sauders *et al.* 2009; Stasiewicz *et al.* 2015; Fagerlund *et al.* 2016) because identifying and subsequently eliminating persistent strains in the processing environment is a key step in reducing consumer risk from *L. monocytogenes* contamination. Leong *et al.*, (2017) determined that out of four food production groups tested, vegetable processing facilities had the highest number of persistent strains and the highest diversity of pulsotypes (a subtype defined by PFGE), which may reflect *L. monocytogenes* presence and distribution in the growing environment for fruit and vegetables.

1.10.2. Potential survival mechanisms of *L. monocytogenes* in the food processing environment

Persistent strains have been demonstrated 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). Some studies suggest that persistent strains form better biofilms than sporadic strains (Nowak *et al.* 2017). Conversely others show 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 contamination (Colagiorgi *et al.* 2017). Another aspect of *L. monocytogenes* physiology which may contribute to persistence in the food processing environment is the ability of the bacteria to enter the VBNC state. The VBNC state may be triggered in response to numerous physiological cues as mentioned previously. Importantly, in the context of the food processing environment, the sanitation procedure (cleaning and disinfection) leads to a loss in culturability of *L. monocytogenes* and appearance of VBNC populations (Overney *et al.* 2017). By entering a protective, VBNC state, *L. monocytogenes* may be able to further resist environmental stresses in the food processing environment (Ayrapetyan and Oliver 2016). Upon entry into a suitable environment (e.g. a harbourage site) VBNC *L. monocytogenes* can subsequently regain culturability and begin to proliferate. Further evidence outlining the potential importance of VBNC *L. monocytogenes* in the food processing environment is demonstrated by work indicating that chlorine stress induces the VBNC state in *L. monocytogenes* and that these VBNC cells remain infectious in a *Caenorhabditis elegans* model (Highmore *et al.* 2018).

Recent evidence has shown that *L. monocytogenes* ST121, a sequence type commonly associated with food and food environments, carries a stress survival islet (SSI-2) that confers increased survival under oxidative and alkaline stresses which are common in the food processing environment (Harter *et al.* 2017). Overall, *L. monocytogenes* is well suited to surviving the various stresses presented by the fresh produce processing environment and may have a competitive advantage over other contaminating

bacteria, facilitated through harbourage sites. Moreover, due to its ubiquitous nature in the growing environment, recontamination of a processing environment in the FLPSC after cleaning and disinfection is possible, meaning that regular sanitation regimes must be undertaken to combat its continuing presence.

1.11. *L. monocytogenes* survival on the product surface: post-harvest

It has been shown that *L. monocytogenes* survives and grows on a range of fresh products including 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* viable cell 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.

1.11.1. 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. 2017a). *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.

1.12. Source tracking *L. monocytogenes* in the fresh leafy produce supply chain

1.12.1. 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 (PFGE) 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 PFGE 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 more specific 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 which have given users the ability to distinguish more closely related strains (Figure 1.5.), 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 stakeholders 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).

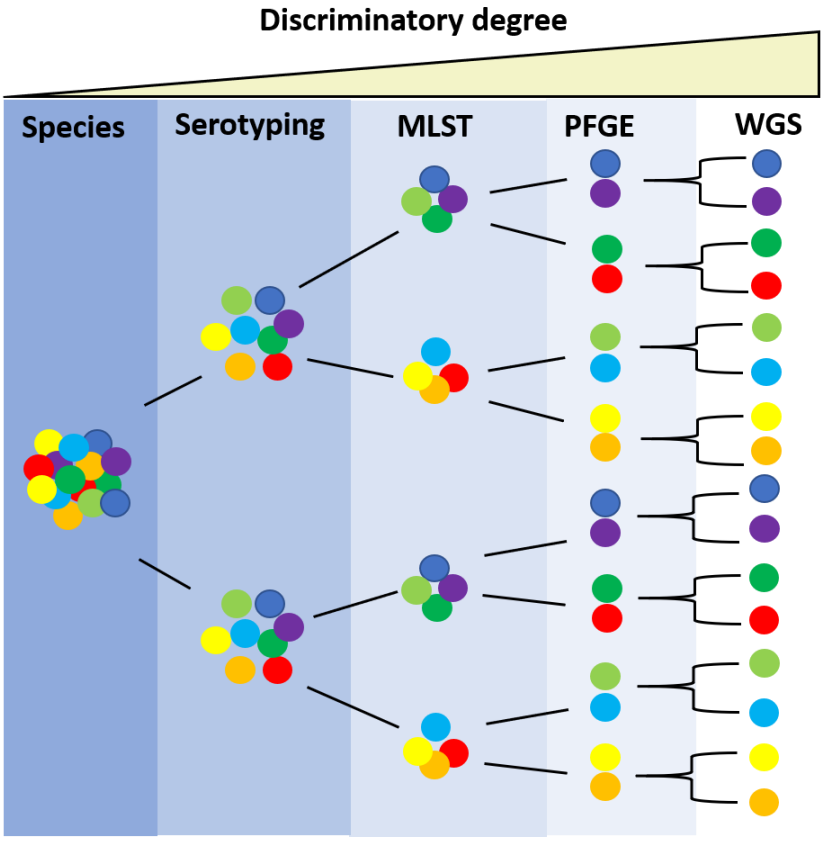

Figure 1.5. **Schematic diagram demonstrating the increase in discriminatory power using different molecular characterisation methods.** Different coloured circles represent different *L. monocytogenes* types with more closely related types being distinguished with increasing discriminatory power.

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 PFGE, 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 remains a significant cost, which is prohibitive for such routine use in the FLPSC.

1.12.2. Subtyping *L. monocytogenes* by WGS can infer the potential disease risk of isolates

In addition to being used as a source-tracking tool, WGS can infer the disease 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.* These include 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). For example, it is widely acknowledged that premature stop codons (PMSCs) in the *inlA* gene which result in a truncated and expressed internalin-A and attenuated virulence exist within the species (Gelbíčová et al. 2015). Observation of this characteristic in strains isolated from food and environmental sources compared to clinical sources has been recognised (Nightingale et al. 2005; Fravalo et al. 2017). However, a recent study from Ireland demonstrated that five out of the six *L. monocytogenes* strains isolated from food did not contain PMSCs in *inlA* (Hilliard et al. 2018), indicating that this characteristic from environmental strains requires further investigation. Determining the presence and functioning of these genes (Table 1.4.) could indicate the potential risk that *L. monocytogenes* isolates found in the FLPSC pose to the consumer, although missing or non-functioning genes do not necessarily confer reduced virulence or avirulence in an isolate highlighting that more research is needed in this area.

Table 1.4. ***L. monocytogenes* virulence factors and the function of their products.** This table is based on information from the VFDB *Listeria* website http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Listeria

Overall function	Gene	Specific product function	Reference
Actin-based motility	*actA*	Actin based motility	(Yoshida et al. 2009)
Adherence	*ami*	May play a role in adhesion to eukaryotic cells	(Asano et al. 2011)
	fbpA	A fibronectin-binding protein present on the listerial surface that can mediate adherence to host cells but also act like a chaperone for two virulence factors, LLO and InlB	(Asano et al. 2013)
	inlF	A *L. monocytogenes*-specific factor involved in mediating enhanced infection in a host cell type/species-specific manner	(Kirchner and Higgins 2008)
	inlJ	A sortase anchored adhesin binds MUC2 (the major component of intestinal mucus) by its internalin domain	(Lecuit et al. 2008)
	lap	Promotes bacterial adhesion to intestinal cells	(Burkholder and Bhunia 2010)
	lapB	Necessary for adhesion to and entry into mammalian epithelial cell lines and for virulence in intravenous or orally infected mice	(Sousa et al. 2010)
Bile resistance	*BSH*	BSH can transform and inactive the bile salts by catalysing the hydrolysis of the amide bond, liberating the glycine/taurine moiety from the side chain of the steroid core.	(Glaser et al. 2003)
Exoenzyme	*mpl*	Zinc-metalloprotease to process PlcB	(Marquis et al. 1997)
	plcA	Phosphatidylinositol-specific phospholipase C, synergizes with LLO and PlcB in the destabilization of primary and secondary phagosomes	(Vazquez-Boland et al. 2001)
	plcB	A broad substrate spectrum including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin,	

		but shows a higher specificity for phosphatidylcholine	
Immune evasion	*oatA*	Critical for the bacterial survival in infected hosts	(Aubry et al. 2011)
	pgdA	Modifying enzyme that deacetylates the N-acetylglucosamine residues of L. monocytogenes peptidoglycan and confers resistance to lysozyme	(Chignard et al. 2007)
Immune modulator	*inlC*	Protrusion formation and dampens innate immune response	(Balestrino et al. 2010)
	inlK	Autophagy evasion	(Wiemer et al. 2011)
	intA	Chromatin remodelling	(Tham et al. 2011)
Intracellular growth	*lplA1*	Necessary for efficient intracellular proliferation	(O'Riordan et al. 2003)
	prsA2	Required for virulence and contributes to the integrity of the *L. monocytogenes* cell wall as well as swimming motility and bacterial resistance to osmotic stress	(Alonzo et al. 2011)
Invasion	*auto*	Required for entry of *L. monocytogenes* into nonphagocytic cells and necessary for full virulence	(Cabanes et al. 2004)
	gtcA	Involved in glycosylation of the cell wall teichoic acids	(Cheng et al. 2008)
	inlA	Mediates the crossing of the intestinal and placental barriers, and invasion of the central nervous system (CNS) may also be mediated by the interaction between InlA and E-cadherin.	(Heinz et al. 2002)
	inlB	Promotes entry into host cells	(Bierne and Cossart 2002)
	inlP	Strongly promotes placental infection	(Faralla et al. 2016)
	lpeA	A novel invasion favouring the entry of *L. monocytogenes* into nonprofessional phagocytes but not its invasion of macrophages	(Pellegrini et al. 2003)
	vip	Interacts with Gp96 localized at the surface of host cells during invasion and	(Cabanes et al. 2005)

		that this interaction is critical for a successful infection in vivo	
Iron acquisition	*svpA*	Haemoglobin binding protein responsible for the acquisition of haem present in low concentrations in the environment	(Borezée et al. 2001)
Metabolic adaption	*hpt*	Hpt mimics the function of the mammalian G6PT to steal fueling metabolites from host cell cytosol for the benefit of the microbe	(Suarez et al. 2002)
Peptidase	*lsp*	A lipoprotein-specific signal peptidase SPase II, responsible for the maturation of lipoproteins in listerial pathogenesis	(Réglier-Poupet et al. 2003)
Regulation	*prfA*	The main switch of a regulon including the majority of the known listerial virulence genes: LIPI-1 genes (with prfA itself) and several genes of the subfamily of secreted internalins (e.g., inlC of *L. monocytogenes* and i-inlE of *L. ivanovii*), are tightly regulated by PrfA	(Vazquez-Boland et al. 2001)
Stress protein	*clpC*	An ATPase promoting early escape form the phagosome of macrophages	(Nair et al. 2000)
	clpE	An ATPase required for prolonged survival at 42	(Berche et al. 1999)
	clpP	Serine protease involved in proteolysis and is required for growth under stress conditions	(Bregenholt et al. 2001)
Toxin	*hly*	Mediates lysis of the primary phagosomes formed after the uptake of extracellular bacteria, and required for the efficient escape from the double-membrane vacuole that forms upon cell-to-cell spead	(Cossart et al. 1989)
Listeria pathogenicity islands (LIPI)			
LIPI-1	*prfA*	Also called PrfA-dependent virulence gene cluster. It comprises six genes (*prfA, plcA, hly, mpl, actA, plcB*), the products of which are required for crucial steps in the intracellular life cycle of the bacteria.	(Vazquez-Boland et al. 2001)
	plcA		
	hly		
	mpl		
	actA		
	plcB		
LIPI-3	*llsA*		

	llsG	Encoding Listeriolysin S (LLS), a post-translationally modified cytolytic peptide	(Hill et al. 2014)
	llsH		
	llsX		
	llsB		
	llsY		
	llsD		
	llsP		
LIPI-4	*lm4b-0232 x 6*	A cluster of six genes encoding a sugar transport system involved in neural and placental infection	(Maury et al. 2016)

Thus, when applied to *L. monocytogenes* isolates in the FLPSC, WGS can only give an indication of risk. 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 help to define more pathogenic types, (e.g. closely related to previous outbreak strains). This information is of clear interest to the companies that operate in the FLPSC and provides value by informing risk assessments associated with *L. monocytogenes* contamination, ultimately reducing the risk to the consumer. However, subtyping by WGS is not routinely done in the UK FLPSC and so information on *L. monocytogenes* strains which may be present is not currently available. Characterising *L. monocytogenes* strains from the UK FLPSC using WGS could help with source tracking of the bacterium (by accurately determining the relatedness of strains) and assessing pathogenic risk. This information can then be used to inform methods to manage or reduce *L. monocytogenes* presence in the FLPSC.

1.13. 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 whilst contamination from the soil is possible,

evidence suggests it is of low risk to consumers due to the small number of bacteria transferred. However, due to fresh leafy produce's close association with soil, *L. monoytogenes* survival in horticultural soils and the environmental and physiological factors that affect its survival requires further investigation. Additionally, data on transfer of the bacteria from the soil to the product surface and survival on the product surface is lacking, and more research is needed to help inform safe practice and regulation in the production of fresh leafy produce.

WGS has the potential to be used to identify and subtype *L. monocytogenes* and is preferable to other typing methods due to the additional valuable data it provides. WGS combined with epidemiological data can be used as a method for source tracking, determination of persistence and to inform stakeholders of the pathogenic risk posed by *L. monocytogenes* isolates. Currently in the UK FPLSC, *L. monocytogenes* strains which have been isolated from product or associated surfaces in the supply chain are only characterised to the species level and are not subject to further characterisation such as MLST or WGS. As such, no information exists regarding the types of *L. monocytogenes* which are present in the supply chain. There is a clear need for this information for stakeholders in the supply chain as it can be used to influence risk assessments and inform regulations/methods to help reduce *L. monocytogenes* presence in the UK fresh produce supply chain.

1.14. Aims & Objectives

The main aim of this thesis is to determine which types of *L. monocytogenes* exist in the UK fresh leafy produce supply chain by characterising strains using phenotypic and DNA based molecular methods (specifically MLST & WGS). Further, by using WGS, to assess the relatedness of isolates and potential virulence according to the presence and intactness of key virulence genes. The secondary aim was to study how these strains survive in typical horticultural soils and on the product. Additionally, to investigate the impact of the wash step on *L. monocytogenes* survival on spinach leaves.

Objectives:

1. To characterise *L. monocytogenes* isolates according to their phenotypic characteristics e.g. biofilm formation, rates of motility, etc. and determine which sequence types are present using MLST
2. Assess the relatedness and potential risk/virulence potential of *L. monocytogenes* isolates using whole genome sequencing and associated bioinformatics
3. To study the ability of selected *L. monocytogenes* isolates to survive in a range of typical horticultural soils
4. To study the impact of the wash step on *L. monocytogenes* populations on fresh produce leaves and subsequently monitor survival

Chapter 2 Identification, multi locus sequence typing and phenotypic characterisation of L. monocytogenes isolates from the fresh produce supply chain

2.1. Introduction

In the food production industry, it is important to identify potential bacterial contaminants quickly and reliably to help inform risk assessments and future policy. Of the pathogenic bacteria found on food, *L. monocytogenes* is of particular concern due to high fatality rates from infection and its ability to survive in food supply chains. The bacteria can survive for extended periods in a range of environments (e.g. soil, processing environment, product surface) and withstand environmental variables which other human pathogenic bacteria such as *E. coli* and *Salmonella* cannot, giving it a competitive advantage. As well as *L. monocytogenes*, other species of the *Listeria* genus are commonly found in food supply chains but are of less concern because they are typically avirulent and therefore pose no danger to human health. Consequently, methods to distinguish *L. monocytogenes* from other *Listeria* spp. need to be fast and reliable.

In addition to species confirmation, determining the sequence type (via MLST) of isolates can infer an evolutionary linkage between *L. monocytogenes* isolates. This characterisation method allows fast inter-laboratory comparison of strains via sharing of DNA sequences, whilst other methods of characterisation such as PFGE and serotyping, do not. Furthermore, MLST can provide accurate phylogenetic characterisation of isolates and lineage assessment (Orsi et al. 2011) which is an important step in identifying more virulent types such as those commonly associated with human disease and outbreaks. Typically, MLST and other subtyping methods are not used by the 3rd party microbiology companies which service the food industry.

Previous research has identified the presence of *L. monocytogenes* on a variety of RTE food products (Little et al. 2009; Wu et al. 2016) and more recent work concentrated on the characterisation of these *L. monocytogenes* strains through phenotypic and molecular methods. As a result, *L. monocytogenes* isolates from food and food supply chains have been found to be genetically diverse (Leong et al. 2017) and exhibit diversity in phenotypic characteristics such as biofilm production (Doijad et al. 2015). Biofilm production is important in the context of the fresh produce supply chain as this characteristic of the bacterium is thought to aid in the persistence and spread of the bacteria in food associated environments (Colagiorgi et al. 2016). Other phenotypic traits of interest are; the ability of isolates to grow in defined media and rates of

motility. Swarming motility is defined as rapid (2–10 µm/s) and coordinated translocation of a bacterial population across solid or semi-solid surfaces. This characteristic of *L. monocytogenes* may, in the context of the fresh produce supply chain, aid in bacterial colonisation of difficult to clean places such as harbourage sites.

Several potential sources of *L. monocytogenes* exist in the fresh produce supply chain (Smith et al. 2018) and data on isolates in a vegetable production facility indicates that diverse populations exist in these type of facilities (Leong et al. 2017). However, currently there is no information present on the variety of *L. monocytogenes* strains which have been isolated from UK fresh produce. Hence, it can be hypothesised that *L. monocytogenes* isolates from the fresh produce supply chain will be genetically (as assessed by MLST) diverse and differ in their phenotypic traits (i.e. their ability to form biofilm and in rates of growth and motility).

Aim: To determine the variety of *L. monocytogenes* strains isolated from the UK fresh produce supply chain according to selected molecular (MLST) and phenotypic characteristics.

Objectives:

- To confirm the identity of *L. monocytogenes* isolates from the UK fresh produce supply chain using a species-specific PCR
- To determine the sequence types of *L. monocytogenes* isolated from the UK fresh produce supply chain using multi locus sequence typing (MLST)
- To determine the growth rate of *L. monocytogenes* isolates in defined media
- To compare the biofilm forming capacity and rates of swarming motility of *L. monocytogenes* isolates

2.1. Materials and Methods

2.2.1. Isolation of *L. monocytogenes* from the UK fresh produce supply chain

A total of 15 *L. monocytogenes* strains were isolated from fresh produce and associated environments in the UK fresh produce supply chain; comprising 9 isolates from spinach, 2 isolates from baby salad kale, 1 isolate from pea shoots, 1 isolate from beetroot, 1 isolate from red leaf lettuce and 1 isolate from an environmental swab taken from a drain in a tray washing facility. Included with analysis was reference *L. monocytogenes* strain EGD-e (Table 2.1). *L. monocytogenes* strains were either isolated directly from product or from swabs of associated areas in the fresh produce supply chain and subsequently identified using ISO 11290-2: 2017 for enumeration purposes or by ELISA detection method, Solus Listeria ELISA AFNOR (Association Française de Normalisation) validation certificate SOL – 37/02 – 06/13 by third party microbial testing companies as part of the routine testing of produce and surfaces in the fresh produce supply chain.

2.2.2. Identification of *L. monocytogenes* from the UK fresh produce supply chain and creation of long-term glycerol stocks

L. monocytogenes was streaked on a nutrient agar slope and sent (in biological substances category B, triple packaged systems, UN 3373) by post, to Edinburgh Napier University, Sighthill Campus before being sub cultured, twice from a single colony at 37 °C for 24 h, on OXFORD agar with modified *Listeria* selective supplement (Oxoid, Basingstoke, UK.). To make long term stocks, a single colony was used to inoculate 10 ml BHI broth (Oxoid) and shaking cultures were incubated at 200 rpm, 37 °C overnight (~16 h) before 1 ml of this culture was centrifuged at 12,000 rpm for 10 min. Resulting bacterial pellets were subsequently washed 3 times in PBS and re-suspended in 500 µl fresh BHI broth + 500 µl of 50 % glycerol. Long term stocks were frozen at -80 °C.

2.2.2.1 Gram stain procedure

Using a disposable inoculation loop, one isolated colony from OXFORD agar plates was mixed with 10 µl of sterilised distilled water (SDW) on a clean microscope slide. This mixture was smeared evenly across the slide before heat fixing for 1 min on a heat block at 200 °C. Staining procedure as follows; crystal violet for 1 min and rinse, Gram's iodine for 1 min and rinse, decolourisation with 95 % EtOH for 5 s and rinse followed by safranin for 1 min and final rinse. Slide was then blotted dry and stain was viewed with a light

microscope under 100 x magnification using oil immersion. Pictures of bacteria were taken using the Canon SU-CA DSLR camera.

2.2.2.2. Species specific PCR and viewing of PCR products

For confirmation of bacterial species, in house, isolates were subject to a species-specific colony PCR. Primers for this assay (referred to as Lm13) were chosen based on their specificity to *L. monocytogenes* compared to other species of *Listeria* as outlined by Tao et al. (2015) and are as follows (5' → 3'): forward primer GTTCGTCGGTCCGTGGTA, reverse primer TTGGCAAGCAAGCAGTTCA. The gene which these primers target is *LMOf2365_2721* (Glycosyl Hydrolase) with a PCR product size of 583 bp. Each PCR reaction was 25 µl and contained; 22.5 µl Biomix Red (Bioline, London, UK), 1.25 µl of 10 mM forward primer, 1.25 µl of 10 mM reverse primer (Eurofins genomics, Ebersberg, Germany), 15 µl Ultrapure water (Invitrogen, Renfrew, UK) and 2.5 µl DNA template from 1 *L. monocytogenes* strain. Template DNA was extracted from *L. monocytogenes* strains by heating 1 isolated colony taken from an agar plate for 10 mins at 100 °C, before centrifuging at 16,000 rpm for 5 mins at 5 °C. The supernatant from this step was used as DNA template. PCR conditions were as follows; (94 °C 5 mins (initial denaturation), 94 °C 30 s (denaturation), 60 °C 30 s (primer binding), 72 °C 40 s (extension) x 35 cycles) 72 °C 10 mins (final extension). PCR products were viewed on a 1.5 % agarose gel where, after the PCR cycle had finished, 10 µl of the reaction mix was loaded into wells with 1.5 kb ladder in the first well. PCR products were electrophoresed on an agarose gel for 45 mins at 100 V for 45 min. *L. monocytogenes* strain EGD-e DNA extracted using GenElute kit (Sigma) was included in reactions as a positive control.

2.2.3. Multi locus sequence typing (MLST) of *L. monocytogenes* isolates

2.2.3.1 Template extraction and PCR

MLST involved sequencing 7 house-keeping genes (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh* & *lhkA)* and subsequent comparison of sequences with an international database of strains. Strains were assigned to sequence types (STs) by a combination of sequenced alleles which were compared to an international database to determine allelic profile (http://bigsdb.pasteur.fr/listeria/listeria.html). Primers used for this assay are also available through the Pastuer Institute website (https://bigsdb.pasteur.fr/listeria/primers_used.html). Primers were synthesised with 'sequencing tails' to allow for sequencing using a universal sequencing primer.

Template extraction for PCR was performed by mixing 500 µl of Ultrapure Water (Thermo Fisher Scientific, Basingstoke, UK) with 1 isolated *L. monocytogenes* colony from a complex streak plate on Oxford Agar (Oxoid, Basingstoke, UK) with Listeria selective supplement (Oxoid, Basingstoke, UK) following 48 h incubation after streaking from glycerol stocks. The colony was then incubated on a heat block at 100 °C for 5 mins. After centrifugation at 13,000 rpm for 5 mins the template was held on ice for use in PCR. PCR reactions were carried out in 50 µl tubes. Except Ultra-pure water (Invitrogen, Renfrew, UK)), all reagents for PCR were provided in Phusion PCR kit (New England Biolabs, Hitchin, UK). This kit was chosen because of its high-fidelity DNA polymerase which has an error rate > 50 -fold lower than traditional Taq polymerase. Reaction mix consisted of 28.5 µl Ultra-pure water, 10 µl Phusion buffer, 1 µl dNTPs, 2.5 µl 10 µmol l^{-1} forward primer, 2.5 µl, 10 µmol l^{-1} reverse primer, 5 µl DNA template and 0.5 µl Phusion Taq polymerase. Thermocycler conditions as follows; heated lid on (105°C), initial denaturation 30 s at 98 °C then 30 cycles of 98 °C for 10 s, 52 °C 10 s, 72 °C 30 s followed by a final extension of 72 °C for 5 mins. Product was held at 4°C until confirmation of bands by gel electrophoresis. To do this, 8 µl of PCR product was added to 2 µl of 5x loading buffer for viewing of PCR products. Products were then electrophoresed on 1.2 % agarose gel containing 5 µl Safe View per 100 ml 1 x TAE buffer. 10 µl of product/loading buffer mixture was added to each well and gel ran at 100 V for 1 h.

2.2.3.2 PCR product extraction, sequencing & determination of sequence type

After confirmation of specific PCR products based on the correct size in agarose gels,, 30 µl of product was loaded into the wells of a gel with larger wells and electrophoresed on a 1.2 % agarose gel 5 µl Safe View per 100 ml 1 x TAE buffer excised using Gel Extracta Kit (Promega, Southampton, UK). PCR products were then purified using Isolate II Kit (Bioline, London, UK) according to the manufacturer's instructions. PlateSeq 96-well sequencing plates for purified PCR products were used to sequence MLST genes (Eurofins Genomics, Ebersberg, Germany). DNA concentration was adjusted to 15 ng/µl using a spectrophotometer (Nanodrop 2000 – Thermo Fisher Scientific) with Ultrapure Water (Thermo Fisher Scientific). Sequence primer concentration was adjusted to 10 pmol $µl^{-1}$ as specified by Eurofins Genomics and sent in separate 1.5 ml Eppendorf tubes. Sequence primers matched the

sequencing tails on primers used in the PCR amplification of genes. Forward and reverse primers for the same PCR product were included to create consensus sequences for 7 housekeeping genes from the two sequences. Genes were sequenced by Eurofins Genomics using the dideoxy chain termination method on ABI3730 XL sequencing machines. Forward and reverse sequences for sequenced genes were aligned using the Muscle algorithm in MEGAX to create consensus sequences. Sequences were then compared to the Pasteur Institute International database to create an allelic profile for housekeeping genes.

2.2.3.3. Phylogenetic analysis of MLST consensus sequences

A consensus sequence (3288 bp) was created for the 7 housekeeping genes and used to determine the phylogeny of the *L. monocytogenes* population. Phylogeny was inferred by IQ-TREE version 1.6.7 (Nguyen et al. 2015) using the Maximum Likelihood method with model finder (Kalyaanamoorthy et al. 2017) option enabled. Branch support was calculated using ultrafast bootstrap support (Hoang et al. 2017) and the SH-like approximate likelihood ratio test (Guindon et al. 2010), both with 1000 iterations.

2.2.4. Determination of haemolytic activity of *L. monocytogenes* strains

2.2.4.1. Preparation of 10 % horse red blood cell suspension

To prepare erythrocytes, 1ml of defibrinated horse blood (Oxoid, Basingstoke, UK) was centrifuged at 3,000 rpm for 10 mins. Supernatant (plasma) and the medium layer (white blood cells) was removed. RBCs were washed 3 times in PBS and resuspended in 1 ml PBS. From this concentrated solution, 1 ml was transferred 9 ml PBS to make a 10 % RBC solution.

2.2.4.2. Incubation of bacterial supernatant with RBC solution

Cultures of *L. monocytogenes* were grown by mixing 1 isolated colony from OXFORD agar in 10ml tryptone soy broth and incubating at 37 °C, 200rpm overnight (16 h). Overnight cultures of *L. monocytogenes* were back diluted to an OD of 0.05 before supernatant was generated by centrifuging at 12,000 rpm for 10 mins. Bacterial supernatants were diluted 1/10, 1/100 & 1/1000 before 5 µl of 5mM DTT (dithiothreitol) was added to bacterial supernatants - DTT is a reducing agent that activates Listeriolysin O (haemolysin) in *L. monocytogenes* supernatant and briefly

vortexed. 500 µl of this supernatant-DTT solution was mixed with 500 µl of 10 % RBC solution. This combined RBC-supernatant mix was incubated at 37 °C for 1 h before centrifuging at 10,000 rpm for 30 mins at 5 °C. Control samples were TSB + 5 mM DTT mixed with 5 % RBC. For each strain, 3 x 150 µl of supernatant after centrifugation was transferred to 3 x wells of a 96-well plate. The absorbance of wells was measured at 540 nm. 3 independent replicates for each strain were completed (n = 3).

2.2.5. Growth rate analysis of *L. monocytogenes* strains in defined media

10ml of BHI broth per strain was inoculated with 1 isolated *L. monocytogenes* colony from OXFORD agar and incubated overnight in a shaking incubator at 200 rpm, 37°C for 16 h. After incubation, absorbance was measured at 595nm before back diluting to an OD 0.05 in 1 ml BHI. 3 x 100 µl of this culture was subsequently transferred to 3 x wells of a 96-well plate per strain. The plate also contained 3 wells of un-inoculated media to serve as control wells and to use as a blank. Plates were incubated at 37 °C for 16 h and a measurement for OD_{595} was taken every hour. Before every measurement, the plate was subject to a brief period of shaking (100 rpm, 5 s). Incubation and measurements were both conducted in the FLUOstar Omega plate reader. Measurements were blank corrected by subtracting the average OD_{595} of control wells from each measurement. Three independent replicates were performed (n = 3) for each time point. From the bacterial growth curve, generation (i.e. doubling) time (G, mins) during the exponential phase was calculated with the following equation:

$$G = \frac{t}{3.3 \log(\frac{b}{B})}$$

where; t = time interval in minutes, b = number of bacteria at beginning of time interval and B = number of bacteria at the end of time interval. To categorise isolates into slow, average and fast growers in defined media, values for generation time, G were placed on a linear scale and the 25th and 75th percentile were determined. Strains with values that were below the 25th percentile were deemed slow growers, strains with values that fell between the 25th and 75th percentiles were deemed to exhibit an average growth rate and those strains with G values over the 75th percentile were

deemed to exhibit a fast growth rate. i.e. (G =) slow < 75.1 mins ≥ average ≤ 82.6 mins > fast

2.2.6. Biofilm formation assay

Long term stocks of *L. monocytogenes* were streaked on OXFORD agar plates with modified *Listeria* selective supplement and incubated at 37 °C for 24 h to obtain isolated colonies. One isolated colony was inoculated into 10ml TSB and incubated at 200 rpm, 37 °C for 16 h. After incubation, absorbance was measured at 595nm before back diluting to an OD of 0.05 in 10 ml of modified Welshimer's broth (HiMedia, India) followed by brief vortexing. 100 µl of this diluted culture was then transferred to 6 x wells of a sterilised, flat bottomed polystyrene 96-well plate per strain. Un-inoculated MWB wells (6) were included in the plate as a control, these wells also served as a blank to use for blank correction during data analysis. Plates were incubated at two different temperatures (20 °C & 30 °C) for 20 h before the turbidity of wells was determined at 595 nm using a microtiter plate reader (FLUOstar Omega). After measurement of cell turbidity, medium was aspirated from wells using a pipette before wells were washed 3 times with distilled water to remove loosely associated bacteria. Plates were air dried for 45 mins before staining with 0.1 % crystal violet solution in water for 45 mins. After staining, wells were washed again 3 times with distilled water. After washing, plates were left to dry for a further 45 mins. At this point, biofilms were visible as purple stains at the bottom of wells. For quantitative analysis of biofilm production, 200 µl of 95% ethanol was added to de-stain wells and left at room temperature on a shaking platform for 45 minutes. 100 µl of this solution was then transferred to a fresh 96-well microtiter plate before the optical density of the de-staining solution was measured on a microtiter plate reader at 595 nm. Results were blank corrected by subtracting the average absorbance from control wells after the staining/de-staining procedure (media only), from the absorbance of test wells.

To ensure that differences in biofilm production were not related to differences in growth rate, average absorbance measurements of cell turbidity versus average absorbance measurements from biofilm CV de-stain underwent a Pearson's test for correlation as tests for normality (D'Agostino & Pearson normality test (Graphpad Prism)) indicated that data conformed to Gaussian distribution. Differences in biofilm production between strains (n=6) were determined using a one-way ANOVA followed

by Tukey's test for multiple comparisons (Graphpad). To categorise isolates into producing low, average and high amounts of biofilm, values for biofilm production (OD_{600}) were placed on a linear scale and the 25th and 75th percentile were determined. Strains with values that were below the 25th percentile were deemed to produce a low amount of biofilm, strains with values that fell between the 25th and 75th percentiles were deemed to produce an average amount of biofilm and those strains with biofilm production values over the 75th percentile were deemed to produce a high amount of biofilm. These categories were determined for 20 °C and 30 °C after 20 h.

2.2.7. Swarming motility assay

To determine the swarming motility of *L. monocytogenes* isolates from the fresh produce supply chain, static cultures were incubated in BHI (Oxoid) at 37 °C overnight (16h). After overnight incubation, cultures were diluted to an OD_{600} of 1. Following dilution, 5 µl was spotted on the middle of 3 % BHI agar plates. Plates were incubated at 22 °C for 72h. After 72 h, the diameter of colonies was measured, and a picture was taken to compare the size of colonies visually. The difference in swarming motility between isolates was determined using a one-way ANOVA. To categorise isolates into categories of low, average and high rates of motility, values for average motility (mm), were placed on a linear scale and the 25th and 75th percentile were determined. Strains with values that were below the 25th percentile were deemed to have low rates of motility, strains with values that fell between the 25th and 75th percentiles were deemed to exhibit an average motility rate and those strains with average colony sizes values over the 75th percentile were deemed to exhibit a fast rate of motility.

2.3. Results

2.3.1. Identification of L. monocytogenes strains isolated from the UK fresh produce supply chain

In total, 15 *L. monocytogenes* strains were isolated and identified from a variety of locations and products within the fresh produce supply chain (Table 2.1.).

Table 2.1. **List of *L. monocytogenes* strains isolated from the UK fresh produce supply chain and associated sample information.** These strains were used in subsequent phenotypic testing. – indicates missing data.

Internal Reference	Sample type	Sample date	Sample location	Stage in supply chain
NLmo1	Lab reference (EGD-e)	-	-	-
NLmo2	Spinach	May-16	West Sussex, UK	Raw product, unwashed
NLmo3	Spinach	May-16	West Sussex, UK	Raw product, unwashed
NLmo4	Environmental swab (drain)	May-16	West Sussex, UK	Tray cleaning facility
NLmo5	Spinach	May-16	West Sussex, UK	Raw Product, unwashed
NLmo6	Red leaf lettuce	Jun-16	Norfolk, UK	Raw product, unwashed
NLmo7	Spinach	Jun-16	West Sussex, UK	Post cooling, unwashed
NLmo8	Spinach	Aug-16	Cambridgeshire, UK	Final product, unwashed
NLmo9	Spinach	Jul-16	Cambridgeshire, UK	Final product, Unwashed
NLmo10	Spinach	Sep-16	Cambridgeshire, UK	Post cooling, unwashed
NLmo13	Spinach	Sep-16	Cambridgeshire, UK	Final product, unwashed

NLmo14	Beetroot	Oct-16	Cambridgeshire, UK	Final product, washed
NLmo15	Pea shoots	Oct-16	Cambridgeshire, UK	Final product, unwashed
NLmo16	Spinach	Nov-16	Cambridgeshire, UK	Final product, washed
NLmo18	Baby salad kale	Apr-17	Cambridgeshire, UK	Post cooling, unwashed
NLmo20	Baby salad kale	Apr-17	Cambridgeshire, UK	Final product, unwashed

Using a species-specific PCR (section 2.2.2.1.) targeting the Glycosyl Hydrolase gene (*LMOf2365_2721* (Tao et al. 2015)), all strains generated PCR product that could be viewed on an agarose gel, confirming that they were *L. monocytogenes*, although different *L. monocytogenes* strains generated different amounts of product as defined by the strength/brightness of the band. For example, Figure 2.1. shows that EGD-e, NLmo5 & NLmo18 all generated substantially less PCR product than other strains.

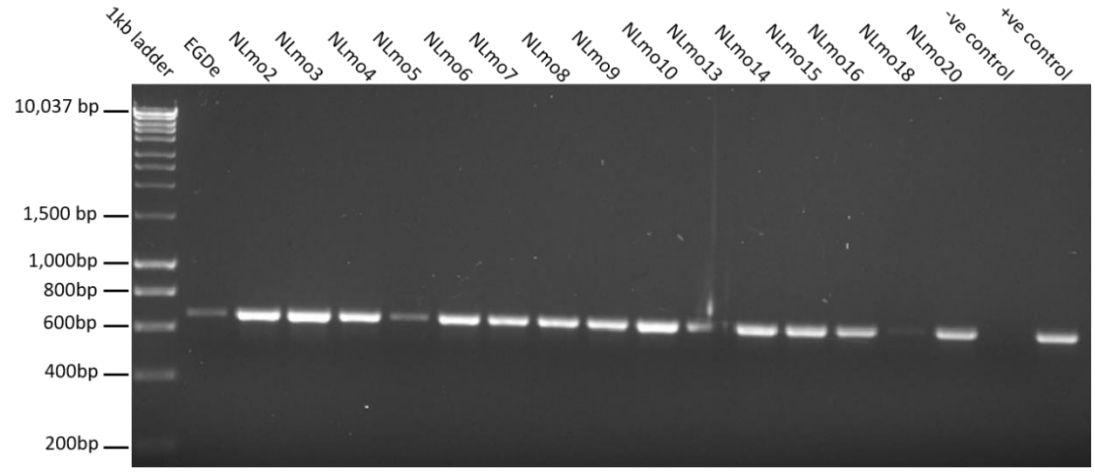

Figure 2.1. **Species-specific PCR identification of *L. monocytogenes* isolates from the fresh produce supply chain.** Strain internal reference can be seen above the agarose gel. Ladder is shown on the left of the figure. *L. monocytogenes* strain EGD-e DNA extracted by GenElute bacterial DNA extraction kit was included as a positive control. bp = base pairs.

L. monocytogenes was also viewed after a Gram stain on a glass slide. Figure 2.2. shows a *L. monocytogenes* Gram stain, where the individual bacteria are rod-shaped, Gram-positive and form short chains which is characteristic of the species. Furthermore, when incubated with sheep RBCs, the supernatant from all strains caused haemolysis of RBCs (Appendix A), an additional characteristic of pathogenic *Listeria* spp.

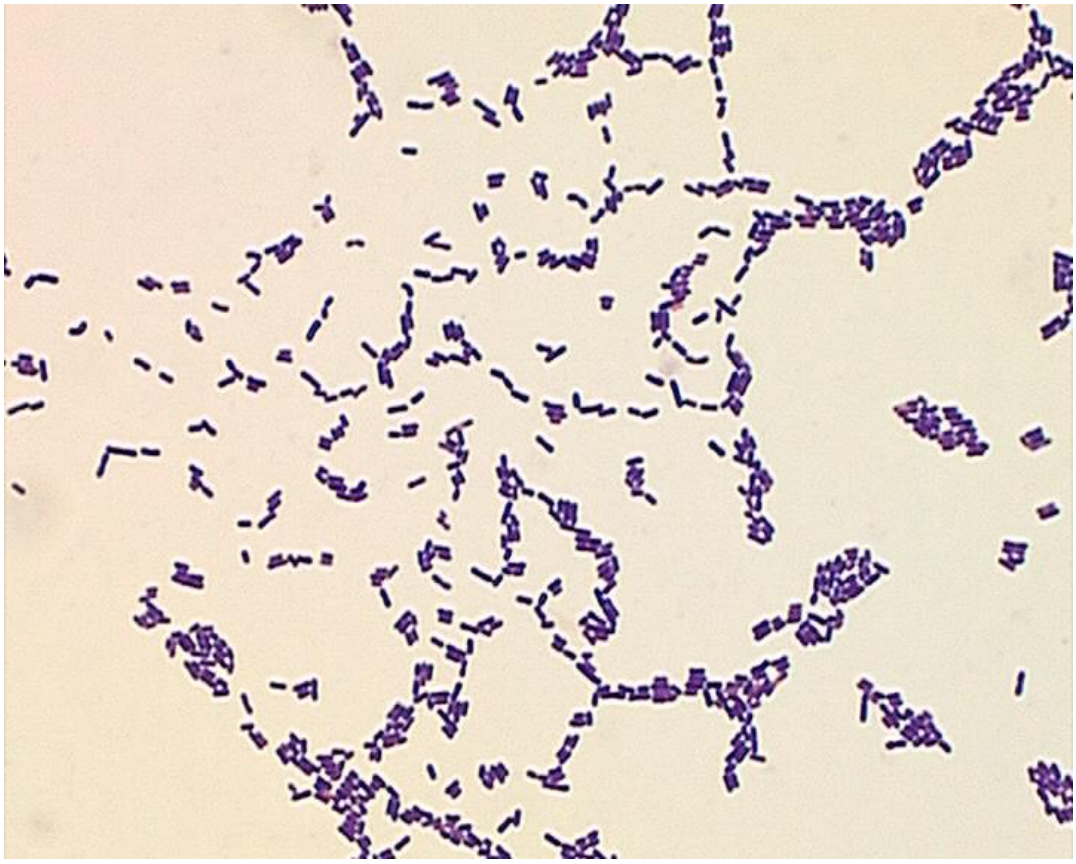

Figure 2.2. **Gram stain of *L. monocytogenes* (NLmo2)**. Individual bacteria appear as singular purple rods which form chains.

2.3.2. Multi locus sequence typing of *L. monocytogenes* isolates from the fresh produce supply chain

2.3.2.1 Confirmation of PCR product and product extraction

PCR products of the 7 MLST housekeeping genes were confirmed on agarose gels for the different strains (for an example of one of these gels see Figure 2.3.). The expected sizes for PCR products were as follows; *abcZ* (535 bp), *bglA* (399 bp), *cat* (486 bp), *dapE* (462 bp), *dat* (471 bp), *ldh* (453 bp), *lhkA* (480 bp). After confirmation of the presence and size of PCR products,, 30 µl of PCR product was added to a gel with larger wells

before products were extracted from the gel, purified and adjusted to 15 ng μl^{-1} for sequencing.

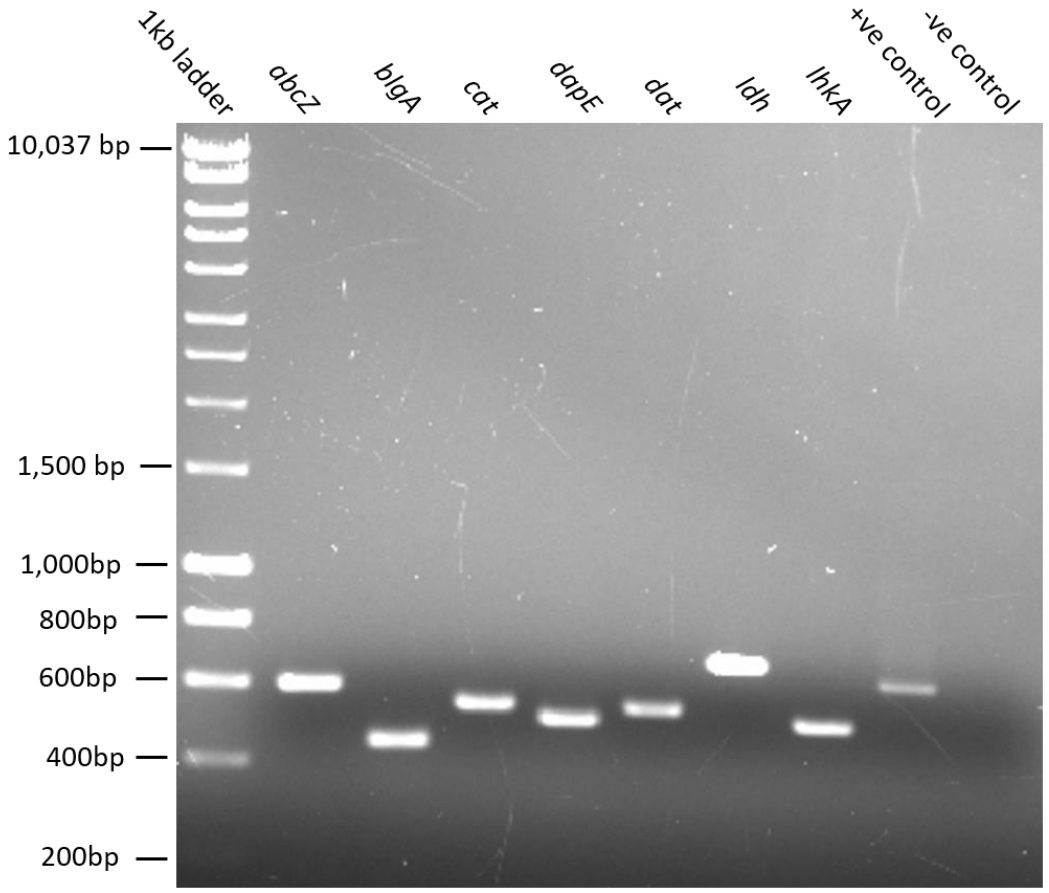

Figure 2.3. **Example of agarose gel detection of PCR products from MLST of *L. monocytogenes* (NLmo2)**. PCR products from NLmo2 template DNA are shown. DNA ladder shown on the left-hand side (bp = base pairs). PCR product name is indicated at the top of each lane; abcZ (ABC transporter), bglA (beta glucosidase), cat (catalase), dapE (succinyl diaminopimelate desuccinylase), dat (D-amino acid aminotransferase), ldh (L-lactate dehydrogenase), lhkA (histidine kinase). Positive control is DNA template extracted from *L. monocytogenes* strain EGD-e.

2.3.2.2 Sequence type of *L. monocytogenes* strains from the UK fresh produce supply chain

Forward and reverse sequences for each gene were aligned to make a consensus sequence, this consensus sequence was then compared to the Pastuer Institute database for MLST sequences to generate an alleic profile for each strain and thus, a sequence type. MLST detected a total of 9 different sequence types from the 15 *L.*

monocytogenes strains which were isolated from the UK fresh produce supply chain (Table 2.2). The most common allelic profile was matched to ST1 (3/15 isolates) and pairs of isolates all belonged to STs 5, 325, 4 & 37. All the other STs contained one isolate. No new sequence types were discovered.

Table 2.2. **Allelic profile of *L. monocytogenes* strains based on the MLST of 7 housekeeping genes.** abcZ (ABC transporter), bglA (beta glucosidase), cat (catalase), dapE (succinyl diaminopimelate desuccinylase), dat (D-amino acid aminotransferase), ldh (L-lactate dehydrogenase), lhkA (histidine kinase). ST = sequence type.

Strain	Alleic profile							ST	Lineage
	abcZ	***bglA***	***cat***	***dapE***	***dat***	***ldh***	***lhkA***		
NLmo2	2	1	11	3	3	1	7	5	I
NLmo3	2	1	11	3	3	1	7	5	I
NLmo4	7	14	10	19	1	8	1	325	II
NLmo5	7	14	10	19	1	8	1	325	II
NLmo6	1	2	12	3	2	5	3	4	I
NLmo7	3	1	1	1	3	1	3	1	I
NLmo8	1	2	9	1	2	5	3	219	I
NLmo9	1	2	12	3	2	5	3	4	I
NLmo10	3	1	1	1	3	1	3	1	I
NLmo13	5	7	3	5	1	8	6	37	II
NLmo14	3	1	1	1	3	1	3	1	I
NLmo15	5	7	6	4	5	4	1	204	II
NLmo16	5	7	3	5	1	8	6	37	II
NLmo18	8	6	15	6	6	2	1	399	II
NLmo20	3	9	9	3	3	1	5	6	I

A phylogenetic tree based on the 7 concatenated MLST sequences (3288bp) shows the relatedness between the *L. monocytogenes* strains isolated from the UK fresh produce supply chain (Figure 2.4). The isolates split into two distinct lineages (I & II) and some isolates fall into the same sequence type meaning that they cannot be distinguished by this method of characterisation. These isolates are; NLmo2 & NLmo3 (ST5), NLmo6 &

NLmo9 (ST4), NLmo7, NLmo14 & NLmo10 (ST1), NLmo4 & NLmo5 (ST325), NLmo13 & NLmo16 (ST37).

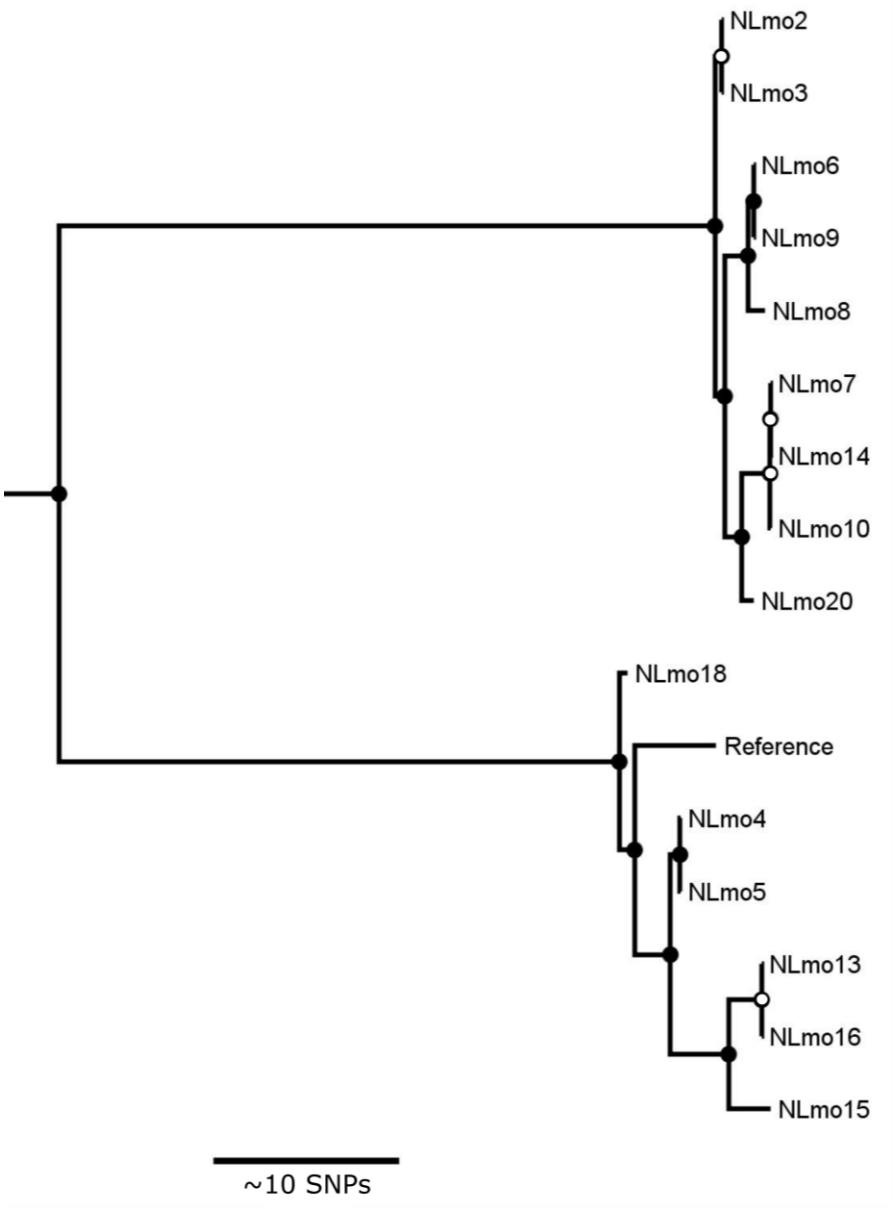

Figure 2.4. **Maximum Likelihood phylogeny of 15 *L. monocytogenes* isolates isolated from the UK fresh produce supply chain based on 3288bp consensus sequence of 7 housekeeping MLST genes.** Reference strain is *L. monocytogenes* strain EGD-e. Tree inferred using IQtree version 1.6.7. Tree is rooted through the midpoint. White circles indicate nodes with ≥ 95% ultrafast bootstrap support and ≥ 80% SH-like approximate likelihood ratio test support. SNPs = single nucleotide polymorphisms.

2.3.3 Growth rate of *L. monocytogenes* isolates in defined media

L. monocytogenes strains took ~2 hours to reach the exponential phase after inoculation at an OD_{600} = 0.05. All strains showed sigmoidal growth in culture, reaching a final OD_{600} of 0.5-0.6 in stationary phase. Some strains exhibited a lack of variation over the 3 independent replicates whilst others (NLmo3, NLmo6, NLmo7) exhibited more variation, as indicated by the relative size of error bars at each time point (Figure 2.5., 2.6.).

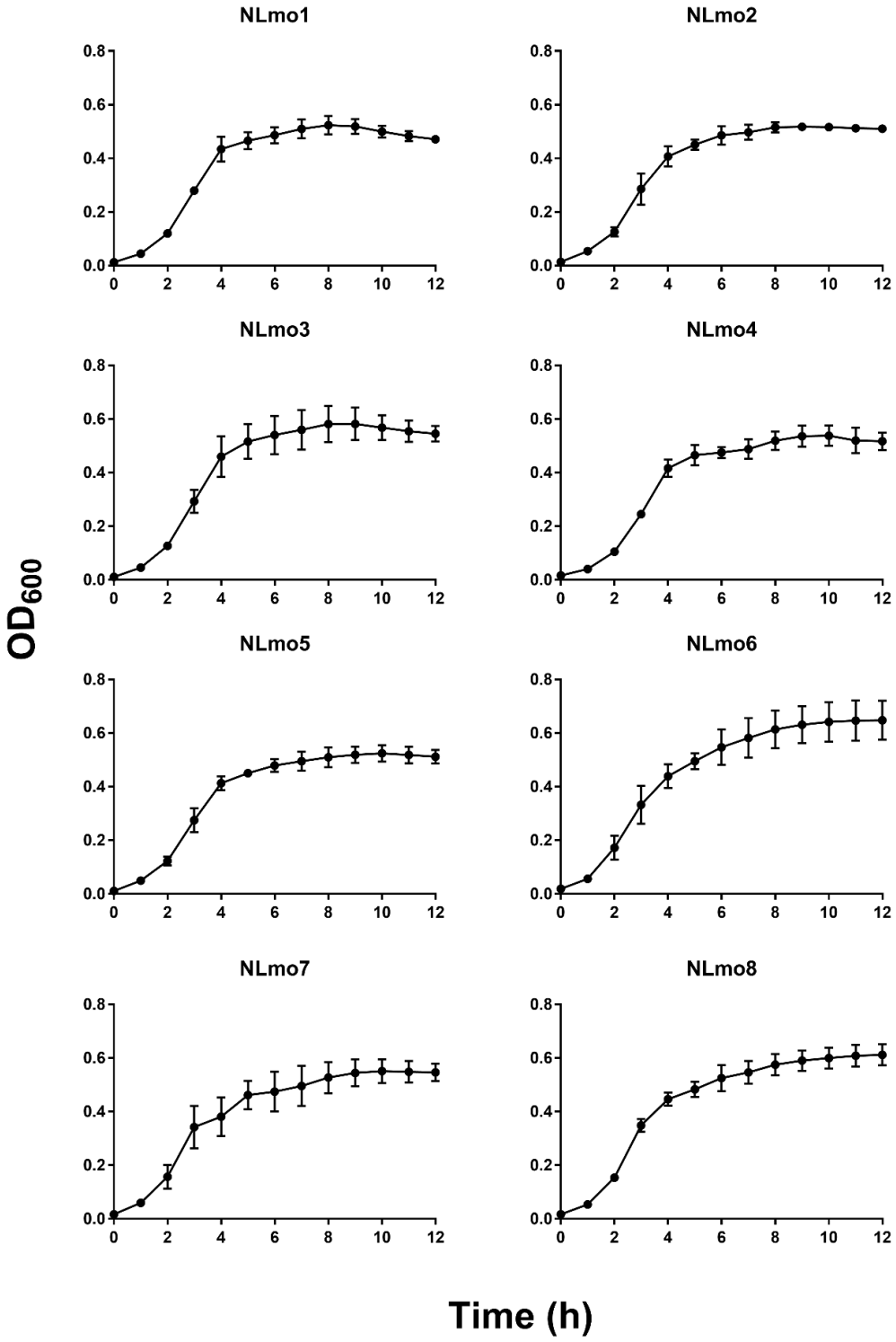

Figure 2.5. ***L. monocytogenes*** **growth in BHI broth.** *L. monocytogenes* strain (internal reference) is indicated above each individual graph. Y-axis shows absorbance (OD_{600}) in relation to the x-axis which is time (hours). Data points are shown with error bars that represent SEM (n = 3).

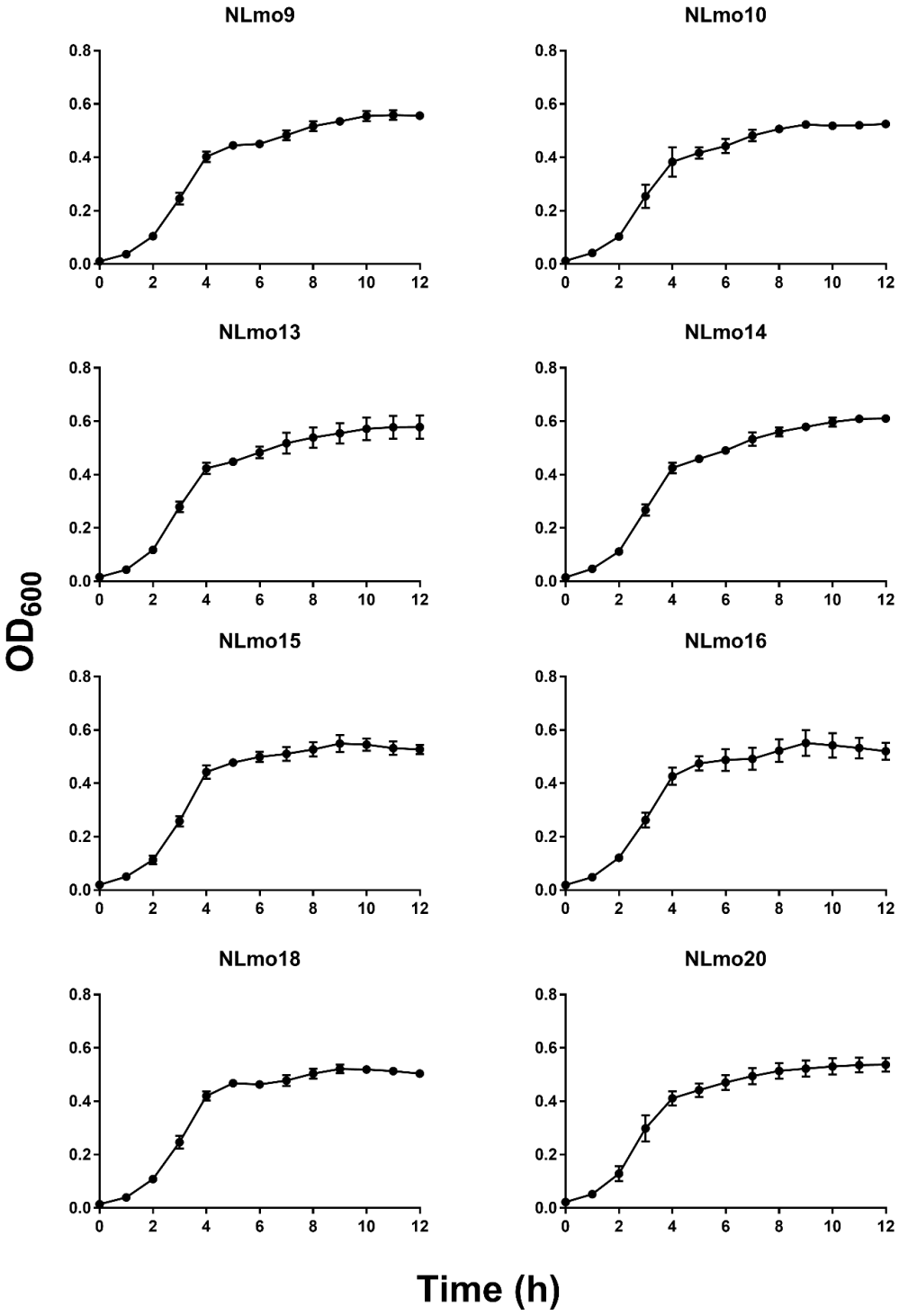

Figure 2.6. ***L. monocytogenes*** **growth in BHI broth.** L. monocytogenes strain (internal reference) is indicated above each individual graph. Y-axis show absorbance (OD_{600}) and x-axis represent time (hours). Data points are shown with error bars that represent SEM (n = 3).

The different strains of *L. monocytogenes* had different average doubling times (G), ranging from 71 minutes (NLmo20) to 90.6 minutes (NLmo2) (Table 2.3.), however, no significant differences were observed in generation time between isolates.

Table 2.3. **Comparison of average generation time (G) between *L. monocytogenes* strains.** G values are given as the mean of 3 independent replicates (n = 3). According to an ANOVA with Tukey's test for multiple comparisons, no significant differences were observed.

	G1	G2	G3	Average G (mins)	SEM
NLmo1	99.8	63.6	60.9	74.8	12.5
NLmo2	142.9	79.0	49.8	90.6	27.5
NLmo3	124.8	60.7	59.6	81.7	21.6
NLmo4	87.9	63.2	76.0	75.7	7.2
NLmo5	97.5	74.8	64.3	78.9	9.8
NLmo6	109.4	69.3	87.4	88.7	11.6
NLmo7	109.1	61.4	85.7	85.4	13.8
NLmo8	97.9	61.6	60.0	73.2	12.4
NLmo9	86.2	60.2	82.9	76.4	8.2
NLmo10	118.3	57.0	63.9	79.7	19.4
NLmo13	90.7	59.5	68.3	72.8	9.3
NLmo14	95.0	61.0	65.5	73.8	10.7
NLmo15	80.6	66.3	80.1	75.7	4.7
NLmo16	105.6	62.2	81.1	83.0	12.5
NLmo18	78.6	68.5	84.6	77.2	4.7
NLmo20	75.4	71.8	65.7	71.0	2.8

2.3.4. Biofilm formation of *L. monocytogenes* isolates

Biofilm production was quantified in all strains at 20°C and 30°C after 20h using a staining/destaining crystal violet method. To ensure biofilm production wasn't related to growth rate, a correlation was calculated between the variables of average final OD

and average biofilm production of *L. monocytogenes* strains under different conditions. The rest of this section describes the results of the quantification of biofilm production with *L. monocytogenes* isolates and compares biofilm production over different temperatures.

2.3.4.1. Biofilm production in 20 h at 20°C

Generally, the optimum conditions for biofilm production by *L. monocytogenes* isolates occurred after 20 h at 20 °C (Figure 2.8). Within this group, biofilm production ranged from OD_{595} values of 0.023 (NLmo5) to 0.258 (NLmo18) (Figure 2.7). An ANOVA of mean biofilm production (n = 6) showed that isolates differed significantly ($P < 0.0001$) in their ability to produce biofilm. Tukey's multiple comparison tests showed that significant differences in biofilm production occurred between individual isolates existed. For example, NLmo1 (EGD-e) produced significantly less ($P < 0.005$) biofilm than NLmo8, NLmo9, NLmo14, NLmo15 & NLmo18. A correlation between average biofilm production and average final OD showed that there was no correlation ($P = 0.804$) between these two variables under these conditions.

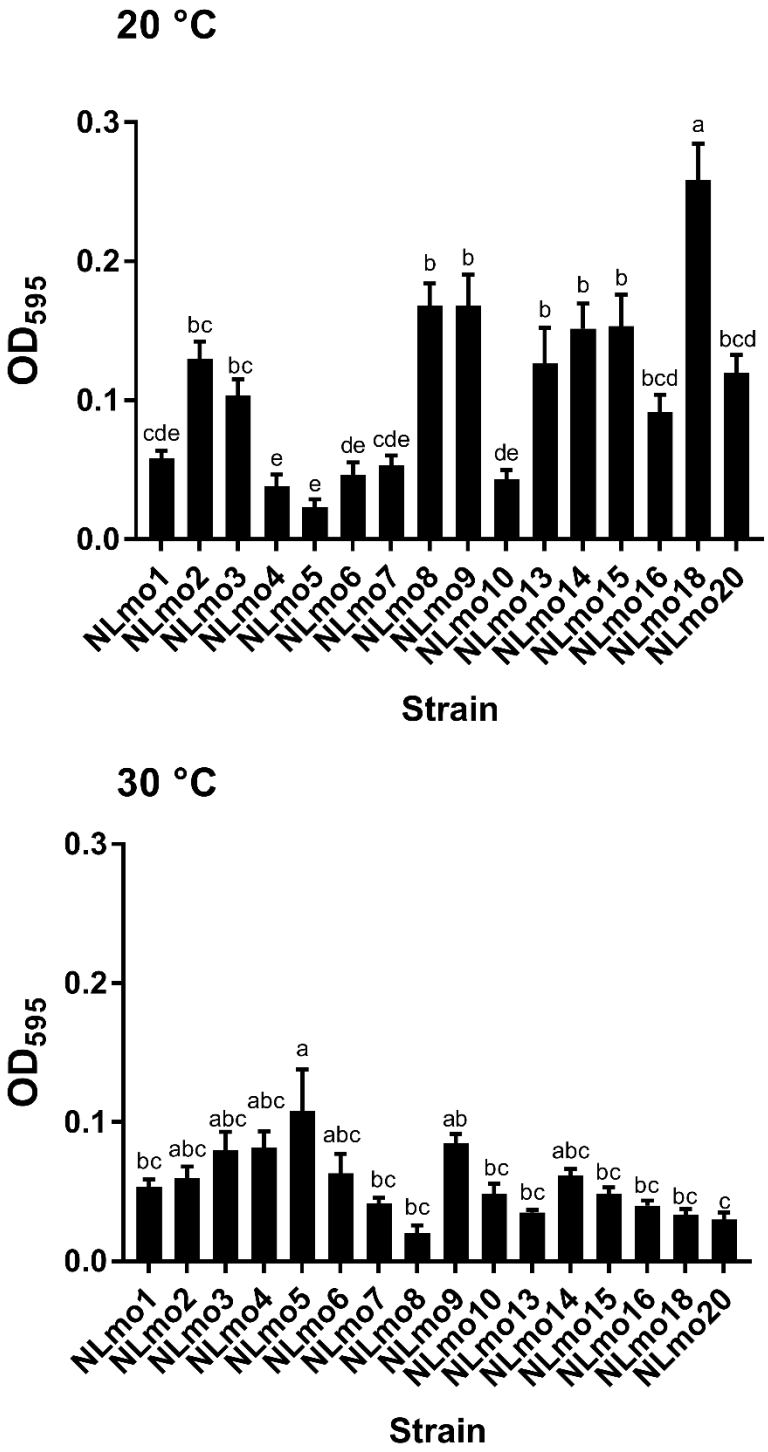

Figure 2.7. **Biofilm production of *L. monocytogenes* strains after 20 h at 20 °C (top chart) & 30 °C (bottom chart) measured by microtiter plate assay (crystal violet staining).** Values are expressed as mean + SEM. Letters above bars in the chart indicate no significant difference between strains according to Tukeys test for multiple comparisons ($P < 0.05$)

2.3.4.2. Biofilm production after 20h at 30°C

Biofilm production in *L. monocytogenes* isolates generally reduced in comparison to 20 °C when exposed to a higher temperature of 30°C, reaching OD_{595} values ranging from (NLmo8) 0.02 – 0.108 (NLmo5) (Figure 2.7.). At 30°C, an ANOVA test of mean biofilm production between isolates showed that, again, isolates significantly differed ($P < 0.001$) in their ability to form biofilm with Tukey's test for multiple comparisons showing differences between individual strains (Figure 2.7.). Average biofilm production decreased as temperature increased (Figure 2.8.). A correlation between average biofilm production and average OD showed that there was no correlation ($P = 0.797$) between these two variables under these conditions.

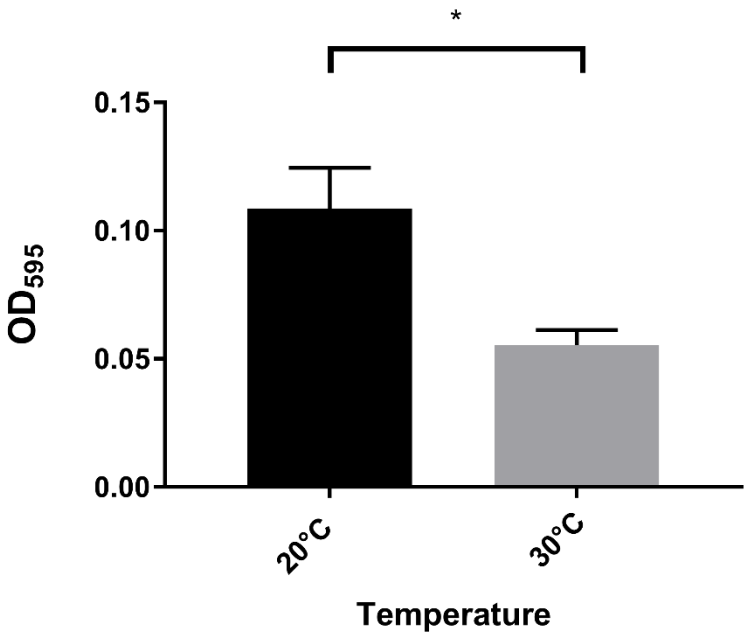

Figure 2.8. **Comparison of average biofilm production of all *L. monocytogenes* strains after 20h at 20°C & 30°C measured by microtiter plate assay (crystal violet staining).** Values are expressed as mean + SEM. (* = $P < 0.05$).

2.3.4.3. Comparison of biofilm production after 20h at 20°C and 30°C

A two-way ANOVA using strain and temperature as factors and biofilm production as an output variable showed that both strain, temperature and the interaction of these two variables had a significant ($P < 0.0001$) effect on biofilm production. Whilst generally, *L. monocytogenes* isolates produced more biofilm production at 20°C than 30°C, Sidak's test for multiple comparisons showed that isolates; NLmo2, NLmo5,

NLmo8, NLmo9, NLmo13, NLmo14, NLmo15, NLmo18 & NLmo20 all differed significantly ($P < 0.005$) in their ability to form biofilm at these two temperatures (Figure 2.9), and with the exception of NLmo5, all these strains produced more biofilm at 20°C.

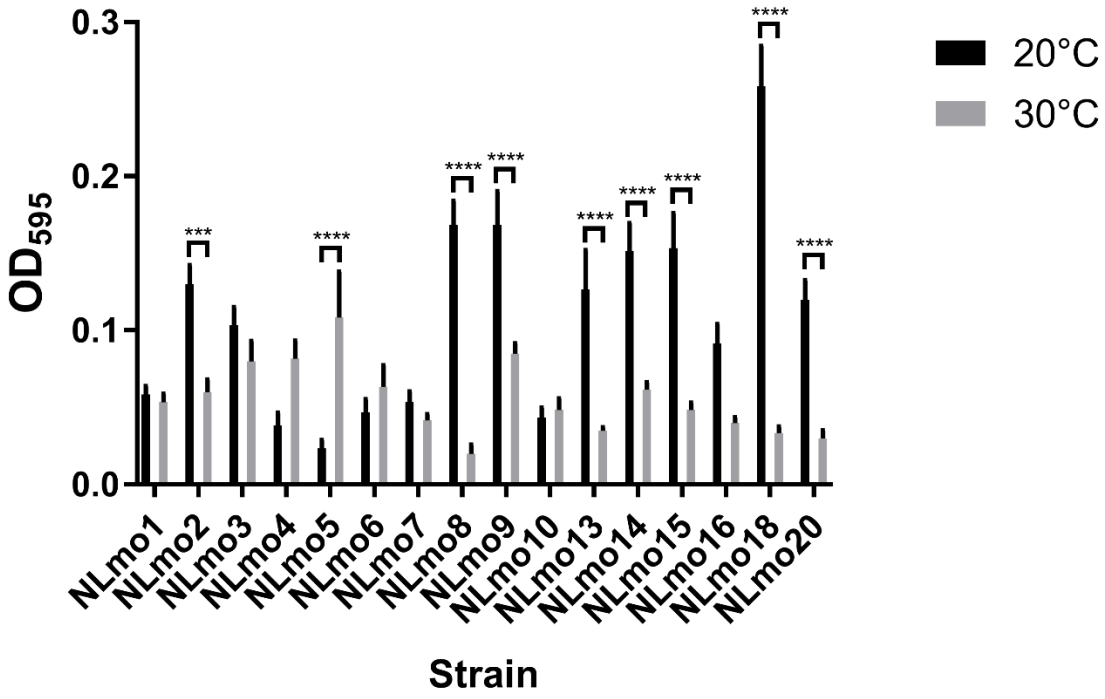

Figure 2.9. **Comparison of biofilm production after 20h at 20°C & 30°C for individual isolates.** Significant differences between biofilm production at different temperatures within strains as assessed by Tukey's test for multiple comparisons are indicated above bars (*** = $P < 0.005$, **** = $P < 0.0001$). Values are expressed as mean + SEM.

2.3.5. Swarming motility of *L. monocytogenes* isolates

Visually, different *L. monocytogenes* isolates produced different sized colonies when grown in 3% BHI agar (Figure 2.10.).

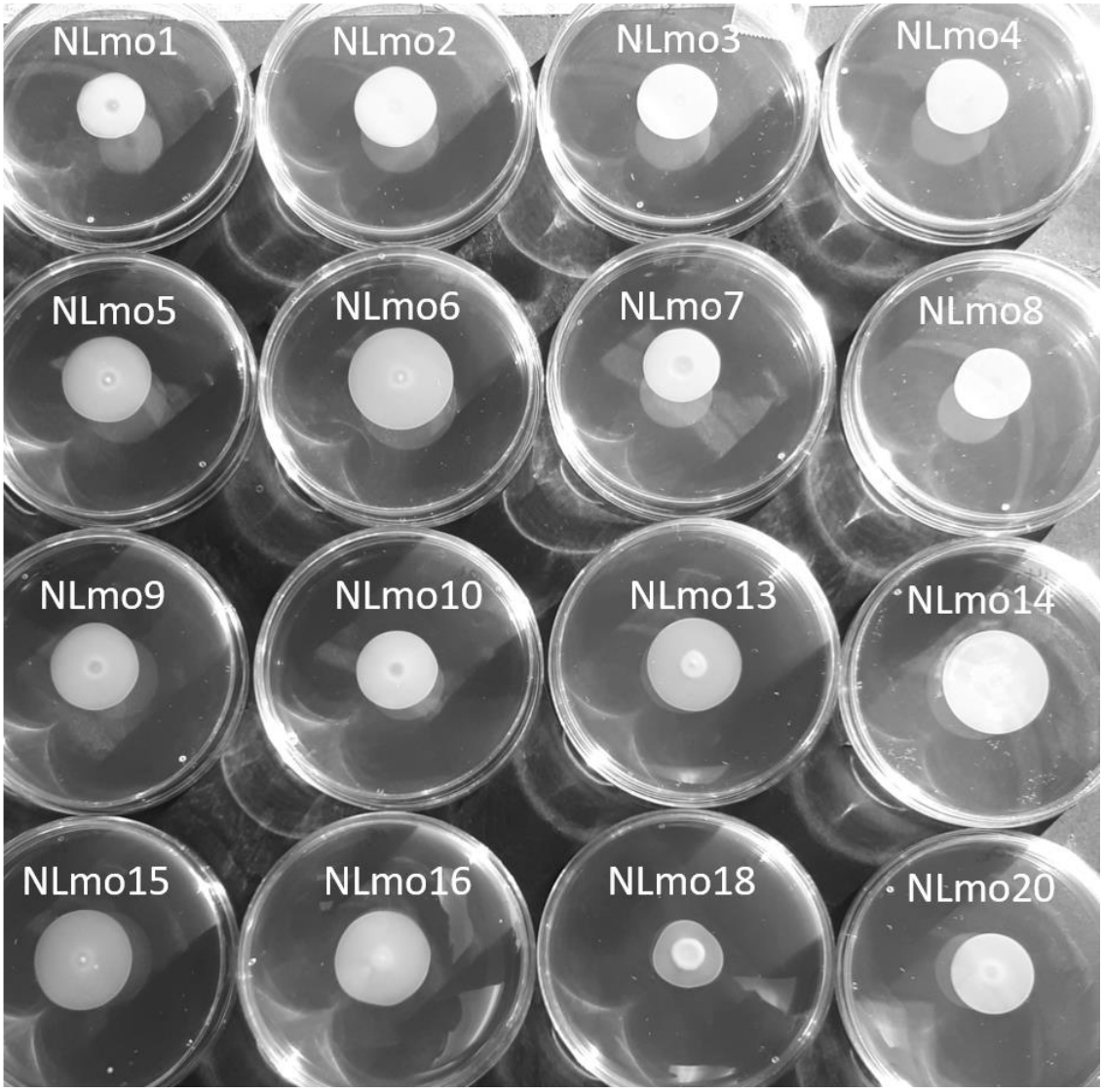

Figure 2.10. **Swarming motility of *L. monocytogenes* isolates on 3% BHI agar.** Internal strain reference is listed above colonies.

These colonies also differed in their morphology with some strains producing large, flat colonies whilst others produced smaller, more raised colonies (Figure 2.10.).

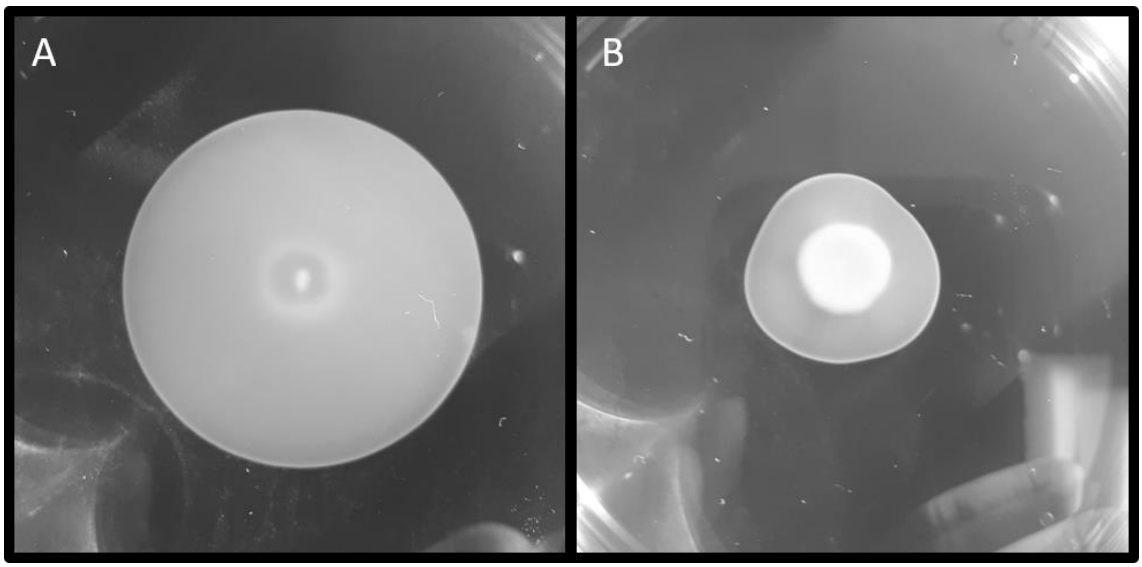

Figure 2.11. **Comparison of *L. monocytogenes* strains that make smooth, large colonies vs. small, raised colonies on 3% BHI agar.** Panel A = NLmo6, Panel B = NLmo18.

When measured (to the nearest mm), an ANOVA between the mean (n = 3) size of colonies showed there was a significant ($P < 0.0001$) difference between the size of colonies. Isolate NLmo18 produced the smallest colonies on average whilst NLmo9 produced the largest average colonies (Figure 2.11.). Tukey's test for multiple comparisons revealed significant differences in average colony size between isolates (Figure 2.12.). A correlation using the variables of average rate of swarming motility (mm) and biofilm production at 20°C, 20h (OD_{595}) determined that there was no significant correlation ($P = 0.872$) between these two characteristics amongst the suite of *L. monocytogenes* strains.

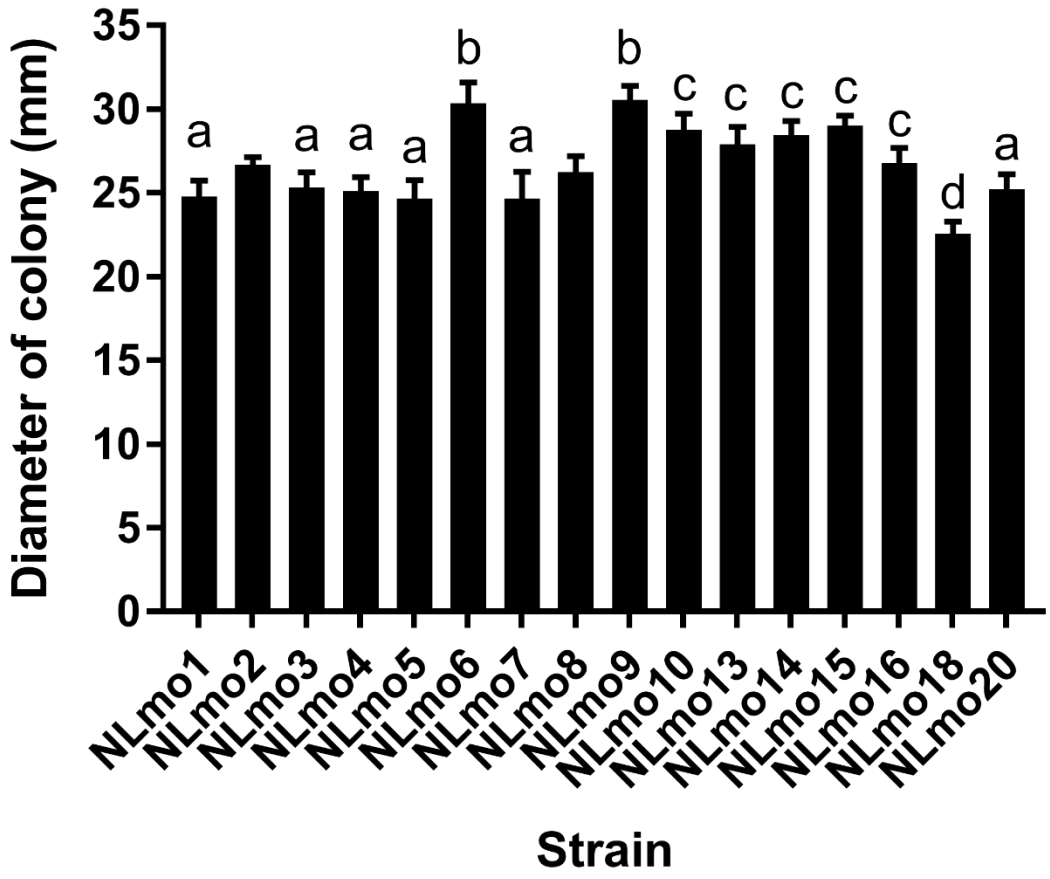

Figure 2.12. **Comparison of colony size of *L. monocytogenes* strains isolated from the UK fresh produce supply chain.** Values are the mean of three independent replicates and error bars represent SEM (n = 3). Groups are shown according to Tukey's test for multiple comparisons. Group a is significantly ($P < 0.05$) to group b. Group c is significantly ($P < 0.05$) different to group d but not groups a & b. Group d is significantly ($P < 0.05$) different to group c & b but not group a.

2.3.6. Summary of results

All strains were identified as *L. monocytogenes* by the species specific (Lm13) PCR. Furthermore, all strains were haemolytic (results not shown) which is indicative of pathogenic *Listeria* spp. MLST showed that the 15 isolates fell into two distinct *L. monocytogenes* lineages (I & II) according to phylogenetic analysis based on a 3288bp consensus sequence from 7 MLST housekeeping genes. Some strains showed high variation in G (as indicated by SEM) e.g. NLmo2, NLmo3 & NLmo10 whilst others showed low variation e.g. NLmo15, NLmo18 & NLmo20. However, there were no significant differences found in average growth rates between strains. Biofilm

production was variable amongst the tested isolates at both temperatures. Good biofilm formers at 20°C were not necessarily good biofilm formers at 30°C. Growth rate not correlated with biofilm production. Isolates also showed a variable degree of swarming motility. Isolates which fell into the same sequence type did not exhibit the same phenotypic characteristics across those tested in this chapter (Table 2.4.).

Table 2.4. **Summary of identification, MLST, and phenotypic traits of L. monocytogenes isolates from the UK fresh produce supply chain.** Y = yes ST = sequence type, Lineage = phylogenetic lineage (according to MLST), Growth rate = G (see section 2.2.4.) Biofilm production = biofilm production at 20h, 20°C. Rate of motility = swarming motility (mm).

Strain	Lm13 PCR	Haemolysis	ST	Lineage	G	Biofilm production	Rate of Motility
NLmo1	Y	Y	35	II	Average	Average	Low
NLmo2	Y	Y	5	I	Slow	Average	Average
NLmo3	Y	Y	5	I	Average	Average	Average
NLmo4	Y	Y	325	II	Average	Low	Average
NLmo5	Y	Y	325	II	Average	Low	Low
NLmo6	Y	Y	4	I	Slow	Low	High
NLmo7	Y	Y	1	I	Slow	Average	Low
NLmo8	Y	Y	219	I	Fast	High	Average
NLmo9	Y	Y	4	I	Average	High	High
NLmo10	Y	Y	1	I	Average	Low	High
NLmo13	Y	Y	37	II	Fast	Average	Average
NLmo14	Y	Y	1	I	Fast	High	Average
NLmo15	Y	Y	204	II	Average	High	High
NLmo16	Y	Y	37	II	Average	Average	Average
NLmo18	Y	Y	399	II	Average	High	Low
NLmo20	Y	Y	6	I	Fast	Average	Average

2.4. Discussion

2.4.1. Identification of *L. monocytogenes* isolates from the UK fresh product supply chain

The first step in this chapter was to confirm that all isolates received from 3rd party microbiology testing companies in the FLPSC were *L. monocytogenes*. This was done because these companies rely on biochemical/ELISA methods which can sometimes lead to false identifications. All isolates were confirmed to be *L. monocytogenes* by using a species-specific PCR using primers which distinguish this species from others in the *Listeria* genus. In addition, all strains were found to be haemolytic, which is a characteristic of pathogenic *L. monocytogenes* strains, as non-haemolytic strains are considered to be avirulent. The next step was to determine if different strains were present in the UK fresh produce supply chain. To do this, the sequence type (ST) of isolates was determined using MLST.

2.4.2. MLST of *L. monocytogenes* isolates provides insight into genetic diversity of the population in the FLPSC

Multi-locus sequence typing of isolates revealed a range of sequence types belonging to lineages I & II (Table 2.4.). Strains from ST1 (3 detected in this study) are globally distributed (Linke et al. 2014b), have been found to contribute to a relatively high number of human listeriosis cases (Henri et al. 2016) and have a strong association with rhombencephalitis in ruminants (Dreyer et al. 2016). These characteristics of strains from ST1 highlight the importance of this subtype and are indicative of this sequence type's supposed hypervirulence. ST4 strains are also globally distributed, are highly correlated with human sources and food and strains from this subtype have been isolated from soil (Linke et al. 2014b). ST5 strains have also been isolated from human sources and have caused outbreaks in the USA, from cantaloupe, chicken and imitation crabmeat, cantaloupe being the cause of the largest ever outbreak in the USA (Lomonaco et al. 2013; Wang et al. 2015). Strains from ST6 have a relatively low incidence of isolation but have been associated with previous outbreaks and have been linked to increasing rates of unfavourable outcome from infection, over time (Kremer et al. 2017) leading to the conclusion that this subtype is also hypervirulent. It is worth noting that the genetic and molecular basis for ST6's hypervirulence requires further investigation (Maury et al. 2016). ST219 is also considered to be a

hypervirulent clone (Calderon-Gonzalez et al. 2016). For these reasons, in this study, strains of interest in terms of public health are strains with the subtypes ST1, ST4, ST5, ST6 & ST219 (Lineage I).

From a public health perspective, other strains isolated in this study are of less concern currently, these are strains with subtypes; ST37, ST204 ST325 & ST399 (Lineage II). Strains from ST37 have been found to be associated with urban, farm and natural environments and are rarely associated with human and clinical cases (Linke et al. 2014b). Strains from ST204 have been found to occupy several niches in Australia, including clinical and non-clinical sources most likely due to the maintenance of mobile genetic elements which enhance the spread of antibiotic and stress resistance genes (Fox et al. 2016). Strains from ST325 have been linked with dairy sources (Jennison et al. 2017) and strains from ST399 are only rarely reported and are associated with environmental and ruminant samples (Dreyer et al. 2016). Overall, MLST is a useful tool for characterising the subtype of *L. monocytogenes* isolates and inferring virulence potential but it lacks the discriminatory power to distinguish amongst closely related strains of the bacterium which is essential for source tracking in clinical and food environments (Lomonaco and Nucera 2012). Furthermore, MLST does not provide data for the presence and function of important virulence genes in the *L. monocytogenes* genome which aids in identifying more virulent types of the bacteria (Liu 2006).

2.4.2. Examination of the phenotypic traits of *L. monocytogenes* shows variation between strains

2.4.2.1. Variation in generation time (G)

Growth of *L. monocytogenes* in culture depends on many different environmental factors including, but not limited to; temperature, pH, water activity (Tienungoon et al. 2000). In addition, growth depends on intrinsic factors such as strain type (Barbosa et al. 1994) With regards to the food industry, challenge tests which determine the growth of *L. monocytogenes* in foods, and thus which regulations must govern said foods, usually must include a strain with known growth characteristics (e.g. from the EU collection), while the other strains can be freely chosen (Álvarez-Ordóñez et al. 2015). The results presented in this work show that there was no significant differences in generation time between strains under the parameters tested. However,

some strains showed a high variation in growth rate over the 3 replicates (based on SEM), which could be a sign of phenotypic heterogeneity within the genetically homogeneous population (Grote et al. 2015).

2.4.2.2. Variation in biofilm production

L. monocytogenes biofilms can be formed on many different surfaces in food supply chains and provide a protective environment for bacterial survival thereby increasing the risk of subsequent contamination (Colagiorgi et al. 2017). Whilst all *L. monocytogenes* in this study formed biofilm, some formed significantly more biofilm than others on polystyrene 96-well plates when grown in MWB, a minimally defined media. No correlation existed between biofilm production and final OD (at 20 °C or 30 °C) suggesting other intrinsic factors, apart from final OD (and thus the number of present) are responsible for biofilm production. Furthermore, that some strains may have a competitive advantage over others in the fresh produce supply chain based on their ability to form biofilm. However, previous research has generated mixed results when determining whether strong biofilm formation is an indicator of persistence in processing environments (Magalhães et al. 2017; Nowak et al. 2017). Moreover, many environmental factors contribute to biofilm production in *L. monocytogenes* including substrate, surface material and temperature meaning a strain that is a good biofilm former in one environment may not necessarily form strong biofilm in another.

In keeping with previous research, biofilm formation of *L. monocytogenes* showed strong strain to strain variation (Colagiorgi et al. 2017). Biofilm production was also affected by temperature with strains producing more biofilm at 20°C than at 30°C on average. Whilst in previous research a correlation between lineage and biofilm production (lineage II isolates producing more biofilm has been reported (Borucki et al. 2003)) these results indicate that phylogenetic lineage does not correlate to biofilm production, in agreement with Di Bonaventura et al., (2008). Overall, further research is needed to examine the intrinsic factors which determine whether stains are strong or weak biofilm formers in the presence of the environmental variables that are representative of environments found in the fresh produce supply chain. Strains of the same sequence type did not necessarily produce the same amount of biofilm.

2.4.2.3. Variation in swarming motility

Bacteria simultaneously grow and spread rapidly over a surface that supplies them with nutrients and direct new cells to the edge of the colony - this swarming reduces competition between cells for nutrients, speeding growth (Kaiser 2007). Swarming motility in bacteria can be operationally defined as rapid multicellular bacterial surface movement powered by rotating flagella and is often oppositely regulated and antagonistic to biofilm formation (Kearns 2010). With regards to *L. monocytogenes* in particular, it has been shown that different strains show variable swarming motility (Roberts et al. 2009), in keeping with the data from this study. It has also been suggested that genes related to motility appear to be less expressed in strains that have a high growth rate (Aravena et al. 2016) but this study found no correlation between generation time and swarming motility (P = 0.552). Overall, like the other phenotypic traits investigated in this chapter swarming motility is variable between strains. Sequence type did not correlate with motility. For example, isolates from sequence type one all produced different sized colonies.

2.5. Conclusion

The first aim of this study was to identify *L. monocytogenes* strains isolated by 3rd party microbiology testing companies servicing the UK fresh produce supply chain. In total, 15 *L. monocytogenes* strains from a range of fresh produce products from different stages within the supply chain were studied. These *L. monocytogenes* strains were initially identified by a third-party microbial testing company and further confirmed to be *L. monocytogenes* by species-specific PCR which discriminates *L. monocytogenes* from other *Listeria* spp. Further characterisation by MLST showed a range of sequence types spanning two lineages (lineages I & II) and demonstrated that some isolates could not be distinguished by this method.

Overall, different *L. monocytogenes* strains exhibited differences in the phenotypic traits tested. Isolates with the same sequence type (as determined by MLST method) did not necessarily exhibit the same amount of biofilm, speed of growth in culture or rates or swarming motility *per se*. No correlation was found between growth rate and biofilm formation, biofilm formation and motility or growth rate and motility. These phenotypic characterisation methods were satisfactory to determine differences between isolates and MLST provided a genetic context. However, as discussed there

are difficulties relating phenotypic traits to survivability/persistence of *L. monocytogenes* in the context of the fresh produce supply chain. Whilst MLST is a sufficient tool for lineage assessment and to examine relatedness of isolates to other *L. monocytogenes* strains in a broad sense, it lacks the accuracy to distinguish between closely related strains and provides no additional information, such as the presence of important virulence genes, which can be used to identify potentially more virulent isolates. The variation in phenotypic traits between isolates of the same sequence type also indicates that there are strains differences, which are not shown by MSLT. Therefore, the next stage of this study focussed on using a more accurate, informative method to characterise strains – whole genome sequencing.

Chapter 3 Assessment of the relatedness and potential pathogenicity of *L. monocytogenes* isolates from the UK fresh produce supply chain using whole genome sequencing

3.1. Introduction

In chapter 2, 15 *L. monocytogenes* strains were isolated from various environments in the UK fresh produce supply chain. These isolates were confirmed as *L. monocytogenes* by species-specific PCR and subjected to a variety of phenotypic trait tests, including; biofilm formation, growth rates and rates of swarming motility. Isolates were also characterised genetically by multi-locus sequence typing (MLST), a DNA-based technique which separates different strains of the bacteria into various subtypes based on a combination of seven sequenced alleles (alleic profile). Results showed that a genetically diverse population exists in the UK FLPSC but also revealed some strains that could not be distinguished from each other using this method. MLST can help to identify more virulent types of *L. monocytogenes*, allows study of the relationships between isolates and is used for lineage assessment (Wu et al. 2016).

While MLST is a powerful tool for these purposes, it is only sufficient to examine the relatedness of isolates in a broad sense and lacks the resolution to distinguish between more closely related isolates (Smith et al. 2019). This discriminatory power is essential for source tracking in outbreak investigations. Phylogenies created with MLST data are based on a concatenated DNA sequence of 7 housekeeping genes which is 3288bp (around 1% of the *L. monocytogenes* genome). The ability to distinguish between closely related isolates is a key part of microbial source tracking in food supply chains and (combined with strong context and epidemiological knowledge and sample information) allows investigators to determine which strains of bacteria are transient within a sampled population, and which are persistent. Additionally, MLST gives no information on the presence and intactness of key virulence genes in *L. monocytogenes* such as the internalin family (*inlA, inlB, inlC, inlF, inlJ, inlK*), Listeria pathogenicity islands (LIPI-1, LIPI-3), etc (Table 3.1). This information is key for determining virulence potential and has potential to be used to inform risk assessments in food supply chains.

In contrast to MLST, whole genome sequencing (WGS) allows the user to create more accurate phylogenies and distinguish between closely related *L. monocytogenes* isolates due to size of sequence being analysed (~3Mb compared to ~3kb). One such type of analysis that can be used to create phylogenies and distinguish between closely related isolates is to compare the number of core genome single nucleotide polymorphisms (cgSNPs) between *L. monocytogenes* isolates and create a phylogeny based on these results. Various bioinformatics tools are associated with this type of processing of WGS data and these tools can be used on a variety of operating systems. Tools for the processing of WGS data range from user-interface 'point and click' programmes which are generally used on Windows and Macintosh operating systems, to bespoke, open source bioinformatics pipelines which are predominantly written and operated on Linux systems. User-interface programmes for the processing of WGS data are generally considered more user-friendly but come with somewhat limited analytical capabilities. Bioinformatics pipelines, on the other hand, can be tailored to the users' needs and take advantage of open source software which is maintained and regularly updated by the authors. A bioinformatics pipeline which has been tailored for the processing of bacterial whole genome sequencing data is the Nullarbor pipeline (Seemann et al. 2019). Nullarbor is operated using the command line in the Linux operating system and processes whole genome sequencing data (illumina paired-end sequencing data) using a variety of built in programmes. From Illumina paired-end sequencing data, Nullarbor (per isolate); assesses the quality of sequence reads (QC), identifies bacterial species, creates *de novo* assembly genomes, annotates these genomes, identifies sequence type (using MLST) and generates a virulome and resistome. For a set of isolates, Nullarbor finds core genome SNPs, infers a SNP phylogeny and generates SNP distance matrix (Figure 3.1.).

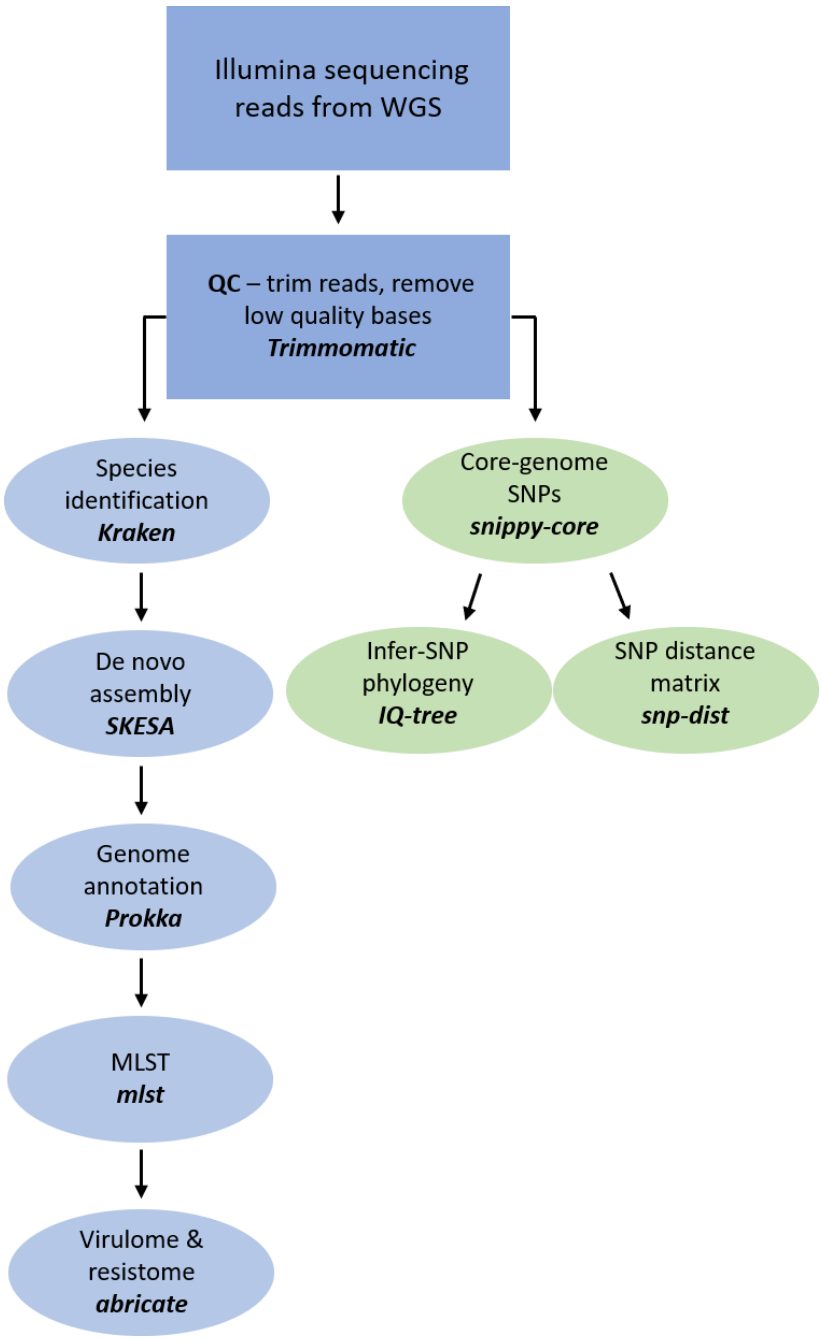

Figure 3.1. **Schematic diagram of the processing of WGS data using the Nullarbor pipeline.** Programmes used for various processes in the pipeline are italicised and bold. Blue ovals represent processes which occur for data per isolate, green ovals represent processes which occur per isolate set (e.g. for 15 genomes of *L. monocytogenes*).

Distinguishing between closely related bacterial isolates using SNP based phylogenies is a methodology that has been used to track gonococcal infections (Kwong et al. 2018), identify persistent *Staphylococcus aureus* bacteraemia (Giulieri et al. 2018) and track *L. monocytogenes* movement in a meat establishment (Nastasijevic et al. 2017). The use of WGS with regards to surveillance of *L. monocytogenes* was pioneered by

Kwong et al. (2016), where the authors analysed genomes from a large number of isolates and found that generation of WGS data combined with cgSNP analysis allowed increased resolution/discrimination of closely related isolates to infer the likelihood of transmission or a point source exposure in an outbreak compared to other typing methods such as PFGE. Thus, the advent of WGS has enabled source tracking of *L. monocytogenes* during outbreak investigations and research, but this technology is not yet used for general surveillance in food supply chains by commercial food testing companies because of its cost (including the purchase of capital and consumable equipment), complexity of analysis and the expertise required to interpret such data. Investigating bodies, in contrast, employ this technology to identify the source of a *L. monocytogenes* outbreak through identifying related cases of listeriosis, identifying the outbreak strain/strains in one or more common food items, and finally (through targeted sampling and traceability of foodstuffs in the supply chain) identifying the offending stage/location in a supply chain (Pouillot et al. 2016; Li et al. 2017). Once the source of the bacteria has been identified, regulatory authorities can take necessary steps to eradicate the source and prevent further contamination.

In addition to source tracking, subtyping of *L. monocytogenes* strains using WGS can reveal pathogenic profiles (virulence potential) and infer relative risk to the consumer. Determining the virulence potential of isolates is important in terms of public health as differences in virulence between *L. monocytogenes* strains may influence infection and clinical outcome. It is worth nothing that all strains of *L. monocytogenes* are currently treated equally for regulatory purposes (Fravalo et al. 2017), however, some strains are highly pathogenic and are more often associated with epidemics of disease, whereas others can be less virulent and are rarely associated with epidemics (Velge and Roche 2010). For example, the majority of clinical *L. monocytogenes* strains fall into phylogenetic lineage I, and hypervirulent strains (from lineage I) belonging to sequence type-6 (ST6) have been implicated recently in two large outbreaks of foodborne listeriosis which caused widespread illness and mortality (European Food Safety Authority 2018; National Listeria Incident Management Team 2018). Further, it is widely acknowledged that differences in virulence potential can arise from premature stop codons (PMSCs) in the *inlA* gene. PMSCs result in a truncated and expressed internalin-A and attenuated virulence (Gelbíčová et al. 2015). Observation

of this characteristic in strains isolated from food and environmental sources compared to clinical sources has been recognised (Nightingale et al. 2005; Fravalo et al. 2017). However, a recent study from Ireland demonstrated that five out of the six *L. monocytogenes* strains isolated from food did not contain PMSCs in *inlA* (Hilliard et al. 2018), showing that this characteristic may not be as widespread in isolates from food as previously thought and requires further investigation. WGS allows researchers to screen the *L. monocytogenes* genomes for important virulence factors like *inlA* and related genes to aid in determining virulence potential while other subtyping techniques do not allow this type of analysis.

To more accurately determine the relatedness of strains and establish a virulence profile, in this chapter, *L. monocytogenes* isolates from the UK fresh produce supply chain were further characterised by WGS and associated bioinformatics tools. WGS data was used to develop a phylogenetic framework using core genome SNPs. In addition, genomes were examined for the presence of virulence and resistance genes and evidence for PMSCs in *inlA*. This information was then used to create a virulence profile for *L. monocytogenes* isolates from the UK fresh produce supply chain and infer relative risk to the consumer. Additionally, data from biofilm formation under optimum conditions (20h, 20°C) from the previous chapter (chapter 2) was used to investigate differences in biofilm formation between isolates that were found to be indistinguishable by WGS.

Aim: To assess the relatedness and virulence potential of *L. monocytogenes* isolates from the UK fresh produce supply chain using WGS

Objectives:

- Sequence the whole genome of *L. monocytogenes* isolates from the UK fresh produce supply chain
- Use associated bioinformatics to create a SNP-based phylogeny and determine the relatedness of isolates
- Inspect the genomes of isolates for the presence and intactness of key virulence & resistance genes

3.2. Materials and Methods

3.2.1. DNA extraction and whole genome sequencing

From long term *L. monocytogenes* stocks (see section 2.2.2.), a lawn of bacteria was grown on BHI agar (Oxoid) before being transferred to a barcoded cryovial (MicrobesNG) containing beads and preparation broth, then briefly vortexed before being sent to MicrobesNG (University of Birmingham, UK) for DNA extraction and whole genome sequencing. For DNA extraction, three beads were washed with extraction buffer containing lysozyme and RNase A, incubated for 25 min at 37°C. Proteinase K and RNaseA were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Eppendorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time increased to 1 minute from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol, with a target 30-fold depth of coverage.

3.3. Bioinformatics methods

3.3.1. De novo assembly, species identification, multi-locus sequence typing, virulome and resistome

Illumina reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger et al., 2014). Trimmed sequencing reads were then processed using a customised, open source bioinformatics pipeline for the handling of sequence data (https://github.com/tseemann/nullarbor). Within the pipeline, *de novo* assembly was performed on samples using SKESA version 2.1 (Souvorov et al., 2018) with the default parameters. Genome annotation was carried out by Prokka (Seemann, 2014). Species identification was carried out by k-mer analysis against a known database (MiniKraken 8GB). Assembled genomes were then scanned for sequence

type using MLST version 2.11 (Seemann https://github.com/tseemann/mlst). Virulence and resistance genes were detected by Abricate, version 0.8 (Seemann https://github.com/tseemann/abricate), which uses BLAST+ & EMBOSS to screen contigs against databases of known sequences of virulence and resistance genes. Virulence and resistance genes were detected by comparison to the Database for Virulence Factors of Pathogenic Bacteria (VDFB) (Chen et al., 2016) and Resfinder (Zankari et al., 2012) databases, respectively. Virulence and resistance genes were considered present when coverage ≥ 95% and identity > 75%, probable when coverage ≥ 36.4% and identity > 75% and missing when undetected. Abricate results were corroborated by manually inspecting Abricate output tables where % coverage, % identity, gene name, accession number and position in a contig were reported. When genes were reported as partial or in two parts, genome annotations (generated by Prokka) were manually inspected, a complete ORF was identified and the annotated nucleotide sequence of the gene was copied and subsequently used in BLAST (Altschul et al., 1990) to ascertain homology with known sequences. When the full-length gene returned coverage ≥ 95% and identity > 75% to *L. monocytogenes*, genes were considered present. If the annotated gene was not found in the genome annotation, as was the case with *ami*, the gene nucleotide sequence was copied from the VDFB Listeria database and used to BLAST against the contig where it was identified by Abricate. *Ami* was considered present when BLAST results returned ≥ 95% and identity > 75%. Occasionally, virulence genes fell between contigs. In these cases, gene presence was considered probable due to the high % identity of these sequences but low % coverage.

3.3.1.1. Determination of PMSCs in inlA

To determine whether strains contained a PMSC in the *inlA* gene, Abricate output tables were used to locate the position of full length *inlA* in (2403bp) in SnapGene Viewer. This sequence was then uploaded to MEGAX for all strains and sequences were subsequently aligned to *L. monocytogenes* EGD-e *inlA* reference using the MUSCLE algorithm. Alignment was then manually inspected for PMSCs in inlA based on those which have been previously reported (Gelbíčová et al., 2015).

3.3.2. Global core genome alignment and construction of Maximum Likelihood phylogeny based on core genome SNPs

To obtain a global alignment of all isolates included in this work, per sample sequence reads were mapped to *L. monocytogenes* EGD-e (NCBI: AL591824.1, 2,9445,28 bp), a ST35, lineage II, L. monocytogenes reference genome. For SNP based analysis, read mapping, and core genome alignment were performed using the Snippy pipeline, version 4.0 (Seemann https://github.com/tseemann/snippy). Sequence reads were aligned to the reference genome and sites that were covered by less than 10 reads were not included in analysis. Any site where at least one of the isolates had a different nucleotide from the other isolates and none of the isolates were absent was considered a core-SNP site. Core-SNPs were used as output to determine the phylogeny of the *L. monocytogenes* population which was inferred by IQ-TREE version 1.6.7 (Nguyen et al., 2015) using the Maximum Likelihood method with model finder (Kalyaanamoorthy et al., 2017) option enabled. Branch support was calculated using ultrafast bootstrap support (Hoang et al., 2017) and the SH-like approximate likelihood ratio test (Guindon et al., 2010), both with 1000 iterations. One *L. ivanovii* isolate isolated from the fresh produce supply chain was included in sequencing, de novo assembly and in core genome SNP analysis. The phylogenetic tree was rooted using *L. ivanovii* as an outgroup. The tree was then modelled and annotated in Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

3.3.3. Determination of genetic relatedness between strains

To establish relatedness between L. monocytogenes strains from the fresh produce supply chain we considered tree topology, MLST and the pairwise SNP distance matrix computed from the core genome alignment to the reference. When determining SNP based phylogenies and examining bacterial outbreaks, different SNP-based subtyping workflows result in variation in the number of core SNPs predicted within the same data set which means that it is not possible to define a universal single cut-off value for delineation of outbreaks or in this case, indistinguishable strains (Saltykova et al., 2018). Therefore, for each workflow this threshold should be estimated separately. Whilst different WGS analytical tools, SNP calling algorithms and reference genomes (Kwong et al., 2016) mean that SNP data is not directly comparable between studies, previous investigations on outbreaks have defined ‘outbreak strains’ that differ by 5,

10, 20 and 28 SNPs (Chen et al., 2017). Additionally, 'plant (i.e. facility) associated' clones have been found to differ by up to 6 SNPs (Morganti et al., 2015). Thus, in this work, it was determined that isolates were undistinguishable i.e. the same strain, when they differed by ≤ 5 SNPs.

3.3.4. Statistical analysis of differences in biofilm production

To determine differences in biofilm production under optimum conditions (20 °C, 20 h incubation) between strains which were determined to be the 'same strain', an ANOVA was used with Tukey's post-hoc test for multiple comparisons.

3.4. Results

3.4.1. Multi locus sequence typing and SNP based phylogeny using whole genome sequencing

WGS generated various numbers of reads, between 488,048 & 4,673,156. The genomes had average depths of coverage between 31 to 308-fold of the reference. *De novo* assembly resulted in genome assemblies of 9 to 21 contigs and the consensus lengths of genomes ranged from 2.87Mb to 3.06Mb. GC content of isolates ranged from 37.4 - 38.9%. All isolates were identified as *L. monocytogenes* and had 88.87-94.64% similarity to *L. monocytogenes* using the Kraken 8GB database (Appendix B, Table B.2.). Using data from WGS, MLST determined 9 distinct sequence types from 15 isolates (Table 3.2.), where the results matched sequence types obtained through PCR based MLST in chapter 2.

Table 3.1. **Allelic profile and number of isolates represented by each sequence type of *L. monocytogenes* isolated from the fresh produce supply chain.** The MLST scheme used to characterise L. monocytogenes isolates is determined by the allelic profile of 7 housekeeping genes, these are; ABC transporter (abcZ), beta-glucosidase (blgA), catalase (cat), succinyl diaminopimelate (dapE), D-amino acid aminotransferase (dat), L-lactate dehydrogenase (ldh) and histidine kinase (lhkA). Clonal complex and lineage information is included.

Sequence type	Clonal complex	Lineage	Number of isolates
1	CC1	I	3/15
4	CC4	I	2/15
5	CC5	I	2/15
6	CC6	I	1/15
37	CC37	II	2/15
204	CC204	II	1/15
219	CC4	I	1/15
325	CC31	II	2/15
399	CC14	II	1/15

Some isolates shared the same sequence type with the most frequent being ST1 (3/15). No new sequence types were discovered. Several strains could not be

distinguished by MLST analysis; these being NLmo10, NLmo14 & NLmo7 (ST1), NLmo6 & NLmo9 (ST4), NLmo2 & NLmo3 (ST5), NLmo13 & NLmo16 (ST37) and NLmo4 & NLmo5 (ST325). These results are in agreement with MLST results from chapter 2.

A phylogeny of the 15 *L. monocytogenes* strains was obtained using core genome SNPs and showed that when compared, isolates from the fresh produce supply chain differ from 0 up to 40,143 cgSNPs. SNP analysis allowed differences between isolates to be established in greater detail than MLST. For example, some isolates from the same MLST groups had no SNP differences while others were different by up to 59 SNPs (Appendix B, Table B.2). SNP analysis highlighted 4 sets of 'indistinguishable' strains, these were; NLmo2 & NLmo3, NLmo4 & NLmo5, NLmo7 & NLmo14 and NLmo13 & NLmo16. Isolates were spread across two genetic lineages from a total of 8 sequence types and 8 clonal complexes (CC's). These isolates were isolated from 4 different types of environment in the UK FLPSC (Figure 3.2.).

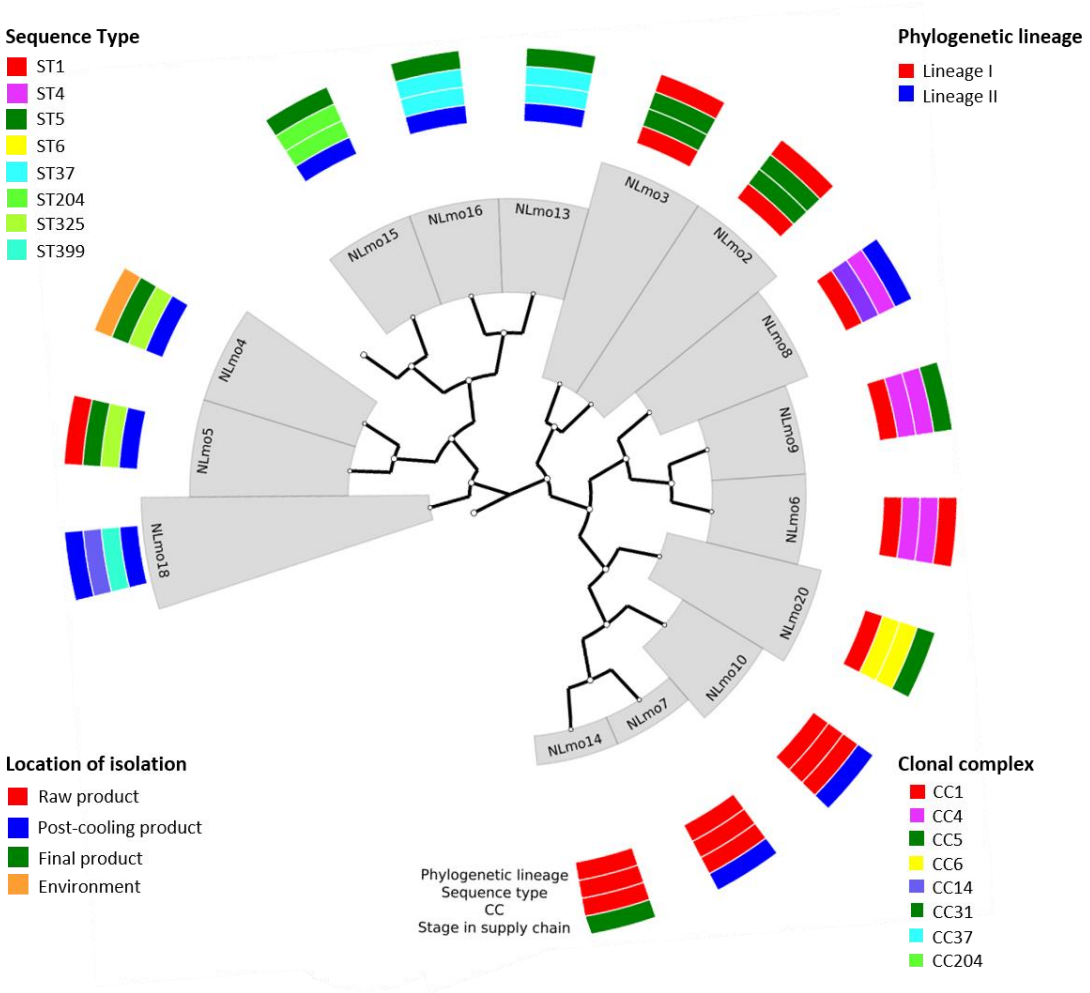

Figure 3.2. **Maximum-likelihood phylogeny of *L. monocytogenes* isolates from the UK FLPSC**. Isolates from raw product, post-cooling product, final product and environmental sources are shown and include comparisons between two evolutionary lineages, 8 clonal complexes (CC), and 8 sequence types. The source of the isolate is indicated in the outer ring by the colours in the legend. The inner rings show phylogenetic lineage and sequence type and clonal complex groupings with the same colour representing the same lineage, clonal complex, sequence type. The phylogeny was inferred using IQTree Version 1.6.7 was constructed using GraPhlAn v0.9.7. (https://huttenhower.sph.harvard.edu/graphlan).

3.4.2. Prevalence of virulence- and resistance-associated genes

The 15 *L. monocytogenes* isolate genomes were analysed for the presence or absence of 42 key virulence factors (genes) by Abricate version 0.8 using the VFDB database (Chen et al. 2016a) and corroborated by manual inspection of genome annotations combined with BLAST of virulence gene sequences against contigs. The presence or

absence of the full range of virulence factors can be seen in Appendix B, Table B.2. Seven out of the 9 isolates in lineage I had 41 virulence factors present whilst 2 out of 9 (NLmo2 & NLmo3) had 34 virulence factors. Of the 6 strains that were in lineage II, 1 isolate (NLmo18) had 33 virulence factors, 3 isolates (NLmo13, NLmo15, NLmo16) had 32 virulence factors and 2 isolates (NLmo4 & NLmo5) had 31 virulence factors present (Figure 3.3). Isolates which had ≤ 41 virulence factors present were all missing the LIPI-3 gene cluster (*llsY, llsX, llsP, llsH, llsG, llsD, llsB, llsA*) which encodes Listeriolysin S. Except for NLmo2 & NLmo3, isolates from lineage I were missing the *ami* gene which codes for an autolysin amidase protein. All isolates contained an intact and full length *inlA* gene, apart from NLmo20 which had a 9-nucleotide deletion in position 2212-2220bp (797 aa's), this version of Internalin A is predicted to be fully functional and isolates with this variant show similar invasion ability compared with strains full length Internalin A (Toledo et al. 2018).

Genomes were also analysed for the presence of resistance genes (Appendix B, Table B.4.) by scanning contigs using Abricate against the ResFinder database. All isolates carried the Fosfomycin resistance thiol transferase (*fosX)* and lincomycin resistance ABC-F type ribosomal protection protein (*lin)* genes whilst 2 isolates (NLmo4 & NLmo5) carried 2 additional, plasmid derived, resistance genes for a quaternary ammonium compound efflux transporter (*bcrB & bcrC*). Figure 3.3. shows the relationship between *L. monocytogenes* strains and the presence of virulence- and resistance-associated genes.

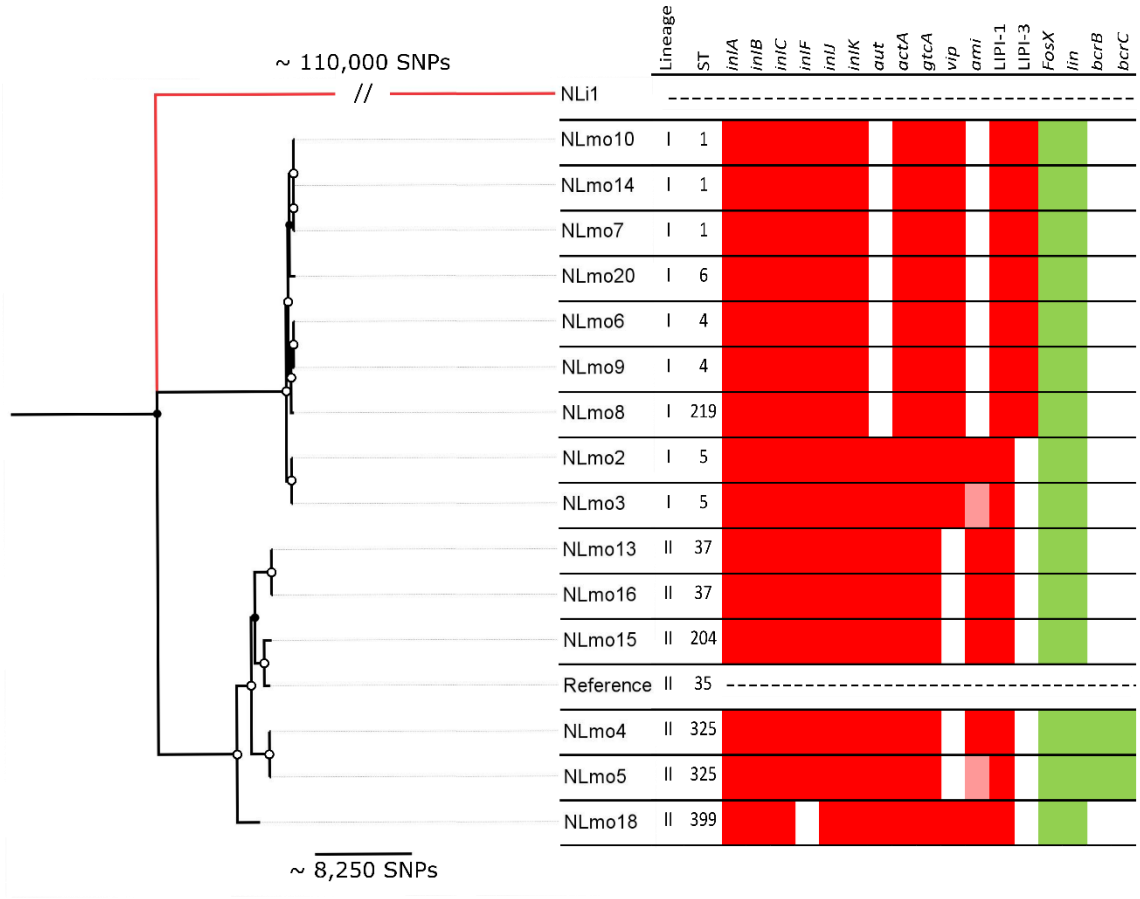

Figure 3.3. **Maximum Likelihood phylogeny of 15 *L. monocytogenes* isolates and 1 *L. ivanovii* isolate from the UK fresh produce supply chain in relation to the presence of virulence and resistance genes.** White circles indicate nodes with ≥ 95% ultrafast bootstrap support and ≥ 80% SH-like approximate likelihood ratio test support. The break (//) in the root branch represents a comparatively long evolutionary distance to the outgroup, *Listeria ivanovii* (NLi1), which is highlighted in red. Approximate branch distance (in SNPs) of this branch is indicated above the break. Columns right of the tree indicate presence (red) probable presence (pink) or absence (white) of L. monocytogenes virulence factors and presence (green) or absence (white) of resistance genes (see Appendix 2 for full lists of genes). Evolutionary distances were computed using the Maximum likelihood method and are in units of SNPs. The analyses involved 17 nucleotide sequences. Evolutionary analyses were conducted using IQTree version 1.6.7. tree was generated using FigTree. Dashed lines indicate strains for which no data on the presence of virulence and resistance genes was collected. ST = MLST sequence type.

3.4.3.3. Comparison of biofilm formation using a SNP based phylogeny

Using the SNP based phylogeny and data from biofilm production under optimum conditions from chapter 2 (20h incubation, 20°C), strains which were identified as 'indistinguishable' were compared by the amount of biofilm they formed. Figure 3.4. shows that some isolates produced a comparatively small (Average $OD_{595} \leq 0.053$) amount of biofilm (NLmo4, NLmo5, NLmo7 & NLmo10), whilst others produced a moderate ($0.053 >$ Average $OD_{595} \leq 0.152$) amount of biofilm (NLmo2, NLmo3, NLmo6, NLmo13, NLmo14, NLmo15, NLmo16, NLmo20) and three isolates produced a comparatively high (Average $OD_{595} > 0.152$) amount of biofilm (NLmo8, NLmo9, NLmo18). Biofilm production was inconsistent between pairs of genetically indistinguishable strains. Pairs of indistinguishable isolates were; NLmo2 & NLmo3, NLmo4 & NLmo5, NLmo7 & NLmo14 and NLmo13 & NLmo16. An ANOVA with Tukey's post-hoc test for multiple comparisons showed that indistinguishable pairs of strains NLmo2 & NLmo3, NLmo4 & NLmo5 and NLmo13 & NLmo16 did not differ significantly ($P > 0.05$) in their ability to form biofilm. In contrast, indistinguishable strains NLmo7 & NLmo14 did differ significantly ($P = 0.0005$) in their ability to form biofilm.

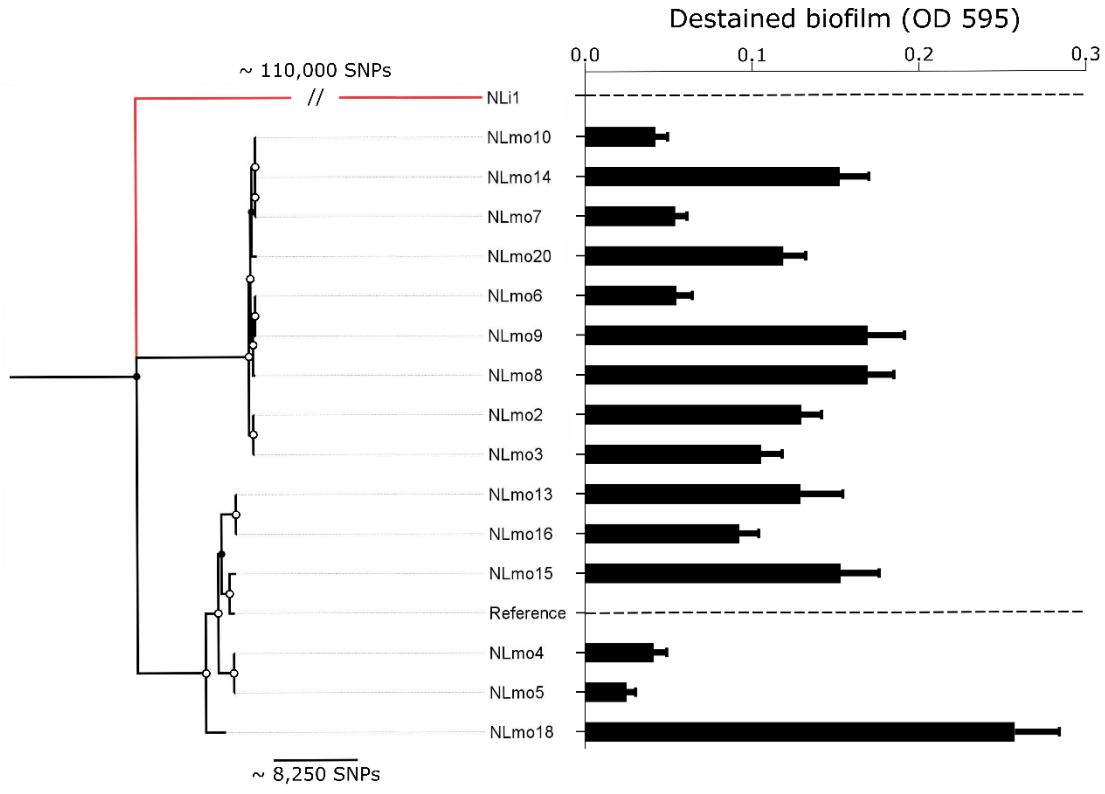

Figure 3.4. **Maximum Likelihood phylogeny of 15 *L. monocytogenes* isolates and 1 *L. ivanovii* isolate from the UK fresh produce supply chain trees inferred using a core-**

SNP based phylogeny in relation to biofilm production. Biofilm production assessed by staining with crystal violet, destaining and measuring absorbance at 595nm. White circles indicate nodes with ≥ 95% ultrafast bootstrap support and ≥ 80% SH-like approximate likelihood ratio test support. The break (//) in the root branch represents a comparatively long evolutionary distance to the outgroup, Listeria ivanovii (NLi1), which is highlighted in red. The approximate branch distance (in SNPs) of this branch is indicated above the break. Dashed lines indicate strains for which no data on biofilm production was collected. Error bars represent SEM of 6 replicates.

3.5. Discussion

L. monocytogenes remains an important foodborne pathogen and is a significant threat to public health in the food supply chain as illustrated by recent outbreaks (European Food Safety Authority 2018; National Listeria Incident Management Team 2018). *L. monocytogenes* has been previously identified in UK foodstuffs (Little et al. 2009), but the virulence and relatedness of strains has not been characterised. This information is important for inferring the potential risk that isolates pose to consumers and in source tracking/highlighting persistent strains. Understanding these aspects of *L. monocytogenes* ecology in food supply chains can help regulators and operators to design more effective microbial surveillance and prevention strategies and inform risk assessments. While it is difficult to draw general conclusions about the whole UK fresh produce supply chain due to the limited number of strains used in this work, these data provide a preliminary insight into the diversity of the *L. monocytogenes* population and virulence potential of *L. monocytogenes* directly isolated from the UK fresh produce supply chain.

MLST of isolates revealed a range of sequence types belonging to lineages I & II (Table 3.2.). MLST is a useful tool for characterising the subtype of *L. monocytogenes* isolates and inferring virulence potential but it lacks the discriminatory power to distinguish amongst closely related strains of the bacterium which is essential for source tracking in clinical and food environments (Lomonaco and Nucera 2012). This observation is corroborated with these results which also demonstrate that phylogenies constructed using concatenated MLST sequences result in a tree with comparably low confidence and low discriminatory power (Chapter 2, Figure 2.4.). Thus, for inter-strain comparison, deeper resolution and higher confidence is achieved with SNP based phylogenies versus those constructed using MLST. SNP based comparisons using the whole genome currently give the greatest discriminatory power available and this method is quickly becoming the gold standard tool for use in outbreak investigations due to its ever-decreasing cost (£70 per genome in this work). Furthermore, based on experience with both methods, the relative effort in comparing isolates using a SNP-based approach vs. MLST is comparable, reinforcing this approach as superior.

In this chapter, 15 *L. monocytogenes* strains spanning two lineages were isolated from various environments in the fresh produce supply chain. According to the presence of SNPs, this work identified isolates that were defined as indistinguishable (<5 SNPs), which also fell into the same sequence type and identified isolates that fell into the same sequence type but differed by up to 59 SNPs. While some isolates were closely related, others were relatively distantly related, suggesting that *L. monocytogenes* contamination of the fresh produce supply chain is a complicated mixture of sporadic contamination, surface-to-surface cross-contamination and/or contamination arising from common sources. With regards to specific cases, indistinguishable strains NLmo2 & NLmo3 were isolated from raw product from the same location (single farm) a week apart. Likewise, isolates NLmo13 & NLmo16 were isolated from raw product in a different common location (holding/packing facility) around 3 weeks apart. These findings may suggest local cross contamination within a given space in the supply chain. In contrast, indistinguishable isolates NLmo7 & NLmo14 came from different locations in the supply chain where product was sampled months apart and isolates NLmo4 & NLmo5 were isolated within a week of each other but at different points in the supply chain. The complexity of the fresh produce supply chain makes it difficult to draw conclusions about these indistinguishable isolates, for example, whether cross contamination has occurred (and in which direction) or whether strains were spread to these locations from a separate, but common source.

While it can be inferred that indistinguishable strains in the supply chain either came from a common source or were the result of cross contamination, it is impossible to confidently elucidate either scenario without the presence of a robust, targeted sampling plan which characterises *L. monocytogenes* using WGS. As well as a robust sampling plan that uses WGS as a subtyping method, investigations of this kind require strong context knowledge and epidemiological data to elucidate contamination mechanisms (Stasiewicz et al. 2015). If comparisons of strains based on the core genome do not provide sufficient resolution for discrimination of strains, the accessory genome of *L. monocytogenes* has been shown to be highly variable between closely related isolates (Casey et al. 2016). Inclusion of the accessory genome during analysis has been used previously to give sufficient

discriminatory resolution between isolates for determination of outbreak strains (Chen et al. 2016b).

Routine commercial sampling of fresh produce and surrounding environments is often infrequent and source tracking in a processing environment remains difficult due to the risk of recontamination, that is, if an indistinguishable strain is found in a processing environment and on a raw material it does not prove that the contamination came from the raw material or vice versa. The current work demonstrates the power of WGS technology in terms of establishing a virulence potential for isolates and discriminating between closely related strains of *L. monocytogenes* and should serve towards designing and implementing a more robust sampling plan for detecting *L. monocytogenes* in the UK fresh produce supply chain. To elucidate a specific source of *L. monocytogenes* in this context, stakeholders would have to embark on a regular sampling regime of both the processing environment and raw (incoming) and processed (outgoing) product combined with WGS and SNP-based analysis. This approach may be able to differentiate sporadic vs. persistent strains and highlight modes of *L. monocytogenes* transmission from the growth environment to inside the processing environment. Within the processing environment, targeted sampling of product contact sites as well as potential harbourage sites may reveal sources of the bacteria. Sampling of processing environments is particularly important as these facilities are 'bottle neck' spaces in food supply chains which come in to contact with all processed produce and thus are potential sites of cross-contamination. Sampling of processed (outgoing) produce is equally important as indistinguishable strains isolated over time, from produce of different origins (growth environments), but passing through the same processing environment would be indicative of cross-contamination and persistence in the processing environment. Overall, more regular sampling and genome-wide strain characterisation is needed to ensure consumer safety but may currently be beyond the scope of the fresh produce supply chain in terms of time and financial investment. For food production and processing companies, where the financial cost and negative reputational impact of a listeriosis outbreak is potentially large (McCollum et al. 2013), this approach should be more

attractive because of the additional information it provides over characterisation techniques which only identify bacteria down to the species level.

Whole genome sequences of isolates revealed that all strains contained the *hly* (listeriolsyin O) gene and all strains had the internalin family of genes present (*inlA, inlB, inlC, inlF, inlJ, inlK*) except strain NLmo18, where *inlF* was missing. This gene codes for a protein that mediates invasion of the brain of the host by binding with vimentin (Ghosh et al. 2018) and suggests that NLmo18 may have reduced virulence in the host based on the lack of *inlF*. Interestingly, a subset of isolates from lineage I (47% of total isolates) contained the *Listeria* pathogenicity island LIPI-3, a virulence factor which has been implicated in severe disease (Kim et al. 2018). LIPI-3 is confined to lineage I strains of *L. monocytogenes* and genes from this pathogenicity island encode Listeriolysin S (LLS), a protein which has been shown to display bactericidal activity and has the ability to modify host gut microbiota in mouse models (Quereda et al. 2017). This protein plays a crucial role in the infection cycle of *L. monocytogenes* and is present in epidemic strains. Importantly, whilst other authors have found that some strains isolated from food and environmental samples express a truncated Internalin-A due to PMSCs in *inlA* (Nightingale et al. 2005) all strains in this work, except NLmo20, contained a full and intact *inlA*. Since the short *inlA* variant that NLmo20 contains is predicted to be fully functional, potential virulence attenuation due to truncated Internalin A (Fravalo et al. 2017) can be ruled out for the strains featured in this work. Whilst all strains had two resistance genes present, NLmo4 and NLmo5 had two additional resistance genes, *bcrB & bcrC*, which encode a quaternary ammonium compound efflux SMR transporter. The presence of the virulence factors discussed above in strains isolated from the fresh produce supply chain coupled with cross referencing of their subtype indicates that they have the necessary genomic prerequisites to cause disease and have many features in common with strains that have previously caused outbreaks of disease. This information is of clear interest to regulators and stakeholders in the UK fresh produce supply chain.

In this work, isolates which were determined to be the same strain (by SNP analysis) did not necessarily produce equal amounts of biofilm, which could suggest that the method for quantifying biofilm in this work was not optimal. On the other hand,

changes in biofilm production within *L. monocytogenes* strains may well be affected by phenotypic heterogeneity (Ackermann 2015) as a strategy to cope with dynamic environments (such as those found in the fresh produce supply chain). For example, strains NLmo7 & NLmo14 were shown to be the same strain by WGS analysis but varied in their ability to form biofilm. These two strains were isolated from different geographical locations, 4 months apart. NLmo14 (isolated later) produced more biofilm than NLmo7, suggesting that while the two isolates can be genetically defined as the 'same strain' NLmo14 may have changed phenotype when adapting to a new environment.

Furthermore, the accessory genome (which was not investigated in this work) of all *L. monocytogenes* lineages is enriched for cell surface-related genes (den Bakker et al. 2013), the products of which may be involved in the EPS matrix of biofilms (Colagiorgi et al. 2016). Also, whilst a correlation between lineage and biofilm production (lineage II isolates producing more biofilm has been reported (Borucki et al. 2003)) our results indicate that phylogenetic lineage does not affect biofilm production (Di Bonaventura et al. 2008). More research is needed to highlight the intrinsic factors which determine whether stains are strong or weak biofilm formers in the presence of the environmental variables that are representative of environments found in the fresh produce supply chain.

3.6. Conclusion

Overall, the *L. monocytogenes* population in the UK fresh produce supply chain is diverse, in line with food isolates other countries such as Ireland (Hilliard et al. 2018), France (Moura et al. 2017), and Australia (Kwong et al. 2015). While some isolates have the necessary genomic components to cause disease and are closely related to outbreak strains, others are distantly related and are of less concern. All isolates formed biofilm but the intrinsic and extrinsic factors affecting this characteristic are complex and require further investigation. This is the first work of its kind in the UK and demonstrates the power of WGS as a subtyping tool for *L. monocytogenes* isolates In terms of the fresh produce supply chain, a more targeted sampling plan is needed to determine whether potentially virulent strains are sporadic (i.e. infrequently isolated) or persistent in a given environment. This is particularly important in processing environments that have the potential to contaminate produce on a broad scale (such

as processing environments). Therefore, in terms of policy, fresh produce supply chains should begin to phase in sampling regimes which implement WGS as standard. Implementing this technology may give customers (retail) and consumers added confidence that growers and processors are informed of the *L. monocytogenes* risk in their supply chain and demonstrates a precautionary, rather than reactionary approach to consumer safety.

Chapter 4 *L. monocytogenes* survival in horticultural soils

4.1. Introduction

In the previous chapters, *L. monocytogenes* strains isolated from the UK FLPSC were characterised by genotypic and phenotypic methods. Results showed that a genetically diverse population exists but that some strains are closely related. Moreover, some isolates were found to be highly related to strains which have caused outbreaks in the past and have the neccassary genetic components to cause disease. Such strains are of more concern to stakeholders in the FLPSC whilst other isolates are not closely related to outbreak strains and are of less concern. Many potential sources of *L. monocytogenes* exist in the UK FLPSC and based on the presence of genetically distinct and 'indistinguishable' strains it was concluded that contamination in the supply chain is a combination of sporadic contamination and contamination arising from common sources/cross contamination. Overall, *L. monocytogenes* contamination in the fresh produce supply chain may be difficult to prevent because of the range of potential sources of the bacterium in the growing and processing environments and this is reflected in the diversity of strains isolated.

As discussed in the introduction to this thesis, soil is an environmental niche for *L. monocytogenes* and is a potential source of contamination due to soil splash. It is important as a potential source of *L. monocytogenes* in the FLPSC due to the intimate association soil has with crops that are grown on a large scale such as spinach and lettuce. Despite the importance of this source, *L. monocytogenes* survival in horticultural soils is poorly understood. Previous research has indicated that *L. monocytogenes* is present in soil samples at low levels ($< 10^4$ CFU g^{-1}) in around 17% of soil samples tested (Locatelli et al. 2013a). Furthermore, that the prevalence and types of *L. monocytogenes* found change in soils from different ecosystems (Linke et al. 2014b). Many factors, including the biotic and abiotic properties of a particular soil contribute to survival rates *L. monocytogenes* in soil. Survival and prevalence in soil (with regards to the production of fresh leafy produce) was discussed at length in the introduction to this thesis and it was concluded that more research is needed to determine survival of the bacterium in horticultural soils used to grow fresh, leafy produce. This information could be used to inform growers of soils which present a higher potential contamination risk from this pathogen, due to increased survival rates.

An understudied aspect of *L. monocytogenes* physiology is the ability of the bacterium to turn VBNC in soil. The VBNC state may be induced in conditions that are facilitated by the soil environment (Maynaud et al. 2016). The VBNC state may be important in contributing to *L. monocytogenes* survival in the FLPSC. VBNC bacteria are metabolically active bacteria that have lost the ability to develop colonies on rich laboratory media. With regards to monitoring survival of *L. monocytogenes* in soil, when traditional culture methods fail to detect the bacteria, molecular methods can be employed to determine its presence. These methods are based on DNA extraction from a sample combined with techniques such as qPCR combined with propidium monoazide treatment or RT-PCR (Ramamurthy et al. 2014). Alternatively, culture methods which involve enrichment (such as those used in ISO11290 – 1: 2017 for the detection of *L. monocytogenes*) can resuscitate VBNC bacteria from a sample (Busch and Donnelly 1992; Dreux et al. 2007).

Due to the lack of data on *L. monocytogenes* survival in horticultural soils used to grow fresh produce it was decided that the survival of *L. monocytogenes* strains from the FLPSC should be investigated in horticultural soils used for growing fresh leafy produce on a large scale. This was carried out using soil microcosms and *L. monocytogenes* survival was monitored by direct plate counts on selective agar. A subset of the soils was sterilised to investigate the effect that endogenous soil microbiota has on *L. monocytogenes* survival in soil. When *L. monocytogenes* number was too low to count on selective agar due to the presence of competitor organisms, molecular tests to determine presence of the bacterium were carried out and included a species-specific PCR and next generation sequencing (NGS). To test whether a proportion of the *L. monocytogenes* population was turning VBNC in soil, a 3-tube MPN enrichment method for quantifying *L. monocytogenes* from a soil sample was developed. Results from this technique were then compared to *L. monocytogenes* quantification using direct plate counts. Comparing the values from both techniques meant the proportion of *L. monocytogenes* population turning VBNC in soil microcosms could be quantified.

This chapter focusses on *L. monocytogenes* survival in horticultural soils and detection of the bacteria in soil using a range of culture and molecular techniques. It was hypothesised that *L. monocytogenes* survival in horticultural soils will be affected by

soil type, strain type and microbiological status of the soil. Further, that a proportion of the *L. monocytogenes* population in soil would be turning VBNC after long incubation.

Aim: To investigate the survival of different *L. monocytogenes* strains isolated from the FLPSC in horticultural soils using a range of culture and molecular methods

Objectives:

- Determine the soil type of different horticultural soils using established methods (e.g. particle size distribution)
- Monitor the survival of different strains of *L. monocytogenes* in *a range of typical horticultural* soil microcosms (3 different soil types)
- Use molecular methods (species-specific PCR and NGS) to determine the presence of *L. monocytogenes* in soil
- Quantify the proportion of *L. monocytogenes* population which may be going VBNC in soil using an MPN-enrichment quantification method

4.2. Materials and methods

4.2.1. Monitoring *L. monocytogenes* survival in horticultural soils

4.2.1.1. Characterisation of horticultural soil samples

Three typical contrasting soil types from fresh produce production farms near Chichester, England were chosen based on texture and their extensive use in commercial production of fresh leafy produce (as advised by industry partners/collaborators). Three separate soil samples (15cm depth) of around 1kg of soil were taken from different areas of these fields and combined to produce homogenous samples of each soil type. Soils were identified as silty loam (50°48'40.1"N 0°37'32.2"W) silty clay loam (50°48'23.5"N 0°37'12.7"W) and sandy loam (50°58'02.9"N 0°36'46.5"W).

The detailed methods used for determining soil type can be seen in the book "Analysis of Agricultural Materials: Manual of the analytical methods used by the Agricultural Development and Advisory Service" by the Agricultural Development and Advisory Service (1986). However, it is useful to provide a brief overview of methods here. Soil particle distribution was determined using peroxide (to dissolve organic matter) combined with gravitational sedimentation (pipette method). Organic matter content was determined by heating dry soil samples in a furnace (550 °C) and measuring the differences in weight between pre- and post-furnace samples. pH was calculated using a calibrated pH meter. Inorganic carbon (C), sulphur (S) and nitrogen (N) content were calculated by combustion analysis in a LECO FP-528 CHNS analyser. This analysis was conducted at Harper Adams University in collaboration with Jim Monaghan and the fresh produce team.

4.2.1.2. Bacterial strains used to inoculate soil microcosms

Isolates NLmo2 (lineage 1, isolated from raw product in the field), NLmo4 (lineage II, isolated from a processing environment) and a laboratory reference strain (EGD-e) were used to inoculate microcosms at around 10^7 cells g^{-1} dry weight (dw) soil and their survival in soil was monitored over 70 days (fresh leafy produce types take from 21 – 70 days to grow). Strains were chosen based on their genetic variety (from genetic lineage I & II) and where they were isolated (from produce, from processing environment and lab reference). Each of the 3 different bacterial strains was

inoculated into the 3 different soil types. Three independent replicates of each strain-soil combination were constructed for each time point to be tested (n=3).

4.2.1.3. Construction of soil microcosms

Soil microcosms were created in 50ml Falcon tubes. Each soil sample was pooled and mixed thoroughly to create a bulk soil for each soil type. The soils were air dried, ground in a pestle and mortar and sieved to a particle size of 1mm. A subset of these soils was sterilised by autoclaving at 121°C 15 minutes, allowing the soil to rest for 24 hours then autoclaving again. This process was repeated twice more. Mesophilic microorganisms in fresh and sterilised soils were quantified by mixing 0.1g of soil with 0.9ml of PBS, plating appropriate dilutions on nutrient agar and incubating plates at 25°C for 24h. Autoclaved soils had a microbial load of ~10^2 CFU g^{-1} soil dw and fresh soils had a microbial load of ~10^7 CFU g^{-1} soil dw.

Air dried soil (2g) was added to a sterilised 50ml falcon tube and soil in the microcosms was adjusted to a representative water holding capacity (80% of the maximum water holding capacity), for fresh produce growing conditions (including inoculum). After inoculation, microcosms were incubated at the average topsoil temperature during a growing season (March-October 2016) for Petworth Farm (baby leaf production fields), Chichester (14.8^{o}C). For purposes of sterility and aeration, microcosms were covered with Parafilm and lids loosely replaced.

4.2.1.4. Sampling procedure for microcosms (culture methods)

4.2.1.4.1. Quantification of L. monocytogenes by viable cell counts

Viable cell counts were determined at days 0, 24 and 70 for fresh and sterilised microcosms. To sample microcosms, a tryptone salt solution (18 mL, 1g/L Tryptone, 8.5g/L NaCl) was added to each microcosm as described in (Locatelli *et al.,* 2013). Microcosms were then mixed well to suspend soil particles in solution and incubated in a shaking incubator at 20°C, 200rpm for 10 minutes. This suspension was then serially diluted up to 10^{-6} and 100µl of appropriate dilutions spread on OXFORD agar plates (Oxoid) with Listeria selective supplement (Oxoid). Three independent replicates for each time point of soil/strain/microbiological status were constructed and sampled. Differences in soil survival between groups were determined using a 2-way ANOVA with Tukey's test for multiple comparisons (GraphPad Prism 7.04). The factors included

in the test were soil type and microbiological status (sterilised/fresh). The two-way ANOVA was repeated at each time point.

All 3 soils were tested for potential background presence of *L. monocytogenes* prior to experimental set up by incubating serial dilutions of soil/tryptone salts suspension in demi-fraser broth (Oxoid) at 30°C, 40h and spreading on OXFORD agar plates with Listeria selective supplement (Oxoid). Plates were incubated for 48h, 37°C (detection limit 10 CFU g^{-1}). If soils yielded characteristic colonies, *L. monocytogenes* presence was confirmed or denied with species-specific primers (see section 2.2.2.1). Soils used were found not to contain any 'background' (detectable) *L. monocytogenes* at all (data not shown).

4.2.1.4.2. Quantification of L. monocytogenes by MPN-enrichment and comparison to viable cell counts

Sterilised microcosms were sampled 257 days after inoculation using the direct plate count method (above) and a 3-tube MPN enrichment method. Out of the 27 microcosms sampled using this method, 12 microcosms (7 sand, 4 silt and 1 clay microcosm) yielded no *L. monocytogenes* using either quantification method. These microcosms were excluded from further analysis. Differences in numbers of *L. monocytogenes* from microcosms that could be quantified by both direct plate count and MPN-enrichment were analysed using a T-test (n=15).

For MPN enrichment, 18ml of half-Fraser broth was added to microcosms and microcosms were briefly vortexed. 200µl of this soil-broth solution was added to 3 x wells of a 96 well-plate before being serial diluted up to 10^{-7}. This primary enrichment was incubated at 30°C for 24h. For secondary enrichment, 2µl of primary enrichment broth was added to 198µl of full fraser broth in a new 96-well plate. Both half-fraser broth and full Fraser broth contained aesculin which turns the broth black in the presence of *Listeria* spp. The secondary enrichment broth was incubated at 35°C for 24h. The last two wells which turned black for every "tube" (see Figure 4.1. for example) were streaked onto OXFORD and *Listeria* Brilliance agar. Characteristic colonies on either of these agars were then subject to a species-specific colony PCR to confirm *L. monocytogenes* presence. An MPN well was positive if a colony was confirmed. MPN values were calculated using MPN Calculator Build 23 by Mike Curiale (http://www.i2workout.com/mcuriale/ mpn/index.html). Quantification of *L.*

monocytogenes number using the MPN enrichment method were then compared to values obtained from the same time point using viable cell counts on selective agar. A T-test (GraphPad Prism 7.04) was carried out to determine whether there was a significant difference in *L. monocytogenes* number between quantification methods.

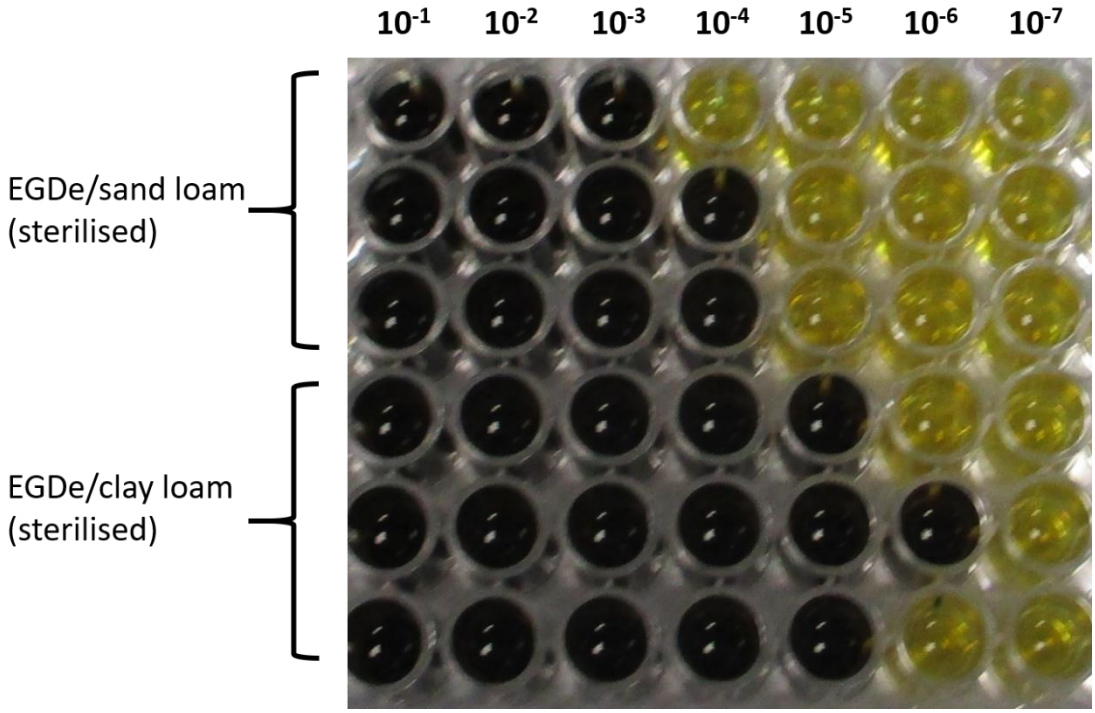

Figure 4.1. **Example of the 96-well plate format used during sampling microcosms with the 3-tube MPN enrichment method.** The serial dilution assigned to each well can be seen at the top of the figure. Strain/soil combinations for each microcosm sample tested are listed on the left of the figure.

4.2.1.4.3. Statistical analysis used during quantification (direct plate counts – CFU) of L. monocytogenes in soil microcosms

At days 24 & 70, a two-way ANOVA was used to determine if differences between treatments and soils were significant (GraphPad Prism 7.04). Two variables were used in the analysis; soil type was used as the row variable and sterilised/non-sterilised was used as the column variable. Tukeys test for multiple comparisons was used to determine if significant differences existed between soil types within the groups of non-sterilised and sterilised soils. GraphPad output showed the effect of the two variables and differences between soil types within the groups of sterilised and fresh soil.

4.2.1.4.4. Identification of competitor species in 3-tube MPN enrichment procedure

The presence of competitor organisms during sampling of fresh soils in the enrichment broth and subsequently on selective agar made it difficult to enumerate *L. monocytogenes* after 70 days (Figure 4.2). Two of the competitor organisms which were frequently identified by their colony morphology on OXFORD agar were subsequently identified a colony PCR with primers targeting the 16S rRNA region followed by 16S rRNA sequencing using the following primers; (forward) 8F - AGA GTT TGA TCC TGG CTC AG and (backward) 1492R - CGG TTA CCT TGT TAC GAC TT and the following PCR conditions: 95°C for 5 mins followed by 35 cycles of; 95°C for 30s, 55°C for 30s, 72°C for 1min 30s then a final extension of 72°C for 10 mins. Following the PCR reaction, products were electrophoresed on a 1.2% agarose gel with 5µl Safe View per 100ml 1 x TAE buffer and excised using Gel Extracta Kit (Promega). PCR products were then purified using Isolate II Kit (Bioline) according to the manufacturer's instructions. DNA concentration was adjusted to 15ng/µl using a spectrophotometer (Nanodrop 2000 – Thermo Fisher Scientific) with Ultrapure Water (Thermo Fisher Scientific). Sequence primer concentration was adjusted to 10 pmol/µl as specified by Eurofins Genomics and sent in separate 1.5 ml Eppendorf's to be sequenced. Sequence primers were the same as primers used for PCR amplification. Genes were sequenced by Eurofins Genomics using dideoxy chain termination method on an ABI3730 XL sequencer. Forward and reverse sequences for sequenced genes were aligned using the Muscle algorithm in MEGAX to create consensus sequences. Sequences were then compared to known sequences using the BLAST algorithm. A Gram stain, catalase test and oxidase test were also carried out on both competitor isolates.

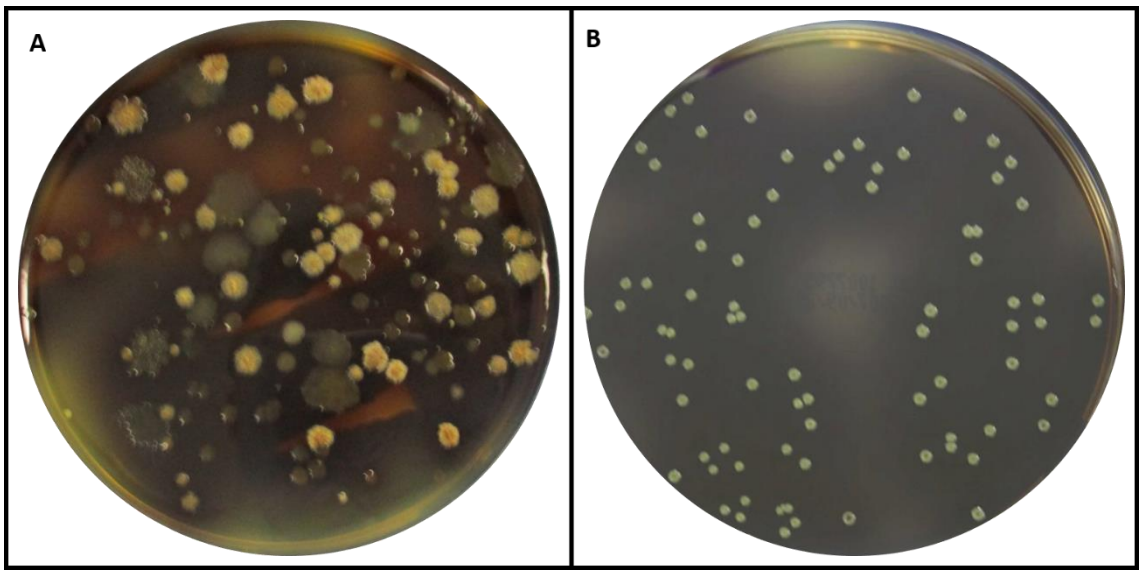

Figure 4.2. **Example of OXFORD agar plates with (panel A) colonies of contaminating bacteria from fresh soil present and (panel B) characteristic colonies of *L. monocytogenes*.**

4.2.1.5. Sampling procedure for microcosms (molecular methods)

4.2.1.5.1. Species-specific PCR for detection of L. monocytogenes in microcosms

As discussed above, after 70 days it became impossible to quantify *L. monocytogenes* presence in non-sterilised microcosms due to the presence of competitors on selective plates. To determine if *L. monocytogenes* DNA could be detected using PCR, DNA was extracted from sterilised and fresh clay soil microcosms at 250 days post-inoculation using a commercial kit (PowerSoil DNA Isolation Kit – Qiagen) following the manufacturer's instructions. Clay soil type was chosen for this method as this soil type yielded the highest CFU g^{-1} of *L. monocytogenes* after 70 days. DNA from microcosms (sterilised and fresh soil) was subject to a species-specific PCR using diluted DNA extracted from clay soil microcosms. DNA was diluted 1 in 5, 1 in 10 and 1 in 20 before use in separate PCR reactions. 10 µl products were then electrophoresed on 1.2% agarose gel containing 5 µl Safe View per 100ml 1 x TAE buffer. 10 µl of product/loading buffer mixture was added to each well and gel ran at 100 V for 1 h. NGS was completed by

4.2.1.5.1. Illumina based NGS for the detection of L. monocytogenes in soil samples

After it was established that *L. monocytogenes* DNA could not be detected in fresh soil microcosms using the method above, it was decided that illumina based next

generation sequencing should be used to determine if this method could detect *L. monocytogenes* DNA in samples. Subsequently, soil DNA was extracted from all fresh soil microcosms after 256 days using the Powersoil DNA extraction method described above. For comparison during NGS, DNA extracted from soil microcosms which had been inoculated with 10^2 CFU g^{-1} of soil were included. The DNA from these microcosms was extracted one day after inoculation with 10^2 CFU g^{-1} of soil. See below section (4.2.2.1.) for full NGS method details.

4.2.2 Bioinformatics analyses for 16S rRNA NGS

4.2.2.1 Sequencing data processing

NGS of DNA from soil samples was conducted by NuOmics (Northumbria University). From raw data, paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASh (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč et al., 2011), and were subsequently called raw tags. Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags (Bokulich et al., 2013) using QIIME (V1.7.0) (http://qiime.org/scripts/split_libraries_fastq.html) quality control process (Caporaso et al., 2010). The resulting high quality tags (sequences) were compared with the RDP training set v16 reference database using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Quast et al., 2013) to detect and remove chimera sequences (http://www.drive5.com/usearch/manual/chimera_formation.html) (Haas et al, 2011).

4.2.2.2 OTU clustering and species annotation

Clustering of OTUs and annotation was performed by UPARSE software (Uparse v7.0.1001, http://drive5.com/uparse/) (Edgar, 2013) using all the effective tags obtained in previous steps. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequences for each OTU was screened for further annotation. Sequences analysis were performed by Blast with QIIME (Version 1.7.0; http://qiime.org/scripts/assign_taxonomy.html) (Altschul et al., 1990) and the RDP training set v16 reference database for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species). To obtain the phylogenetic relationship of all OTU representative sequences. Subsequent analysis of alpha

diversity and beta diversity metrics were all performed based on this output normalized data.

4.2.2.3. Alpha diversity

To analyse the diversity within each sample, Alpha diversity metrics were calculated for 6 diversity indices. These included community diversity indices (Shannon, Simpson), community richness indices (ACE, Chao1), Observed-species and Good's coverage indices. The analysis was performed using QIIME (Version 1.7.0). Wilcox and Tukey tests were performed to examine if the differences in the indices were significant between groups. 'Groups' were defined as different soil types as well as a 'group' of spiked (with *L. monocytogenes)* soils.

4.2.2.4. Beta diversity

To compare microbial communities between soil samples, β-diversity was measured. Because the experimental set recorded species abundances within samples (individual frequencies), differences between these samples were calculated by the variance analysis of Bray-Curtis abundance-based measures to calculate P-values and test for significant differences between soil groups. β-diversity was visualised in a dendrogram constructed using FigTree. This dendrogram was generated based on species abundances within samples.

4.2.2.5. Data visualisation

Microbiome Analyst (Dhariwal et al. 2017) was used to estimate the relative abundances of taxonomical groups for all individual samples and visualise the taxonomic composition of each sample. This tool allows for the detailed phylogenetic analysis and interactive display of the complex phylogenomic data in a hierarchical context. It also provides the abundance percentages for each taxon in each amplicon sequencing, and thus a more informed interpretation. It was also used to follow specific changes in the relative abundance of the dominant species in soil microcosms. MicrobiomeAnalyst output (% relative abundance for different samples) was used to create Figure 4.12. in Microsoft Excel.

4.3 Results

4.3.1. Abiotic characteristics of soil used in survival studies

3 separate soil types were suggested by farm managers from fields which had previously been used to grow fresh leafy produce. To confirm that these soils were different, a sample of the bulk soil was collected for further analysis. This included tests for determining soil texture (including the proportion of different particle sizes) and analysis of the chemical components of soil. Table 4.1. shows information associated with the different abiotic characteristics of the three soils used in *L. monocytogenes* soil survival studies. Soils were determined to be texturally different and were assigned to the following soil type; silt loam, silty clay loam, sandy loam. For purposes of clarity in the graphs in the following sections, soil types are denoted as silt, clay and sand.

Table 4.1. **Characteristics of the 3 different soils used in *L. monocytogenes* soil survival studies.**

Soil Type	pH	Organic matter (%)	Nitrogen content (%)	Carbon content (%)	C:N ratio	Sulphur content (%)	Silt (%)	Clay (%)	Sand (%)
Silt loam (silt)	7.04	3.72	0.20	1.53	7.5	0.0205	54.9	26.6	18.5
Silty clay loam (clay)	7.77	6.60	0.35	3.15	9.13	0.0252	43.8	39.9	16.3
Sandy loam (sand)	6.70	3.42	0.18	1.15	6.36	0.0184	19.0	18.7	62.3

4.3.2. Assessment of *L. monocytogenes* survival in soil using culture methods

4.3.2.1 Monitoring *L. monocytogenes* survival using viable cell counts

L. monocytogenes survival was assessed in soil microcosms by counting viable cells on selective agar. In general, *L. monocytogenes* numbers declined over time (up to 70 days) in all soil/strain/microbiological status combinations tested (Figure 4.3.). In soils

sterilised by autoclaving, average (across soil and strain types) numbers of *L. monocytogenes* were higher at 24 days by 4 Logs and at 70 days by 3 logs. Strain type was not found to affect *L. monocytogenes* survival in soil, but soil type did affect soil survival with *L. monocytogenes* surviving at a higher rate, for longer in silty clay loam than silt loam or sandy loam soils. These results are explained in more detail in the following sections.

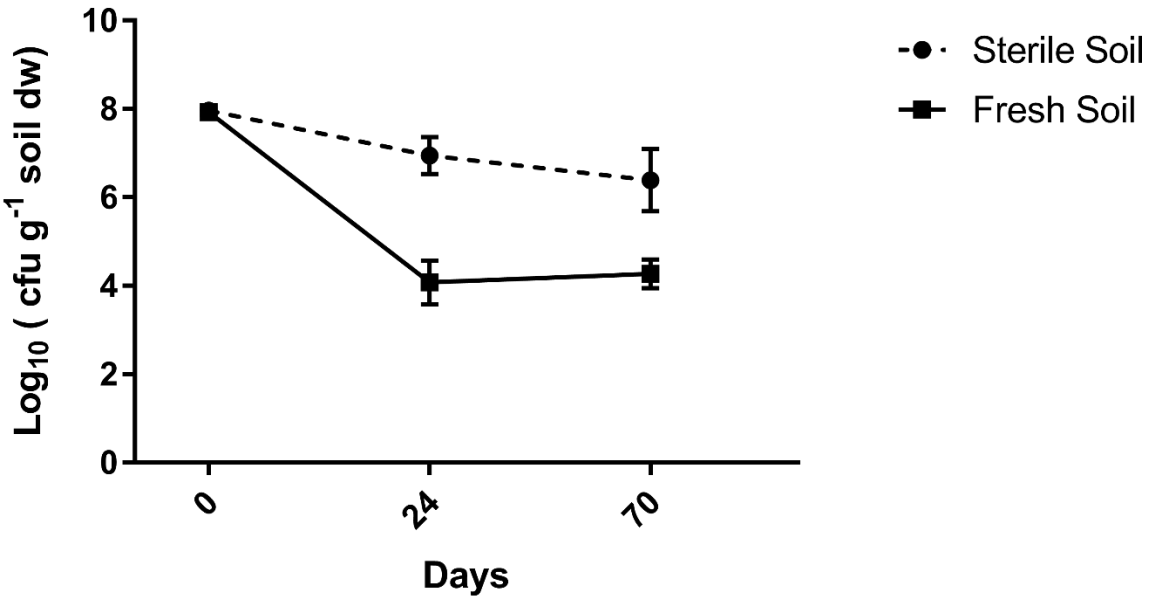

Figure 4.3. **Comparison of average *L. monocytogenes* number across soil and strain types over 70 days.** Graph is not showing interactions with other factors. For each data point n = 27.

4.3.2.1.1. Comparison of L. monocytogenes survival in sterilised and fresh soil using 3 soil types

In general, *L. monocytogenes* declined over time both in sterilised and fresh soils (Figure 4.3.). The following section takes a more detailed look at differences between fresh and sterilised soils across 3 soil types at different time points. At the point of inoculation all soils showed the same number of *L. monocytogenes*, ~10^8 CFU g^{-1} soil dw (data not shown). At 24- and 70-days points, a two-way ANOVA was used to determine if differences between treatments and soils were significant.

After 24 days (Figure 4.4.), *L. monocytogenes* was significantly (F (1, 45) = 80.6, P <0.001) higher in soils that had been sterilised vs. fresh soils. Soil type also had a

significant (F (2, 45) = 17.08, P < 0.001) effect on *L. monocytogenes* numbers in soil after 24 days. The interaction of soil type and soil microbiological status was also significant (F (2, 45) = 16.99, P <0.001). Tukey's test for multiple comparisons showed that amongst sterilised soils after 24 days, CFU of *L. monocytogenes* silty clay loam soil were significantly (P = 0.01) higher than in silt loam and sandy loam soils. However, there were no significant differences in CFU of *L. monocytogenes* amongst fresh soils.

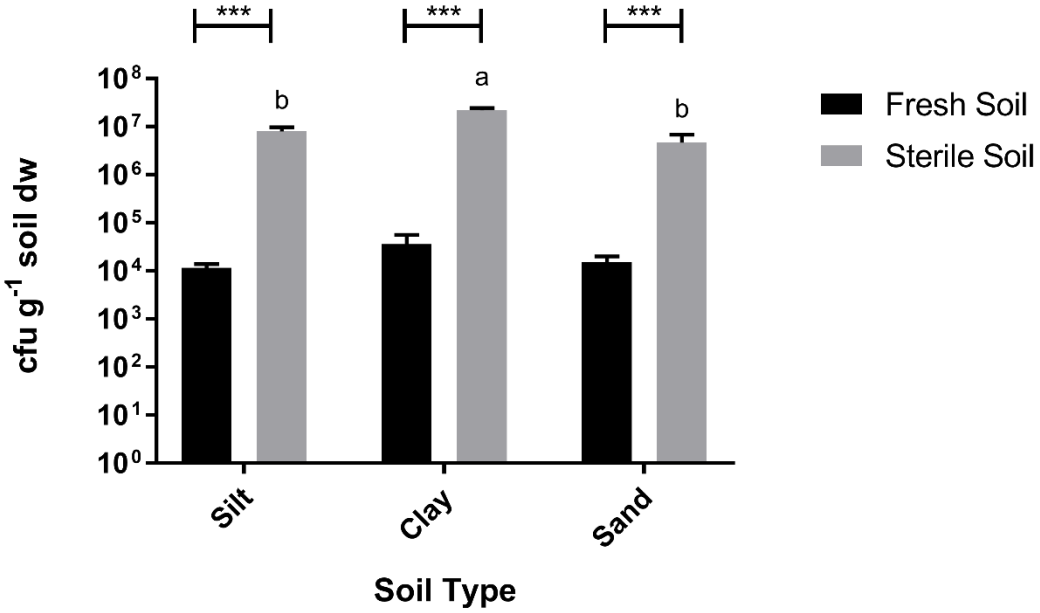

Figure 4.4. **Comparison of *L. monocytogenes* (CFU g^{-1} soil dw) in soil microcosms after 24 days.** Data from fresh soil is indicated by black bars, number in sterilised soil is indicated by grey bars. Soil type is indicated on the x-axis and number of *L. monocytogenes* is indicated on the y-axis. Error bars represent the SEM of 9 independent replicates (n = 9). *** = P <0.001 and show significant differences between sterilised and fresh (fresh) soils. Within the group of sterilised soils, bars marked with (a) are significantly (P = 0.001) different from (b).

Likewise, after 70 days (Figure 4.5.) *L. monocytogenes* CFU in soil was significantly (F (1, 44) = 38.44, P < 0.001) higher in soils that had been sterilised vs. fresh soils. Soil type also had a significant (F (2, 44) = 14.51, P < 0.001) effect on *L. monocytogenes* numbers in soil after 70 days. The interaction of the two variables was also significant (F (2, 44) = 14.63, P < 0.001). Tukey's test for multiple comparisons showed that amongst

sterilised soils after 70 days, *L. monocytogenes* number was significantly ($P < 0.001$) higher in silty clay loam soil than in the other two soil types.

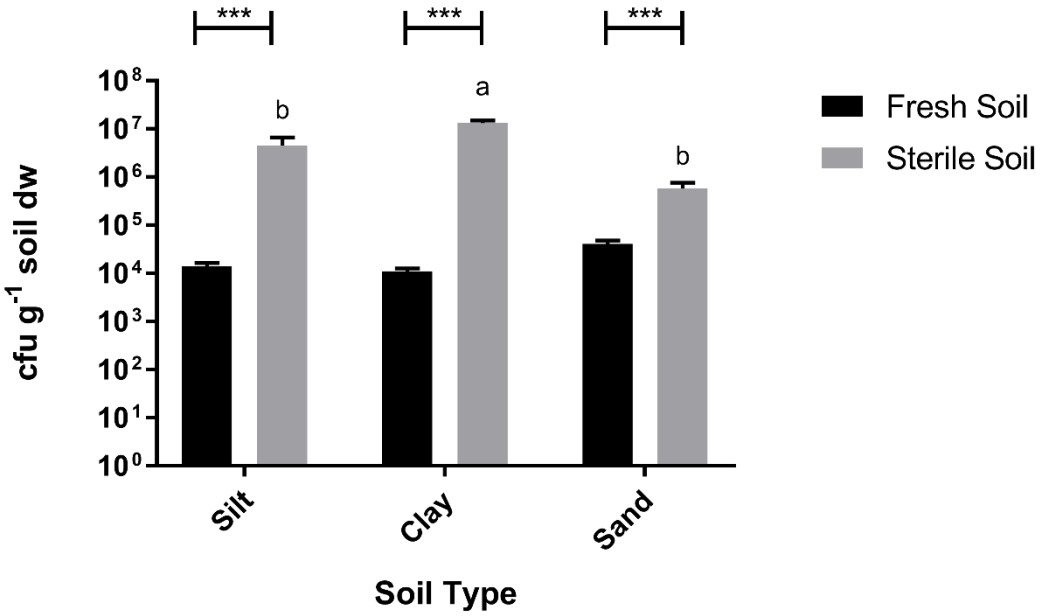

Figure 4.5. **Comparison of *L. monocytogenes* number in soil microcosms after 70 days.** Data from fresh soil is indicated by black bars, number in sterilised soil is indicated by grey bars. Soil type is indicated on the x-axis and number of *L. monocytogenes* is indicated on the y-axis. Error bars represent the SEM of 9 independent replicates (n = 9). *** = $P < 0.001$ and show significant differences between sterilised and fresh (fresh) soils. Within the group of sterilised soils, bars marked with (a) are significantly ($P = 0.001$) different from (b).

4.3.2.1.2. Effect of strain type on L. monocytogenes survival in soil

No significant differences were found between the survival of different strains across all three soils at the different time points tested in either fresh or sterilised soils (Figure 4.6.).

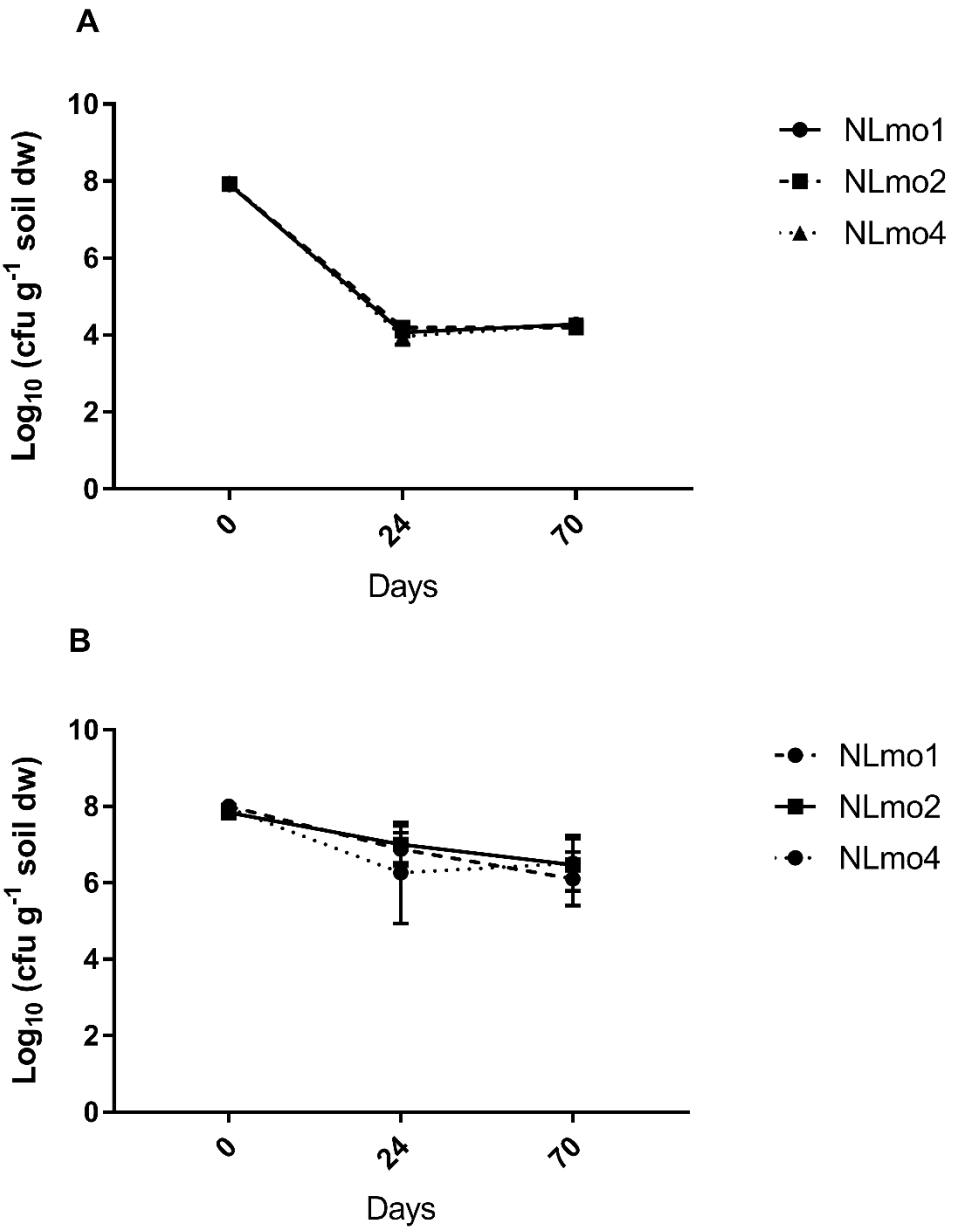

Figure 4.6. **Comparison of different *L. monocytogenes* strains survival in fresh (panel A) and sterilised (panel B).** Data points show the average ± SEM for 9 replicates across all 3 soil types. Day of sampling is indicated on the x-axis and *L. monocytogenes* (Log_{10} (CFU g^{-1} soil dw)) is indicated on the y-axis

4.3.2.1. Quantification of *L. monocytogenes* in soil microcosms using an MPN enrichment method – time point 257 days

L. monocytogenes in soil microcosms could only be quantified in soil that had been sterilised using the MPN enrichment method (described in section 4.2.1.4.3.). This was due to the presence of competitor organisms in the enrichment broth which were

subsequently transferred to and grew on selective agar plates, making it impossible to distinguish *L. monocytogenes* colonies. The two most common colony types were isolated and were identified (section 4.3.2.2.1). However, in sterilised soil microcosms, *L. monocytogenes* number could be quantified by MPN enrichment and compared to direct plate viable cell counts.

4.3.2.2.1. Identification of competitor organisms growing in the enrichment broth

Two competitor organisms were isolated from enrichment broths during sampling of fresh soil microcosms using the enrichment MPN method. Competitor micro-organism 1 was catalase negative, oxidase negative and Gram negative (Figure 4.7.). The 16S rRNA sequence of competitor 1 was compared to known sequences using the BLAST nucleotide online tool (Altschul et al. 1990) and was found to have a 98.4% sequence similarity to 16S rRNA sequence from *Sporosarcina contaminans*, a soil bacterium which has been previously isolated from an industrial clean-room floor (Kämpfer et al. 2010). The uploaded sequence also had high similarity with 'uncultured compost bacterium clone'.

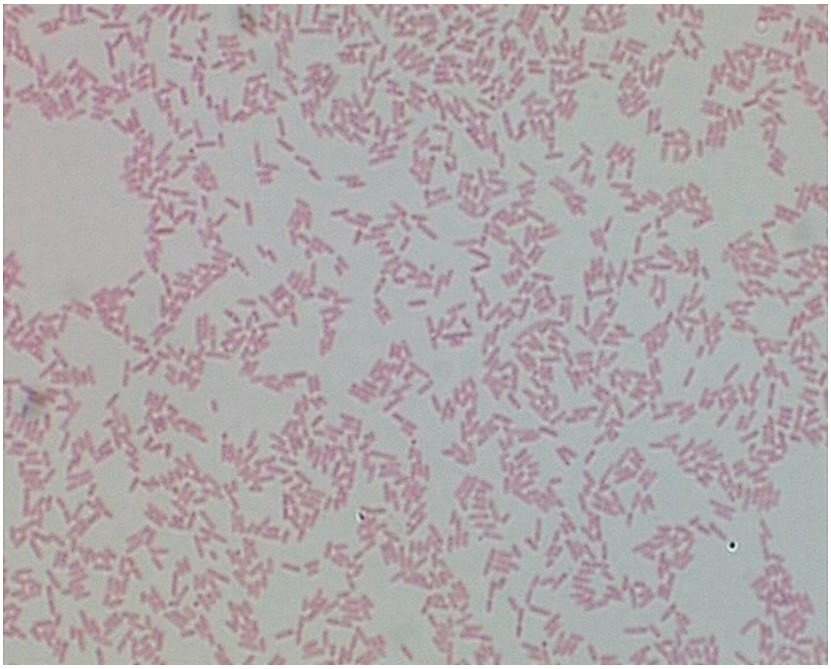

Figure 4.7. **Gram stain of competitor micro-organism 1 isolated during MPN enrichment of fresh soil microcosms.** This bacterium was subsequently identified as *Sporosarcina contaminans* by sequencing the 16S rRNA gene.

Competitor micro-organism 2 was catalase negative, oxidase positive and gram positive (Figure 4.8.). The 16S rRNA sequence of competitor 2 was compared to known sequences using the BLAST nucleotide online tool (Altschul et al. 1990) and was found to have a 98.33% sequence similarity to a 16S rRNA sequence from *Bacillus licheniformis*, a Gram-positive, saprophytic organism that occurs in plant and soil (Lee et al. 2017b). Both competitor species are endospore-forming.

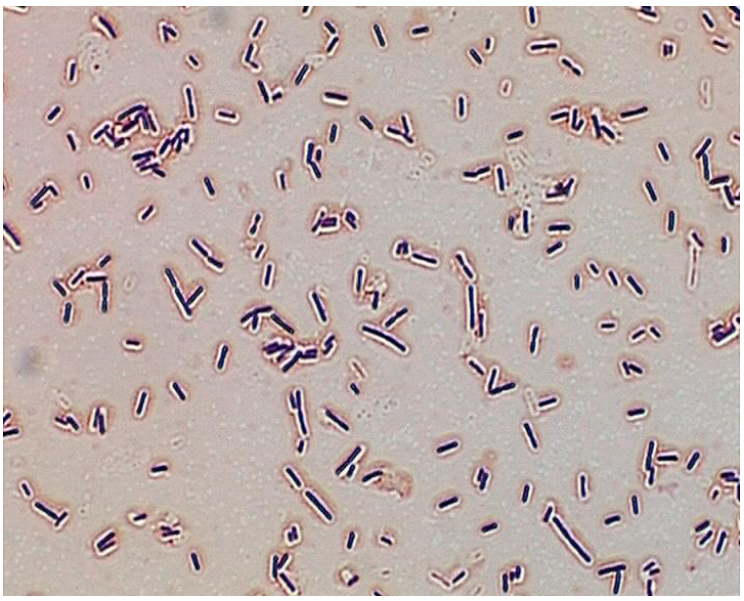

Figure 4.8. **Gram stain of competitor micro-organism 2 isolated during MPN enrichment of fresh soil microcosms.** This bacterium was subsequently identified as *Sporosarcina contaminans* by sequencing the 16S rRNA gene.

4.3.2.2. Comparison of *L. monocytogenes* number from sterilised soil microcosms using an MPN enrichment method vs. direct plate counts

L. monocytogenes number was quantified in sterilised soil microcosms using the MPN enrichment method and direct plate counts after 257 days incubation at 14.8°C. The average number of *L. monocytogenes* quantified by the direct plate method was 1.16 x 10^6 CFU g^{-1} soil dw while the average number quantified by MPN method was 4.06 x 10^6 CFU g^{-1} soil dw. Meaning that on average, there was a significant ($P < 0.01$) increase in *L. monocytogenes* number using the MPN method (Figure 4.9.).

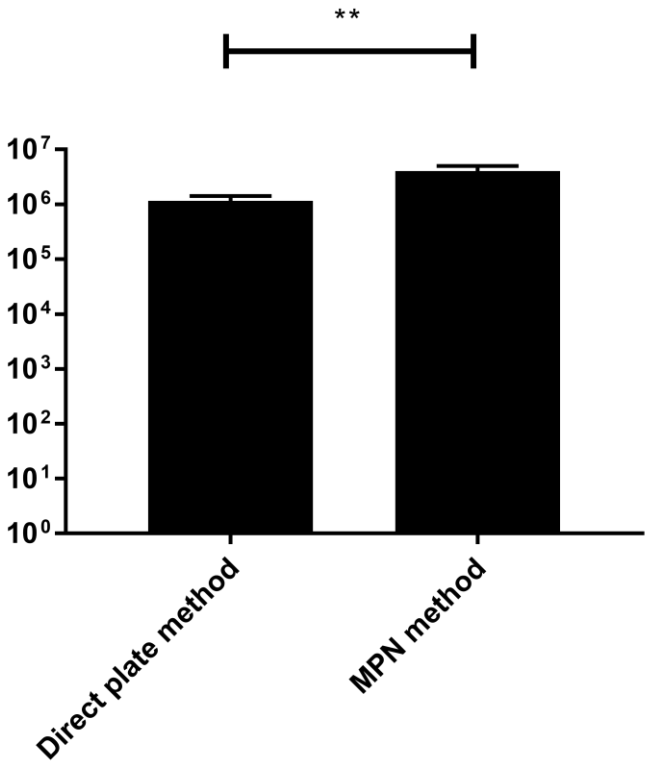

Figure 4.9. **Comparison of *L. monocytogenes* quantification methods when sampling sterilised soils after 257 days including data from all strain and soil types.** Error bars represent SEM of 15 independent replicates (n = 15). ** indicates significant difference ($P < 0.01$).

4.3.3. Assessment of *L. monocytogenes* presence in soil using molecular methods

4.3.3.1. Detection using a species-specific PCR

To determine *L. monocytogenes* presence in soil microcosms after 250 days, DNA was extracted from soil and this DNA was subsequently subjected to a species-specific PCR. *L. monocytogenes* DNA could be detected in sterilised microcosms but not fresh soil microcosms. The product of the species-specific PCR was 583bp. Figure 4.10. shows the agarose gels used to confirm the presence/absence of *L. monocytogenes* DNA in soil microcosms.

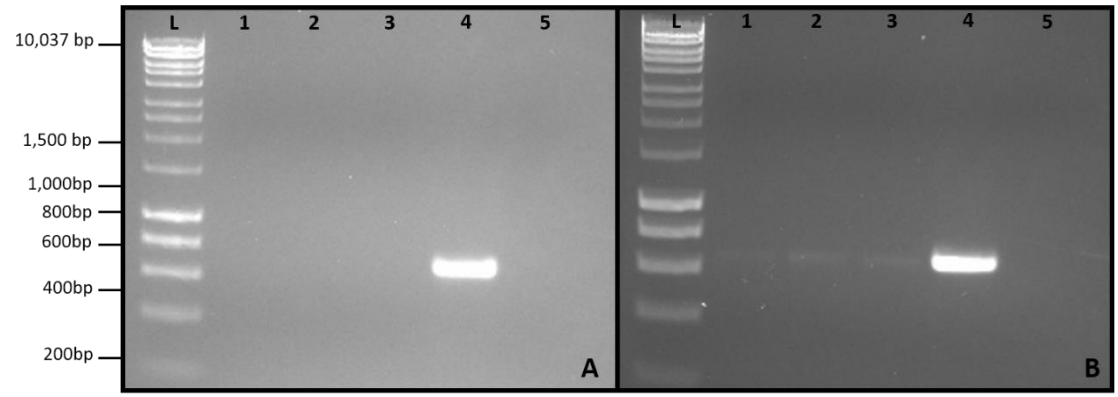

Figure 4.10. **Agarose gels of PCR product from reactions using *L. monocytogenes* specific primers.** Panel A shows PCR products where DNA from fresh microcosms was used in reactions, panel B shows PCR products where DNA from sterilised microcosms was used. Lanes as follows; 1 – DNA diluted 1 in 5, 2 – extracted DNA diluted 1 in 10, 3 – extracted DNA diluted 1 in 20, 4 – positive control using *L. monocytogenes* DNA, 5 – negative control. Ladder size is indicated on the left side

4.3.3.2. Detection of L. monocytogenes in soil microcosms using NGS (16S rRNA) and comparison with 'spiked' microcosms

In this study, NGS was used to determine whether *L. monocytogenes* was still present after a long incubation period post inoculation to these soils (256 days). Fresh soils 'spiked' with *L. monocytogenes* (at a level of 10^2 CFU g^{-1} soil and sampled one day after) were included to determine if NGS could resolve this species amongst the soil microbiota at this level. In general, bacterial community structure at a phylum level exhibited some changes related to relative abundance depending on soil type and whether the soil was spiked with *L. monocytogenes*, or not (Figure 4.11.). Firmicutes (which include *L. monocytogenes*) tend to be similar in abundance amongst soil types. Spiked microcosms had a higher relative abundance of Firmicutes than non-spiked soils. Sandy soil samples 1 and 2 had a higher relative abundance of Firmicutes than the other soil types which were not spiked with *L. monocytogenes*. The most dominant bacterial phyla across soil samples are Acidobacteria, Actinobacteria and Proteobacteria. OTUs could be resolved to family-level, and many to genus. This meant that across the samples, the OTU which represented *Listeria* spp. (OTU3 in this analysis) could be detected in all previously inoculated samples as well as in uninoculated sandy soil and spiked sandy soil (Table 4.2).

Table 4.2. **Number of OTU reads and percentage relative abundance of OTU3 which represents *Listeria* spp. in tested samples.** Samples where this OTU was not detected are not listed in the table.

Sample	Replicate	Initial *L. monocytogenes* inoculum (CFU g^{-1})	Day post-inoculation on which microcosm was sampled	# of OTU 3 reads	% relative abundance
Silty clay loam	1	10^8	256	6	0.007
	2	10^8	256	1	0.001
	3	10^8	256	1	0.001
Clay loam	1	10^8	256	6	0.004
	2	10^8	256	51	0.074
	3	10^8	256	5	0.006
Sandy loam	1	10^8	256	2458	2.539
	2	10^8	256	1532	1.473
	3	10^8	256	5	0.009
Sand loam (control, uninoculated)	-	10^2	1	143	0.342
Sandy loam (control, spiked with *L. monocytogenes*)	-	10^2	1	1	0.002

The sequence represented by this OTU had 100% identity with *L. monocytogenes* 16S rRNA sequences when analysed by BLAST (Altschul et al. 1990).

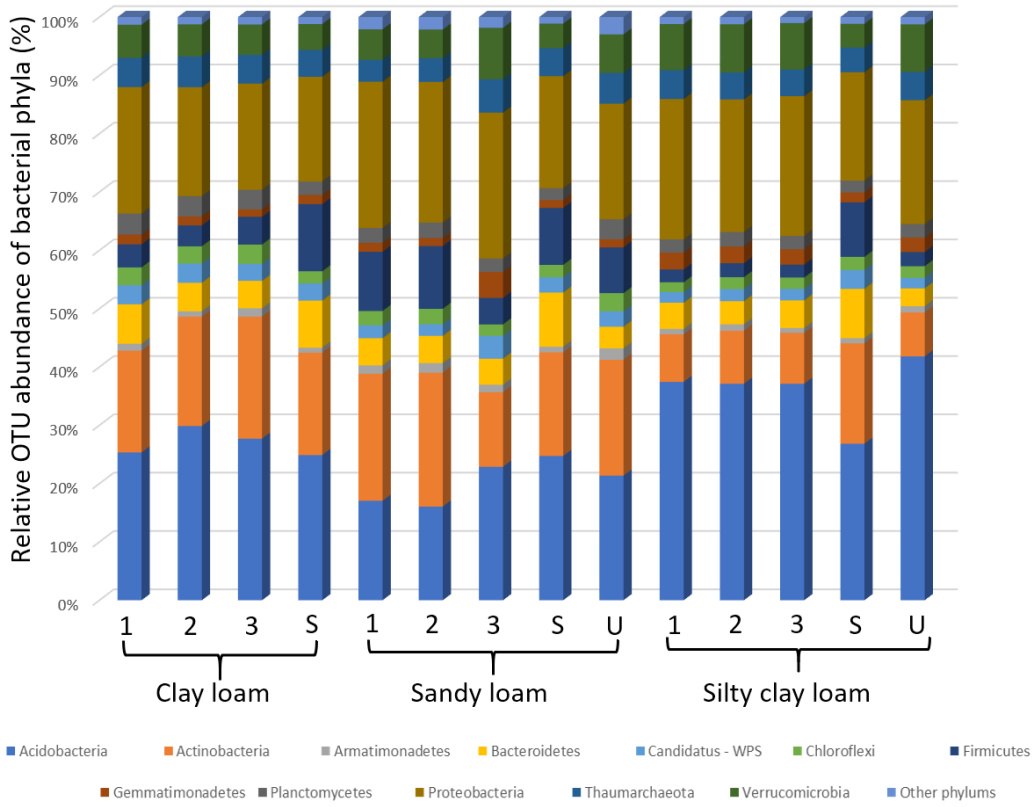

Figure 4.11. **Bacterial community structure in different soil samples.** Samples are denoted by replicates (1, 2 & 3 (sampled 256 days post inoculation)) and with sub-level 'S' *(L.* monocytogenes spiked soils (sampled 1 day post inoculation)) and 'U' (uninoculated soils). 'Other phyla' represents the microbial diversity with < 1% of relative OTU abundance at the phylum level.

α-diversity was calculated for the different soil samples. This diversity term was introduced by Whittaker (Whittaker 1972), representing the mean species diversity in sites at a local scale. The Shannon index was used as a measure of α-diversity and results are shown in Table 4.3.

Table 4.3. **α-diversity of bacterial community structure in soil samples from *L. monocytogenes* soil microcosms.** U = uninoculated soils (no *L. monocytogenes*) S = spiked soils (with 10^2 cfu g^{-1} soil dw).

Soil type	Repeat	Shannon index (Log_e)	Average
Silty clay loam	1	6.38	6.41
	2	6.44	
	3	6.4	
Clay loam	1	6.51	6.46
	2	6.42	
	3	6.46	
Sandy loam	1	6.32	6.34
	2	6.33	
	3	6.36	
Silty clay loam (u)	-	6.36	6.37
Sandy loam (u)	-	6.37	
Silty clay loam (s)	-	6.18	6.15
Clay loam (s)	-	6.13	
Sandy loam (s)	-	6.13	

Diversity level was significantly lower on average in sandy soil microcosms compared with clay loam but not when compared to silty clay loam soil. Clay loam soil had the

highest average bacterial community diversity of the tested soils. Soils which had been spiked with *L. monocytogenes* had a significantly ($P < 0.001$) lower mean diversity level than other soils (Table 4.3.).

B-diversity was calculated for the determination of bacterial communities' similarity between samples as shown in Figure 4.12. According to measurements of B-diversity (Bray-Curtis algorithm), all samples have different bacterial communities and no significant similarities are observed between soil samples. However, bacterial communities from same soil types were more like each other than communities from different soil types and spiked also soils clustered together.

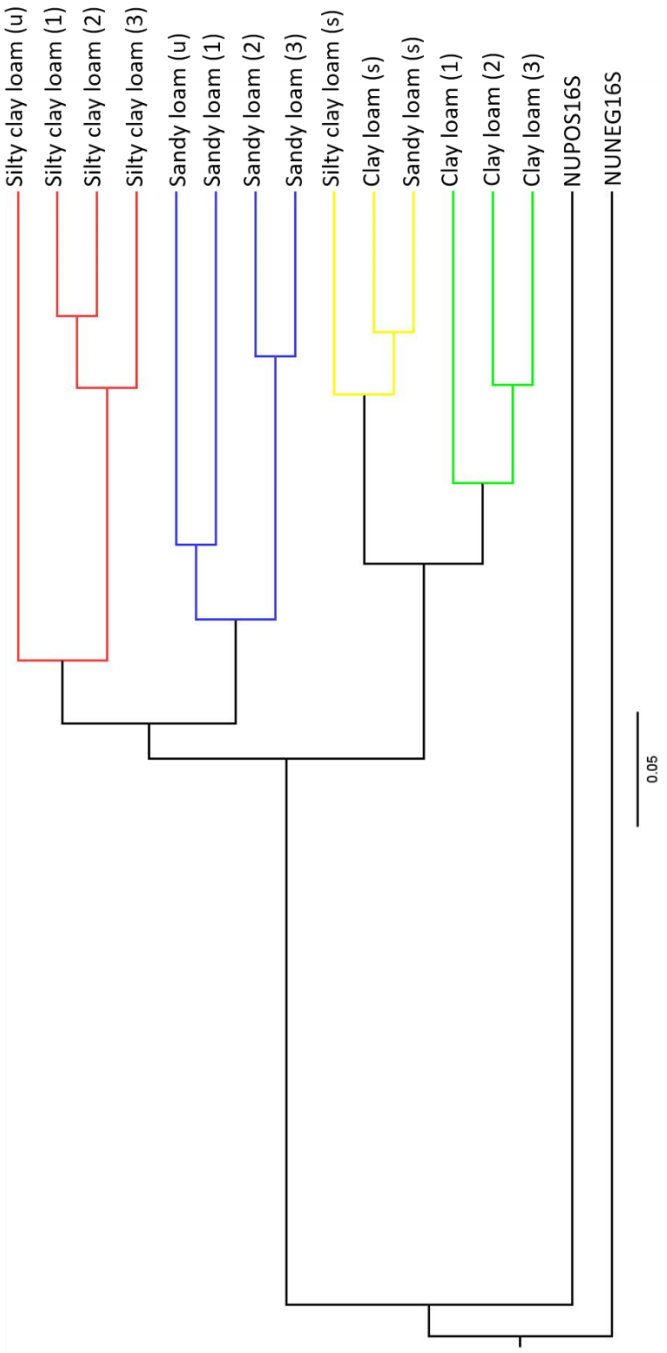

Figure 4.12. **β-Diversity level given by the Bray Curtis algorithm obtained for all samples tested.** Tree was obtained using Figtree. Four sample groups and respective branches are represented by different colours: Silty clay loam (red); Sandy loam (blue); Clay loam (green) and spiked soil samples (yellow). Samples are denoted by replicates (1, 2 & 3 (sampled 256 days post inoculation)) and with sub-level 'S' *(L.* monocytogenes spiked soils (sampled 1 day post inoculation)) and 'U' (uninoculated soils).

4.4. Discussion

L. monocytogenes survival in soil is complex and is influenced by many biotic and abiotic factors (Locatelli et al. 2013b). In these experiments *L. monocytogenes* survival was determined by direct plate counts in soils which are used to grow fresh leafy produce on a large scale. A subset of soil was sterilised to determine the effect of endogenous soil microbiota in these soils on *L. monocytogenes* survival. *L. monocytogenes* isolates from the fresh produce supply chain were selected for study based on their genetic differences and location which they were isolated in the supply chain. Their survival in soil was compared to a laboratory reference strain (EGD-e).

L. monocytogenes number in soil fell over time in both sterilised and fresh soils. In fresh soils *L. monocytogenes* number fell sharply before remaining relatively stable at a level of 10^4 CFU g^{-1} soil dw from 24 to 70 days across soil types. In sterilised soils, *L. monocytogenes* numbers didn't fall below ~10^6 CFU g^{-1} soil dw and persisted at this level until sampling at 257 days. *L. monocytogenes* survival in soil was significantly affected by soil microbial status (sterilised/non-sterilised) showing that the endogenous soil microbiota had a significant suppressive effect on *L. monocytogenes* survival, in agreement with McLaughlin et al., (2011) and (Falardeau et al., (2018). 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. In fresh soil microcosms, *L. monocytogenes* survival was not significantly affected by strain or soil type. However, in sterilised soil microcosms, survival was significantly affected by soil type with *L. monocytogenes* surviving at a higher number, for longer in clay loam soil than the other two soil types tested. From similar studies investigating *L. monocytogenes* survival in soils, this result agrees with the consensus that *L. monocytogenes* survives better in clay type soils compared to other soils (Dowe et al. 1997; Vivant et al. 2013). It has been proposed that this effect is due to clay type (finer) particles being able to harbour more negatively charged particles, meaning that soils of this type contain more base cations essential for bacterial life (Locatelli et al. 2013b).

In these experiments, after 70 days, growth of competitor bacterial species on *L. monocytogenes* selective plates made it difficult to quantify *L. monocytogenes* by

direct plate counts in fresh soils. Additionally, growth of competitor bacterial species in *L. monocytogenes* selective broths made it difficult to quantify the bacterium in fresh soil using an MPN enrichment method. The growth of competitor bacterial species in *L. monocytogenes* enrichment broths when detecting *L. monocytogenes* has been noted previously (Dailey et al. 2016) and may interfere with the food sampling process by generating false positives. To overcome this problem, molecular methods such as qPCR may be used to quantify *L. monocytogenes* in soil but molecular methods have been shown to have a high minimum detection limit in soil (~10^4 CFU g^{-1}) (Locatelli et al. 2013a) most likely due to the presence of high amounts of non-*L. monocytogenes* DNA and PCR inhibitory compounds in the soil such as humic substances than inhibit *Taq* DNA polymerases (Braid et al. 2003). Therefore, in this chapter, this method of quantification was of no use since *L. monocytogenes* levels fell below this number. However, it should be noted that there are now a number of DNA extraction kits and PCR master mixes that state they overcome these problems.

As such, while detection of *L. monocytogenes* in complex matrices such as soil is straightforward, quantifying *L. monocytogenes* at low levels in soil may well be unachievable with current technologies. During attempts at quantification of *L. monocytogenes* by the MPN-enrichment of fresh soil, competitor organisms growing in enrichment broth meant that *L. monocytogenes* survival in soil could not be quantified in fresh soils after 70 days. The two main competitors were identified and are both soilborne bacteria. While fresh soil microcosms could not be quantified by this method, MPN-enrichment quantified *L. monocytogenes* in sterilised soil microcosms at a higher number than direct plate counts. This result suggests that long incubation in the soil environment is causing a portion of the *L. monocytogenes* population to turn VBNC similar to incubation in manure microcosms (Desneux et al. 2016). In this study, around 75% of the total *L. monocytogenes* population was determined to be in the VBNC state. However, this is a preliminary result which needs to be confirmed. The VBNC state in bacteria is thought to be a response to stress which in the context of bacterial survival in soil is easily experienced. Osmotic stress, nutrient limitation and desiccation may all be encountered in the soil environment, causing a portion of the *L. monocytogenes* population to enter the VBNC state. Upon entering a culture media which provided the neccassary components for *L. monocytogenes* growth, VBNC *L.*

monocytogenes may have reversed the VBNC state to begin metabolising again. Little is understood about the metabolic pathways associated with switching to the VBNC state and back again. Recently, VBNC bacteria have been gaining more attention in the scientific and wider community due to the additional challenges they present to cleaning/sampling regimes and regulators and more research is needed in this whole area.

After a long incubation in non-sterilised (fresh) soil, *L. monocytogenes* DNA could not be detected with a species-specific PCR. However, using NGS targeting the 16s rRNA gene region the genus *Listeria* could be detected in 11 out of 15 soil samples tested. When the sequence for this OTU was compared to other available sequences through BLAST it had full coverage identity with *L. monocytogenes* sequences suggesting that this OTU did represent *L. monocytogenes* rather than other species of the *Listeria* genus. However, the OTU representing the *L. monocytogenes* was only counted >10 times in 4 out of 15 samples. These samples were; one sample of clay loam soil, two samples of sandy loam soil (both of which had been inoculated with ~10^8 *L. monocytogenes* CFU g^{-1} soil dw 256 days prior) and one 'control' sandy soil that had not been inoculated. This indicates that *L. monocytogenes* was present at very low levels in the control soil and that NGS can detect *L. monocytogenes* at low levels in soil (down to 0.074% of the total bacterial community). Finally, these results may suggest that *L. monocytogenes* was surviving at a higher level in sandy loam soils than other soil types after 256 days, however, Shannon index of α-diversity indicated that the bacterial community in sandy soils was the least diverse meaning that *L. monocytogenes* DNA could represent a potentially higher proportion of the total bacterial community. Whether *L. monocytogenes* survived at a higher rate or was simply present at a higher relative abundance in soil due to a less diverse microbial community remains an open question.

4.5. Conclusion

Overall, *L. monocytogenes* survives in soil for long incubation periods (>70 days) and survival in soil is influenced by soil type and endogenous soil microbiota. Quantification of *L. monocytogenes* in sterilised soil after 257 days using two different culture methods (MPN-enrichment and direct plate count) showed that a higher

number of *L. monocytogenes* can be quantified by the enrichment method vs. direct plate counts suggesting a portion of the population is turning VBNC in soil.

These results can help fresh produce growers to choose soil types where *L. monocytogenes* survival is worse, and the contamination risk is thus less likely. These results also highlight the need for a diverse microbial community in soil, commonly an indicator of good soil health (Schloter et al. 2018). Whether a more diverse soil population in this study (as indicated by Shannon's index of α-diversity) has resulted in better suppression of *L. monocytogenes* in clay and silt loam soils compared to sandy soils remains an open question and requires further investigation. This chapter has shown that *L. monocytogenes* strains found in the FLPSC can survive in soil over a typical growing season and is therefore a potential source of contamination if this soil is transferred to the product. Therefore, the next chapter investigates detection rates of *L. monocytogenes* on leaves spoilt with contaminated soil, *L. monocytogenes* survival on the leaf and the effect of the wash step on *L. monocytogenes* populations on the leaf.

Chapter 5 The impact of the wash step on *L. monocytogenes* populations on spinach leaves

5.1 Introduction

Soil spoilage of fresh leafy produce is thought to be a significant source of human bacterial pathogen contamination in the supply chain (Monaghan and Hutchison 2012) and soil splash has been shown to be a route of contamination to leafy produce for the *L. monocytogenes* surrogate, *L. innocua* (Girardin et al. 2005). Despite this evidence, contamination of fresh produce by *L. monocytogenes* via the soil is poorly understood. . Chapter 4 showed that *L. monocytogenes* can survive in horticultural soils used to grow fresh leafy produce over the course of the growth of the crop from seed to final product. This means that any contamination of soil by *L. monocytogenes* could result in subsequent contamination of the crop from soil splash or other soil spoilage. This type of contamination is likely to be difficult to prevent, random and sporadic. Additionally, soil is a potential contamination route to fresh produce for *L. monocytogenes* due to the close contact between soil and the product during the growing process. Therefore, the first part of this chapter looks at factors that may affect the detection of *L. monocytogenes* (from soil) on leaves. Inoculum size, time that inoculum spent in soil and different amounts of soil are factors which were tested.

Currently in the FLPSC, there is a clear focus on reducing *L. monocytogenes* (and other human pathogenic bacteria) contamination of fresh leafy produce. To do this, stakeholders in the FLPSC use preventative measures such as not watering crops in the days before harvest, thus reducing the risk of bacterial contamination on fresh leafy produce from soil splash (Figure 5.1.) or contaminated water.

Figure 5.1. **Soil splash on baby red leaf lettuce**. Photograph taken in a baby leaf production field. Barway, UK. October 2016.

Since it is difficult or impossible to stop all soil contamination of fresh produce in the growing environment, further processing of spoilt leaves is required to remove dirt and debris. The main tool employed by stakeholders in the FLPSC to reduce microbial contamination of fresh produce is the wash step. During the wash step, fresh produce undergoes manual/mechanical cutting before mechanical washing in the presence of sanitizers and rinsing with potable water. This is followed by portioning and packaging (Figure 5.2.).

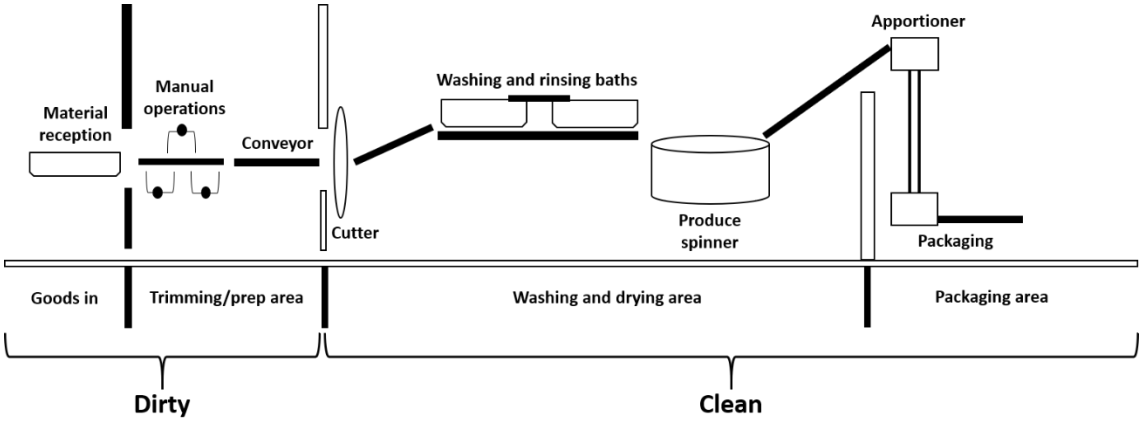

Figure 5.2. **Schematic of a fresh cut produce washing system.** Adapted from Allende and Artes (2005).

The wash process involves light agitation so as not to damage the product and helps to remove dirt and foreign bodies from the product surface. Sanitisers are often included in the wash water mainly to prevent bacterial growth in wash water tanks but also

reduce the microbial load on fresh produce (Gil et al. 2009). A 1-2log (90-99%) reduction of microorganisms on produce during washing is generally accepted as effective sanitation for fresh produce. The most widely used sanitizers in the food industry today are chlorine products such as sodium hypochlorite (Kaczmarek et al. 2019). When used at recommended levels (50-200ppm free chlorine) chlorine is an effective sanitizer and maintains the sensory quality of fresh produce (Allende et al. 2008). This means that chlorine does not have a detrimental effect on consumer perception of produce quality (such as wilting and discoloration). The wash step adds monetary value to fresh cut products that are subsequently marketed as ready-to-eat (i.e. requires no further washing by the consumer). It is also important to note that in the UK FLPSC for products which have delicate leaves that can be more easily damaged (like spinach and rocket), the highest concentration of free chlorine in wash water tends to be limited to 60ppm.

Specifically for *L. monocytogenes*, chlorine wash at 50ppm for 50 to 90 seconds has been found to reduce *L. monocytogenes* number on artificially inoculated leafy greens by ~1'2 logs and there is many data support the sanitising effect of chlorine wash (Stopforth et al. 2008; Hoelzer et al. 2014). In this chapter, to confirm that chlorine is an effective sanitizer using operational values for time and sanitiser strength for the UK FLPSC, spinach was artificially contaminated with *L. monocytogenes* and subsequently washed with different levels of chlorine wash up to the maximum accepted level for baby leaf products (60ppm). Spinach leaves were washed 24- and 48-hours post contamination to determine the effect that time has on the effectiveness of the chlorine wash.

Recently, concerns have been raised about the efficacy of chlorine products (Highmore et al. 2018) and their effect on human and environmental health due to the formation of carcinogenic halogenated disinfection by-products, like trihalomethanes (THMs) and haloacetic acids (HAAs) (Ölmez and Kretzschmar 2009). Furthermore, the reduction of large volumes of process water is a priority due to water consumption and wastewater discharge concerns. This means that regulatory bodies like the EU are looking for alternatives to the chlorine wash for reducing microbial load (European Commission 2014). Other processes are available to reduce microbial contamination of fresh produce in the FLPSC but are less commonly used in the UK e.g. Ozone, organic acid

compounds and electrolysed water (EW). Other methods are effective at reducing microbial loads on produce but it has been proposed that EW may be a suitable alternative for the treatment of leafy greens due to its effective sanitization effect, supposed lower production of residues and low impact on produce sensory quality (Rahman et al. 2016). Whilst it's mechanism of action is not entirely understood, the production of EW involves electrolysis of water and NaCl which generates a high amount of available chlorine concentration (ACC) and is significantly more environmentally friendly than chlorine and its derivatives (Kaczmarek et al. 2019). This makes EW an attractive alternative to chlorine wash for fresh leafy produce and a promising non thermal food sanitizer. To investigate EWs viability as a chlorine replacement in the fresh produce supply chain, the effect of EW on *L. monocytogenes* number on the leaf was determined. This was carried out by following operational values (contact time, free chlorine concentration, etc.) for washing spinach in the FLPSC.

Whilst the effect of chlorine wash on *L. monocytogenes* number on the leaf is well understood, little data exists on the effect that chlorine wash has on *L. monocytogenes* survival on leaves that have been contaminated post wash. The effect of temperature in this scenario is also poorly understood. Therefore, in this chapter, *L. monocytogenes* was inoculated on the leaf at room temperature (20°C) and refrigeration temperature (4°C) after a chlorine wash at operational levels to study how the *L. monocytogenes* population changed over time, for the shelf life of the spinach (4 days). Initially, this was done with ready-to-eat and unwashed spinach from a local retailer (pilot study) followed by spinach that had been washed in-house following washing protocols from a processing company in the UK FLPSC (Nature's Way Foods). In addition, the leaf microbiome of leaves that had been treated via chlorine wash was studied to determine the effect that the washing process has on leaf microbiota and whether a change in level of microbiota may be a reason for different levels of *L. monocytogenes* survival on the leaf.

Aim 1: Investigate soil as a source of *L. monocytogenes* contamination on spinach leaves

Objective:

- Determine the effect that inoculum size, time inoculum has spent in soil and the amount of soil has on detection rates for soil that has been spoilt with *L. monocytogenes* contaminated soil.

Aim 2: Study the effect that the wash step has on *L. monocytogenes* populations on spinach leaves.

Objectives;

- Determine the effect of chlorine wash at operational levels on *L. monocytogenes* populations and investigate the effect of time post-contamination in this scenario.
- Compare the effect of chlorine wash with effect of electrolysed water (EW) on *L. monocytogenes* population wash at operational levels of free chlorine.

Aim 3: Investigate the effect that chlorine wash has on *L. monocytogenes* survival on spinach leaves post-contamination i.e. contamination after leaves have been washed

Objectives;

- Investigate *L. monocytogenes* survival post-wash at different (room and refrigeration) temperatures in a pilot study of shop bought spinach (ready to eat & unwashed).
- Study the effect of chlorine wash following washing protocols from a vegetable processer in the UK FLPSC. Also, determine the effect that chlorine wash has on the leaf microbiota.

5.2. Methods

5.2.1. Determining the effect of inoculation level, time that inoculum has spent in soil and amount of soil contamination on *L. monocytogenes* detection rates on spinach

5.2.1.1. Construction of *L. monocytogenes* stock culture and inoculum

L. monocytogenes strain NLmo2 (see section 2.3.1. for further details) was used to inoculate clay loam type soil (see section 4.3.1. for further details). Strain NLmo2 was grown overnight (16 h) in a 250ml Erlenmeyer flask with 50ml of TSB at 25 °C, 200 rpm. 1.5 ml of this culture was centrifuged 10,000 g for 2 mins before being washed twice in PBS and resuspended in 1.5 ml PBS & 50% glycerol. Aliquots were then frozen for long term storage before being subsequently defrosted and serial diluted before inoculated into soil.

5.2.1.2. Inoculation of soil and experimental design

Soil was adjusted to 80 % water holding capacity (excluding inoculum) before inoculating the soil with *L. monocytogenes* at 3 different levels. These were; low (10^2 CFU g^{-1} soil dw), medium (10^4 CFU g^{-1} soil dw), and high (10^7 CFU g^{-1} soil dw). Subsets of these soils were left to incubate for one and two months at 14.8 °C. Using these inoculated soils, spinach was spoiled by transferring either 1.5 g (1 % w/w) or 7.5 g (5 % w/w) of soil to a 125 g bag of shop bought unwashed spinach (Tesco, UK). This was followed by thorough hand mixing for 1 minute. Spoiled spinach was then left overnight in a fridge (4 °C) before sampling the next morning. Controls using uninoculated soil and shop bought spinach (no *L. monocytogenes* contaminated soil added) were included with every sampling time point (0, 28 & 56 days). Inoculum was mixed in 1 ml of water before mixing with soil.

5.2.1.3. Sampling method and statistical analysis

Spinach was sampled following industry standard protocols (ISO 11290-1: 2017). Briefly, 25 g of previously spoiled spinach was placed in a stomacher bag before adding 225 ml of half-Fraser broth. This mixture was stomached on the high setting for 1 minute. This primary enrichment broth was incubated for 24 h at 30 °C. After primary enrichment, 0.1 ml of primary enrichment broth was transferred to Fraser broth. This secondary enrichment broth was incubated for 48 h at 37 °C. After incubation,

secondary enrichment broth was streaked on to ALOA ((brilliance) Oxoid) and OXFORD agars. Characteristic *L. monocytogenes* colonies were confirmed using a species-specific colony PCR (see section 2.2.2.1). A total of 5 x 25 g samples were used for each inoculum size/incubation time/soil amount combination and converted to percentage detection rate based on the amount of positive results. The effects of different factors (in terms of direction, size and significance) were determined using a binary logistic regression in SPSS.

5.2.2. Effect of different levels of chlorine wash and electrolysed water on *L. monocytogenes* populations on spinach leaves

5.2.2.1 Experimental design, inoculum preparation and washing process

Previously constructed NLmo2 stock cultures (see section 5.2.1.1) were diluted to 10^7 CFU ml^{-1} in PBS and used to inoculate spinach leaves by placing 25 g of spinach leaf in a stomacher bag and applying 1 ml of inoculum. This made the final inoculum on spinach leaves ~10^6 CFU g^{-1}. To make chlorinated water for washing spinach, Sodium Hypochlorite solution (Merck) was diluted in tap water to levels of 20, 40 & 60 ppm. Tap water was included as a control. The free chlorine concentration of a stock chlorine solution using the CL200 ExStik Waterproof Chlorine Meter (Extech) according to the manufacturer's instructions. For the spinach washing process: in stomacher bags, 225 ml of ddH_20 was added to the leaves and the mixture was shaken (by hand) for 45 s (primary wash). Following the primary wash spinach leaves were subject to a chlorine wash at the 4 given concentrations for 45 s. This secondary wash was also discarded before the sampling (quantification) procedure started. After the optimum level of chlorine had been determined (60 ppm) on spinach that had been contaminated 24 h previously and left to incubate at 20 °C, this level of chlorine was also tested on spinach that had been contaminated and left to incubate for 1 h and 48 h at 20 °C. The effect of chlorine wash was compared to 'control' leaves which were washed in tap water. The effect of chlorine wash was also compared to electrolysed water, which was diluted from a stock solution to 60 ppm free chlorine. To determine differences between the treatments and times, a two-way ANOVA with Tukey's tests for multiple comparisons was used. Free chlorine in electrolysed water was again measured using the CL200 ExStik.

5.2.2.2. Sampling procedure

After pouring off the secondary wash, inoculated spinach (25 g) was sampled by adding 225 ml of sterilised PBS to the stomacher bag. This mixture was homogenized in a Stomacher on high for 1 min before serial diluting to appropriate dilutions and playing on OXFORD agar with *Listeria* selective supplement. Units are expressed in CFU per gram of leaf (CFU g^{-1}).

5.2.3. Monitoring *L. monocytogenes* survival on spinach leaves after post-wash contamination

5.2.3.1. Pilot study

For details of the *L. monocytogenes* stock culture that was subsequently inoculated onto spinach leaves for these experiments see section 5.2.1.1. Prior to inoculation on the leaf surface, stock aliquots of *L. monocytogenes* culture were washed twice in PBS and diluted to a level of 10^6 CFU ml^{-1} in PBS. A total of 1 ml of this inoculum was used to inoculate 1g of spinach leaves. Bags of baby leaf spinach were bought from a local retailer (Lidl, UK). These were ready to eat and unwashed. A subset of unwashed leaves for 5 time points were also surface sterilised in 70 % ethanol and left to air dry in a class 2 microbiological safety hood.

To inoculate spinach, 1 g of spinach leaves were placed in a stomacher bag (10 ml), inoculated with 1 ml *L. monocytogenes* inoculum and left to incubate at 20 °C for 5 days. Spinach leaves were sampled every 24 h for over the course of the experiment. To do this, 3 x 1 g of leaf (n=3) was sampled for each leaf treatment, every 24 h, by adding 9 ml of PBS and homogenising in the stomacher for 1 minute on the high setting before serial diluting and plating appropriate dilutions on OXFORD agar with *Listeria* selective supplement (Oxoid). Levels of *L. monocytogenes* are expressed as CFU g^{-1} spinach leaf.

5.2.3.2. Effect of chlorine wash on microbial populations on spinach leaves

To determine the bacterial communities present on spinach leaves, washed (ready to eat) and unwashed spinach was purchased from a local retailer (Lidl, UK). Microbial DNA was extracted from 3 x 1g of uninoculated leaves using the PowerSoil Kit (Qiagen). 3 x 1g of leaves that had been sterilised by submersing in 70% ethanol for 1 minute were included as 'control' leaves. For the full method of bioinformatics

analyses for 16S rRNA NGS, see section 4.2.3. Bacterial load was also quantified directly from infected spinach leaves. 1g leaves from each treatment were placed in a stomacher bag with 9ml PBS per 1g leaf. Leaves were homogenised in the stomacher set on the high setting for 1 minute before plating appropriate dilutions on Nutrient agar which were incubated at 25 °C for 48 h. Colony forming units were counted and expressed as CFU g^{-1} dw. 3 independent replicates were taken (n=3) A leaf imprint was taken by pressing leaves from the different treatments on nutrient agar and incubating the plates at 25 °C for 48 h to culture mesophilic bacteria

5.2.4.3. Leaf treatment based on operational values during fresh produce washing in industry

Following the results of the pilot study described above (section 5.2.3.1.) unwashed spinach bought from a local retailer (Lidl, UK) was washed following washing protocols from food processers in the FLPSC. Briefly, unwashed spinach was submerged in 60ppm chlorine wash (the maximum accepted concentration for fresh leafy produce) for 45 seconds and mixed gently before a subsequent 45 second wash/mix in tap water. After washing, spinach was left to airdry at room temperature for 30 minutes before inoculation. Spinach was incubated at two different temperatures (20 °C & 4 °C) and sampled every 24 h from the time of inoculation for 4 days. The methods for inoculating and sampling spinach remained the same as above (section 5.2.3.1.).

5.3. Results

5.3.1. The effect of inoculation level, time inoculant has spent in soil and amount of soil contamination on *L. monocytogenes* detection rates on spinach

The effect of different factors on detection rates (%) was determined using a binary logistic regression model. Detection rate significantly increased with increasing inoculum size (10^2 – 10^7 CFU g^{-1} soil dw; $P<0.001$), and with increasing soil contamination rate (1% - 5% w/w; $P<0.05$). Conversely the detection rate significantly reduced over time (0 – 56 days; $P < 0.001$) (Figure 5.3.). When sampling immediately after adding fresh inoculum to soil, *L. monocytogenes* could be detected in all but one sample regardless of inoculum size or level of soil contamination. After 28 days, *L. monocytogenes* could not be detected on spinach spoiled with soil inoculated with 10^2 CFU g^{-1} soil dw. Detections of *L. monocytogenes* from leaves with 1% (w/w) soil contamination at a level of 10^4 CFU g^{-1} soil dw also reduced from 100 to 60% after this time, however detections from 5% (w/w) contamination and both contamination levels of soil inoculated with 10^7 CFU g^{-1} soil dw remained at 100%. After 56 days, *L. monocytogenes* again could not be detected on spinach contaminated with soil inoculated with 10^2 CFU g^{-1} soil dw and was only detected 20% of the time in both levels of soil contamination at an inoculum level of 10^4 CFU g^{-1} soil dw. At an initial level of 10^7 CFU g^{-1} soil dw after 56 days, *L. monocytogenes* was detected in 100% of samples with 5% soil contamination but only 40% with 1% soil contamination.

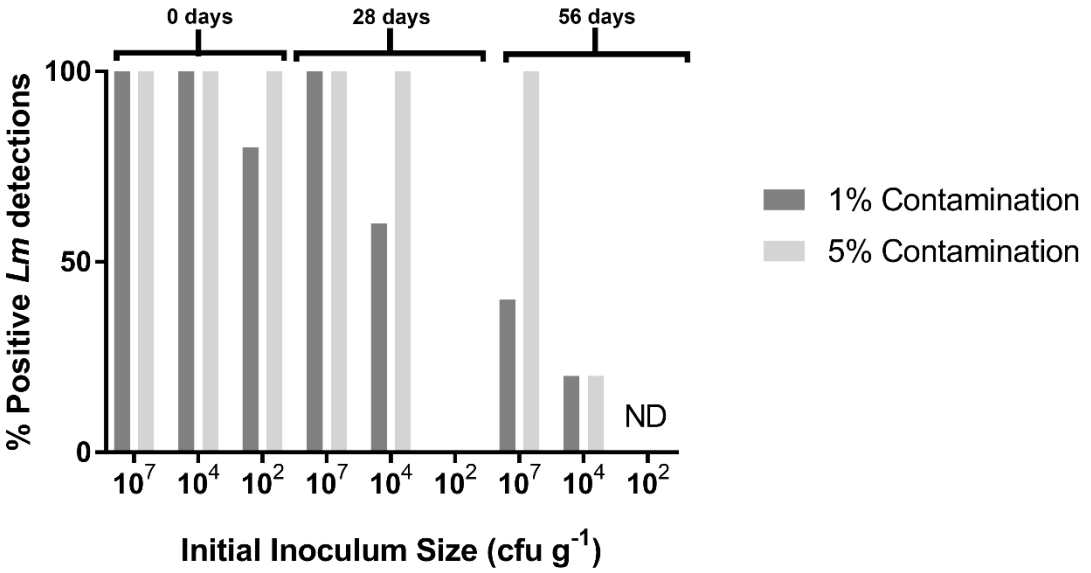

Figure 5.3. **Effect of soil inoculum size and time on *L. monocytogenes* detection rates on spinach leaves carrying varying amounts of *L. monocytogenes* contaminated soil.** Dark grey bars represent spinach spoilt with 1% w/w soil contamination and light grey bars represent spinach samples spoilt with 5% w/w soil contamination.

5.3.2. The effect of chlorine wash on *L. monocytogenes* populations on spinach leaves

L. monocytogenes was inoculated onto spinach leaves and left to incubate at 20°C for 24h. Following this, spinach was washed using different levels of chlorine wash and subsequently sampled to enumerate the remaining *L. monocytogenes* on the leaf. From an initial inoculum of 10^6 CFU g^{-1} spinach leaf, washing with tap water alone for 45 seconds showed no effect on the number of *L. monocytogenes* on the leaf. (Figure 5.4.). Increasing concentrations of free chlorine increasingly lowered *L. monocytogenes* populations on spinach leaves up to 60ppm - the maximum accepted strength of chlorine wash solution for baby leaf spinach in the UK fresh produce supply chain. At this level, *L. monocytogenes* was reduced in number by ~1Log (from Log 6.25 to Log 4.98; $P < 0.05$). An ANOVA with Tukey's test for multiple comparisons showed that 40ppm & 60ppm free chlorine in wash water significantly ($P < 0.05$) reduced the number of *L. monocytogenes* on spinach leaves.

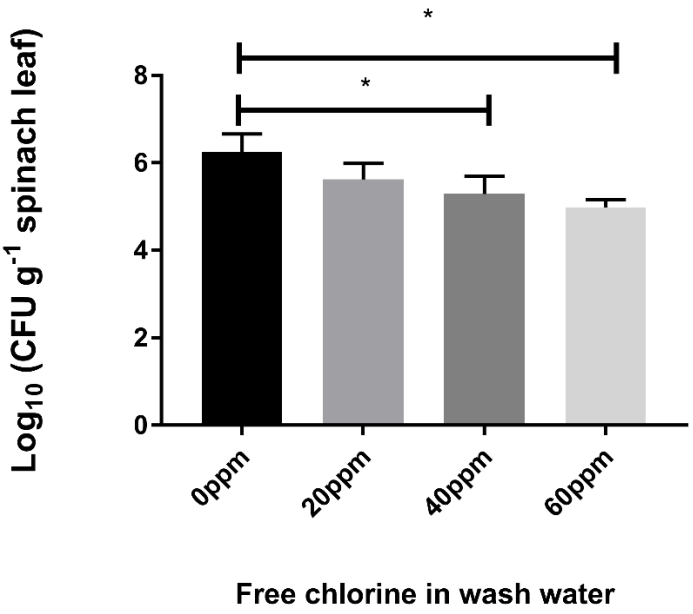

Figure 5.4. **Effect of different levels of free chlorine (made by diluting sodium hypochlorite) on *L. monocytogenes* populations on the leaf.** * = P < 0.05. Error bars indicate SEM of 3 independent replicates (n = 3).

After it had been established that 60ppm free chlorine in wash water provided the most effective sanitation compared to lower levels of chlorine, the effect of time post contamination on the effectiveness of chlorine wash was investigated (Figure 5.5.). This was compared to washing only with tap water. After an initial inoculum of 10^6 CFU g^{-1} spinach leaf, both tap water and 60ppm chlorine wash reduced *L. monocytogenes* number by 1.5 logs. After 24h and 48h tap water wash was ineffective at reducing *L. monocytogenes* number. 60ppm chlorine wash was more effective at 24h (reduction of 1.26 Logs) than 48h (reduction of 0.69 Logs) (Table 5.1.).

Table 5.1. **Mean difference between 0ppm and 60ppm chlorine wash after different times post contamination.** All values are in Log *L. monocytogenes* CFU g^{-1} spinach leaf.

		0ppm	60ppm	Average difference	SEM
Time	**1h**	4.78	4.67	0.10	0.171
	24h	6.25	4.98	1.26	
	48h	6.17	5.48	0.69	

A two-way ANOVA with Tukey's test for multiple comparisons showed that the effectiveness of the chlorine wash was significantly reduced after 24h ($P < 0.001$) and 48h ($P < 0.005$)

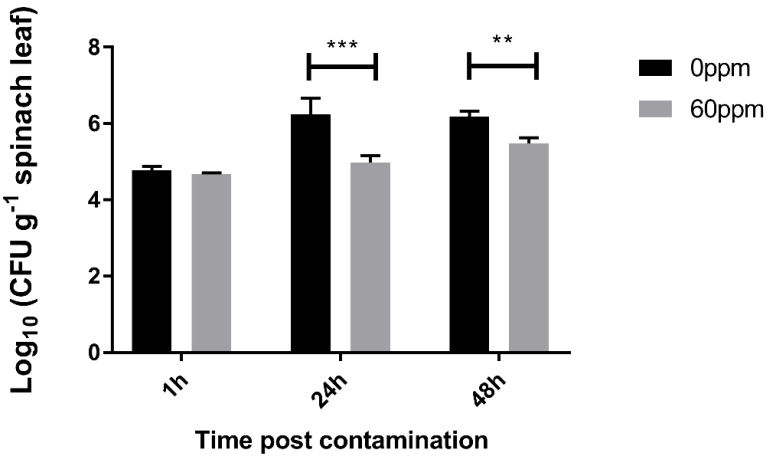

Figure 5.5. **Effect of length of time post contamination before washing spinach leaves with 0ppm (tap water) and 60ppm chlorine wash on *L. monocytogenes* viable counts.** ** = $P \leq 0.005$, *** = $P < 0.001$. Error bars indicate SEM of 3 independent replicates (n = 3).

In summary, chlorine wash became less effective as the amount of time post-contamination increased.

5.3.3. Comparison of the efficacy of electrolysed water wash to chlorine wash using industry standard wash practices

Using industry standard wash practices, the efficacy of electrolysed water wash (at a level of 60ppm free chlorine) was compared to a chlorine wash using spinach that had been contaminated with *L. monocytogenes* 24 hours before. Figure 5.6. shows that the chlorine wash was more effective than electrolysed water wash at reducing the size of the *L. monocytogenes* population on the leaf. An ANOVA which analysed differences between the average number of *L. monocytogenes* left on the spinach leaf after treatment showed that while 60ppm chlorine wash significantly ($P < 0.005$) reduced the *L. monocytogenes* population, reduction of *L. monocytogenes* by electrolysed water wash was non-significant.

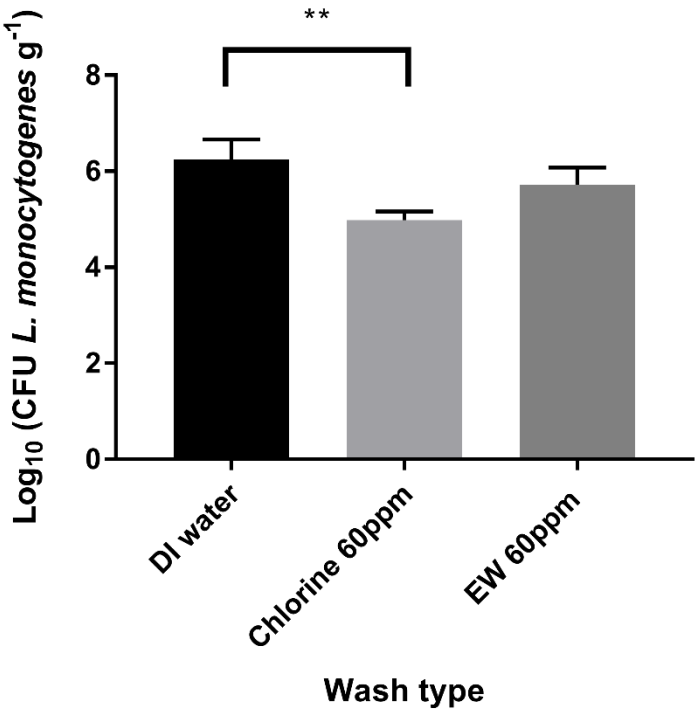

Figure 5.6. **Comparison of washing efficacy with 3 different wash types after 24-hour post contamination with *L. monocytogenes* on spinach leaves.** ** indicates significant difference according to an ANOVA of $P < 0.005$. Error bars represent the SEM of 3 independent replicates (n = 3).

5.3.4. Monitoring *L. monocytogenes* survival on spinach leaves from post-wash contamination

5.3.4.1. Pilot study

5.3.4.1.1. Differences in bacterial communities between unwashed, washed and EtOH treated spinach leaves

Spinach leaves were pressed against nutrient agar and subsequently incubated for 48h at 20°C. Leaf prints can be seen in Figure 5.7. where the print from an unwashed leaf yielded the most bacterial colonies, followed by the ready to eat leaf. The EtOH leaf yielded the least bacteria from a leaf print on nutrient agar plates.

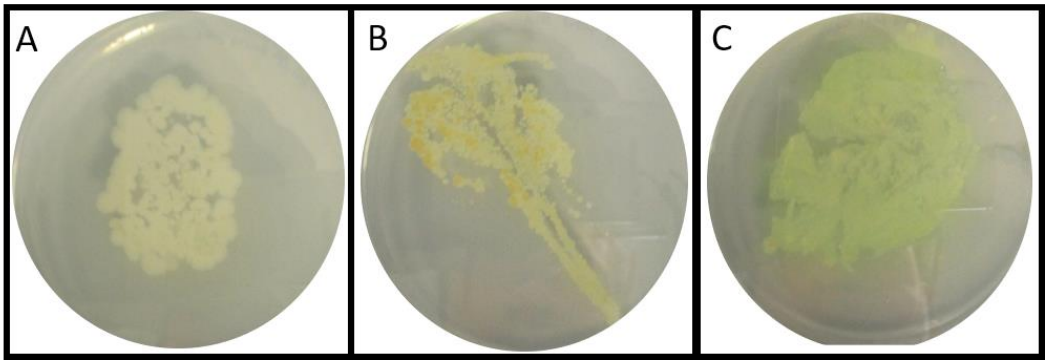

Figure 5.7. **Comparison of leaf prints on nutrient agar with spinach leaves from 3 different treatment groups.** A – leaf print from spinach leaf treated in 70% EtOH for 1 minute, B – ready to eat leaf, C – unwashed leaf.

To determine the number of culturable bacteria on spinach leaves bought from a local retailer, spinach leaves were mixed (stomached) with PBS before plating appropriate dilutions on nutrient agar. Plates were left to incubate at 20°C for 48h. Total culturable bacteria (CFU g^{-1} spinach leaf) was higher on average on unwashed leaves than ready-to-eat leaves and significantly higher ($P < 0.005$) higher than leaves that had been sterilised in 70% EtOH for 60 seconds (Figure 5.8.).

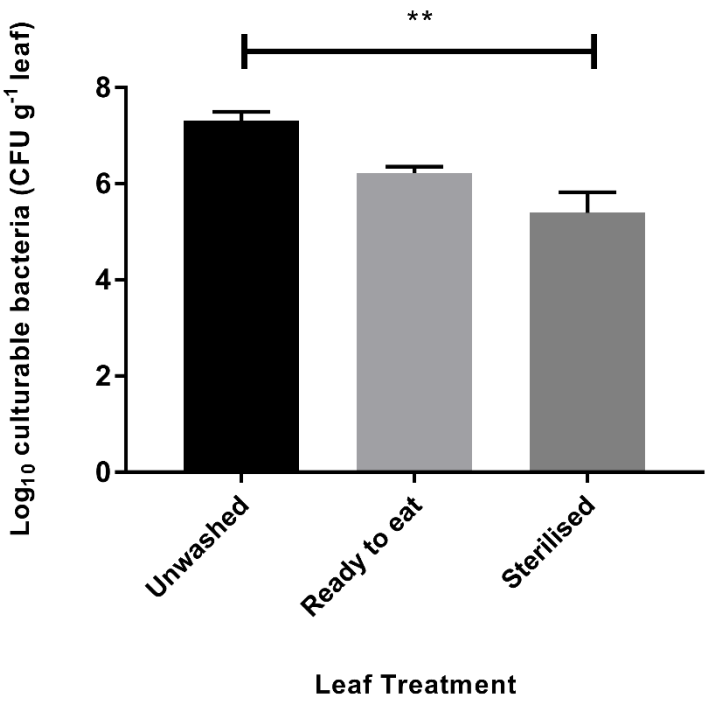

Figure 5.8. **Number of culturable bacteria on shop bought ready to eat and unwashed spinach compared to 'sterilised' leaves.** ** indicates significant difference between

unwashed and sterilised leaves according to an ANOVA (with Tukeys test for multiple comparisons) using 3 independent replicates (n = 3).

The bacterial communities on these leaves were also determined using NGS targeting the 16S rRNA gene. Low primer specificity for targeting the bacterial 16S rRNA gene meant that chloroplast DNA from spinach leaves was detected during analysis and made up a large proportion of final relative abundance of species in samples. To view bacterial communities on leaves without chloroplast DNA the operational taxonomical unit (OTU) representing chloroplast DNA was removed from the analysis. Percentage relative abundances of different bacterial orders in the three different sample types can be seen in Figure 5.9.

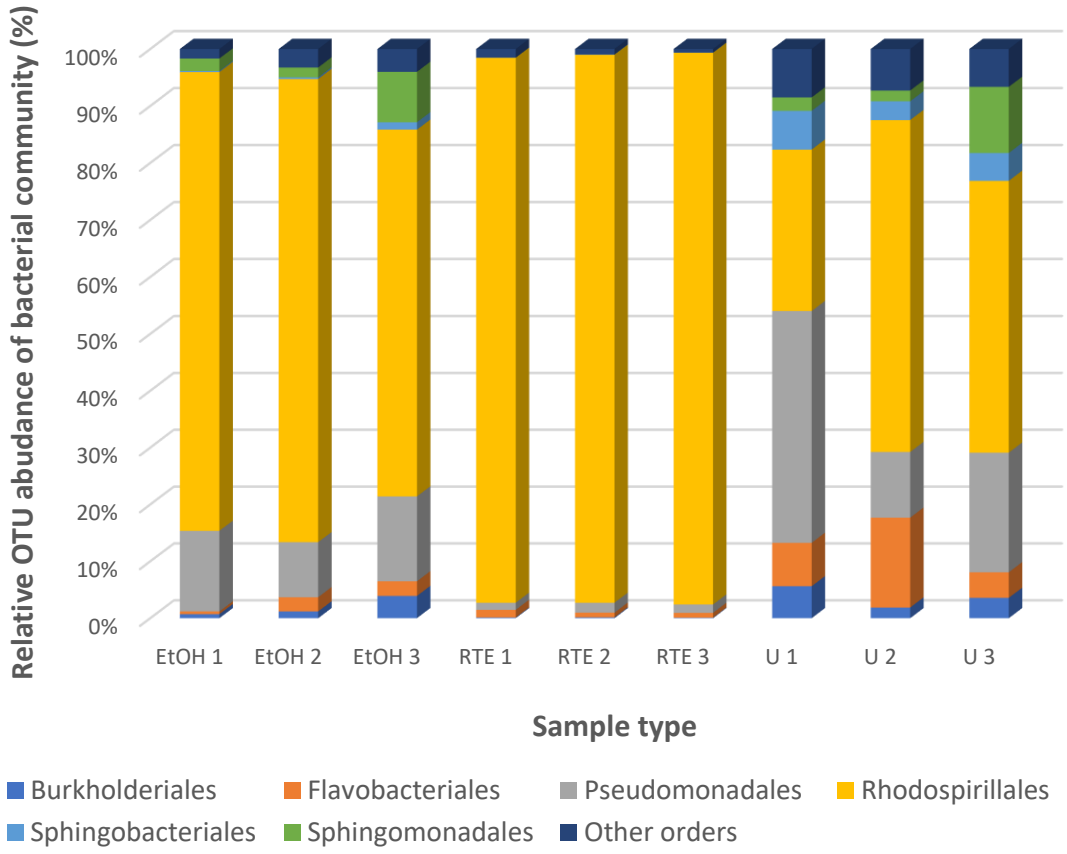

Figure 5.9. **Bacterial community structure in spinach samples from different treatment groups.** Sample type as follows; EtOH – unwashed spinach submerged in 70% Ethanol for 1 minute, RTE – ready to eat spinach bought from a local retailer, U – unwashed spinach bought from a local retailer. 'Other orders' represents microbial diversity of orders containing bacterial order that represented < 1% of the total bacterial community.

In general, bacterial community structure at order level exhibited some changes related to relative abundance depending on leaf treatment. Rhodospirillum made up a large proportion of all samples but made up a higher proportion of the bacterial community in RTE spinach samples. Unwashed leaves and leaves treated in EtOH had a higher proportion of Pseudomonad(ale)s as well as bacteria representing other bacterial orders. According to the Shannon index for α-diversity (Figure 5.10.), unwashed spinach samples contained the most diverse bacterial community (average 1.83) followed by leaves treated in EtOH (average 1.09) and then RTE leaves (average 0.26).

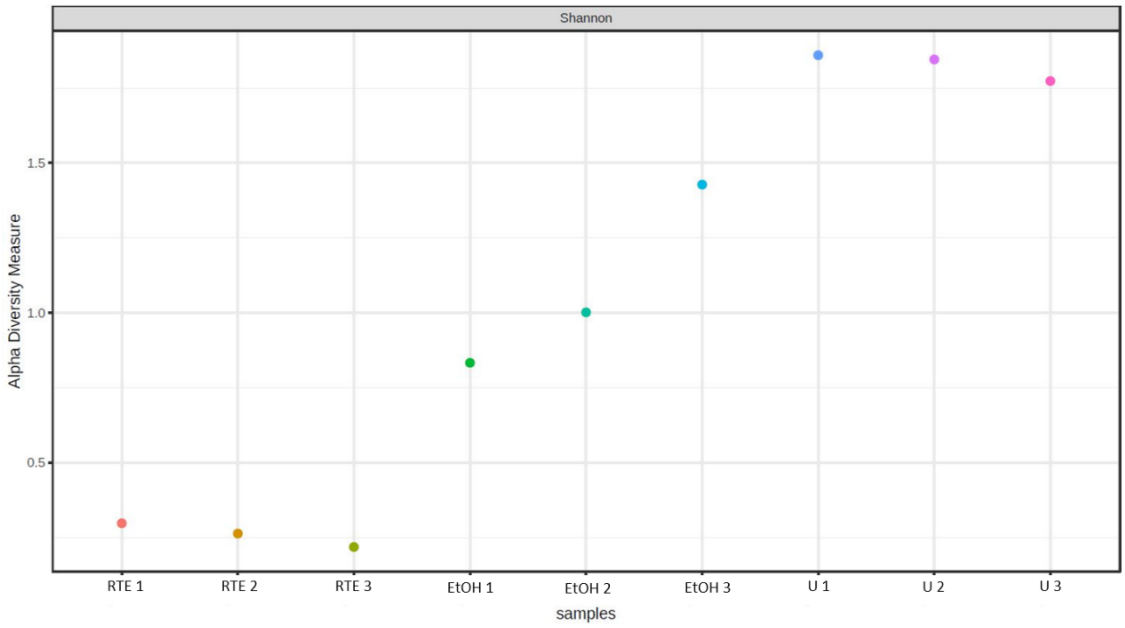

Figure 5.10. **α-diversity (Shannon index) of bacterial communities on spinach leaves from different treatment groups**. Sample type as follows; EtOH – unwashed spinach submerged in 70% Ethanol for 1 minute, RTE – ready to eat spinach bought from a local retailer, U – unwashed spinach bought from a local retailer.

5.3.4.1.2. L. monocytogenes survival on ready to eat, washed and EtOH treated spinach leaves at room and refrigeration temperatures

Survival of *L. monocytogenes* populations from an initial number of ~10^6 CFU g^{-1} spinach leaf were monitored for 4 days on spinach leaves from 3 different treatment groups. This process was repeated at two different temperatures. At 20°C (Figure 5.11.), the *L. monocytogenes* population on unwashed spinach leaves reached a maximum size of ~$10^{7.5}$ CFU g^{-1} after 3 days before slightly reducing after 4 days. On

ready to eat spinach, the population reached a similar size after 1 day and maintained this size until 4 days. EtOH treated leaves saw the largest increase in *L. monocytogenes* population size from the initial inoculum to 10^8 CFU g^{-1} after 1 day rising to $10^{8.5}$ CFU g^{-1} after 2 days and maintaining this size until 4 days.

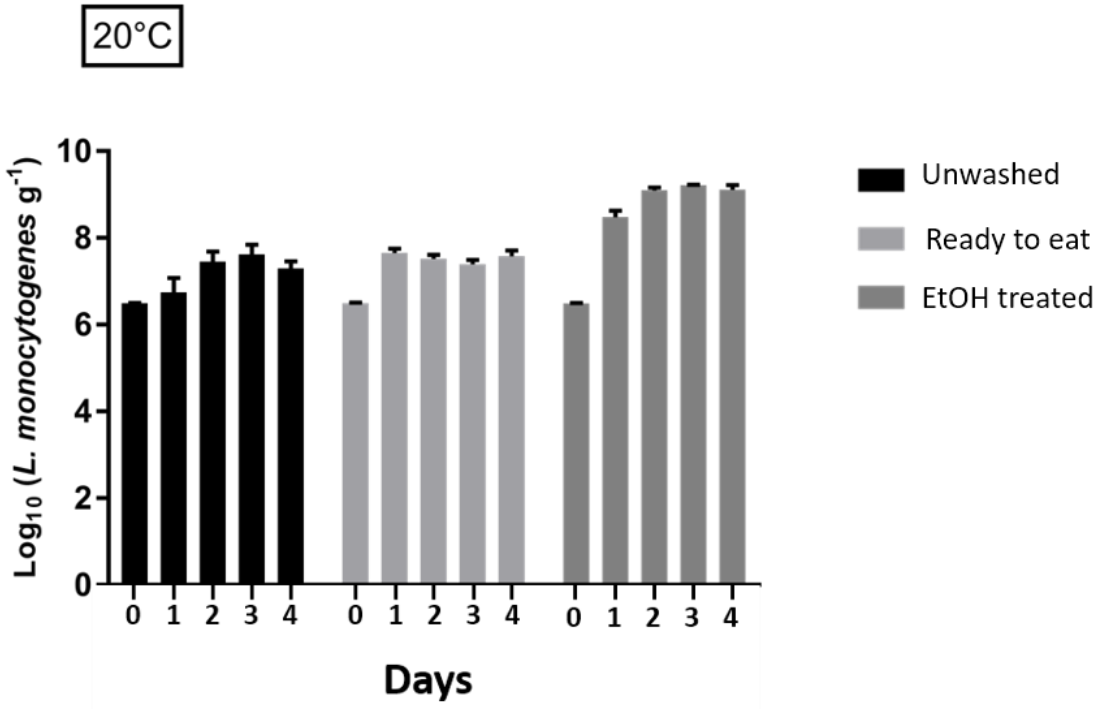

Figure 5.11. ***L. monocytogenes*** **survival on spinach leaves from 3 different treatments at 20°C.** Black bars represent unwashed spinach; light grey bars represent ready to eat spinach and dark grey bars represent EtOH treated spinach. Error bars represent the SEM of 3 independent replicates (n = 3).

At 4°C (Figure 5.12.), the *L. monocytogenes* population on unwashed spinach leaves fell on every day spinach was sampled to a final population size on day 4 of 10^5 CFU g^{-1} spinach leaf. On ready to eat spinach, population size rose steadily every day until a final population size of ~$10^{6.5}$ CFU g^{-1} spinach leaf. EtOH treated leaves saw a larger increase every day meaning that the final *L. monocytogenes* population size on day 4 was ~10^7 CFU g^{-1} spinach leaf.

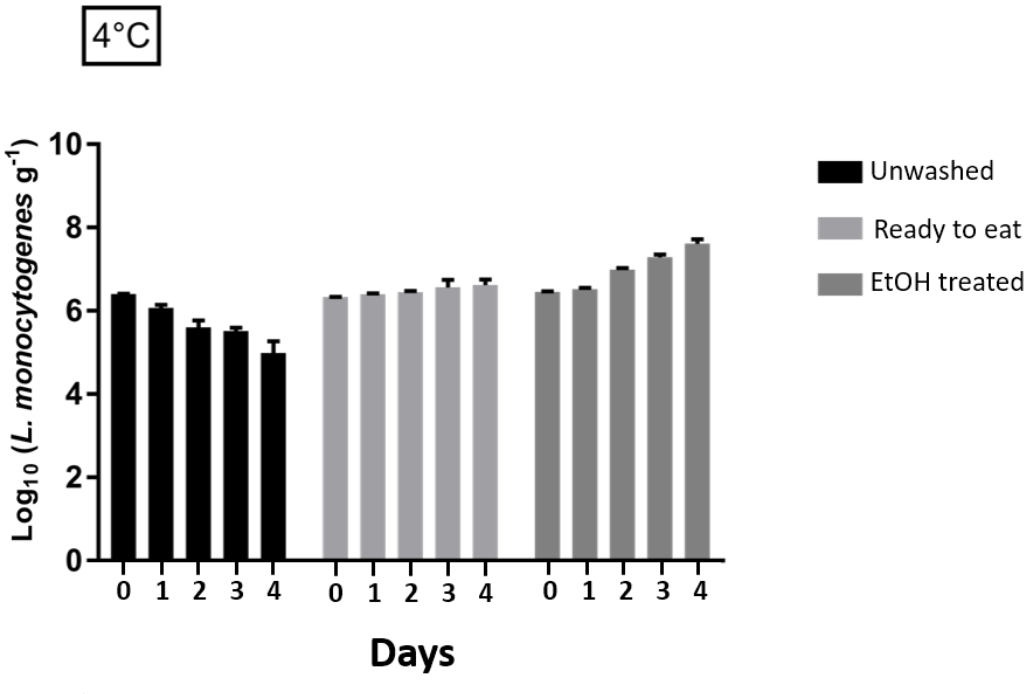

Figure 5.12. ***L. monocytogenes*** **survival on spinach leaves from 3 different treatments at 4°C**. Black bars represent unwashed spinach, light grey bars represent ready to eat spinach and dark grey bars represent EtOH treated spinach. Error bars represent the SEM of 3 independent replicates (n = 3).

5.3.4.2. Monitoring survival of *L. monocytogenes* on spinach washed using industry standard practices

To determine the effect of an industry standard chlorine wash on culturable bacteria on spinach leaves, leaves were mixed (stomached) with PBS before plating appropriate dilutions on nutrient agar. Plates were left to incubate at 20°C for 48h. Total culturable bacteria (CFU g^{-1} spinach leaf) was significantly ($P < 0.001$) higher on average on unwashed leaves (Figure 5.13). The chlorine wash reduced total bacterial populations by ~1 Log.

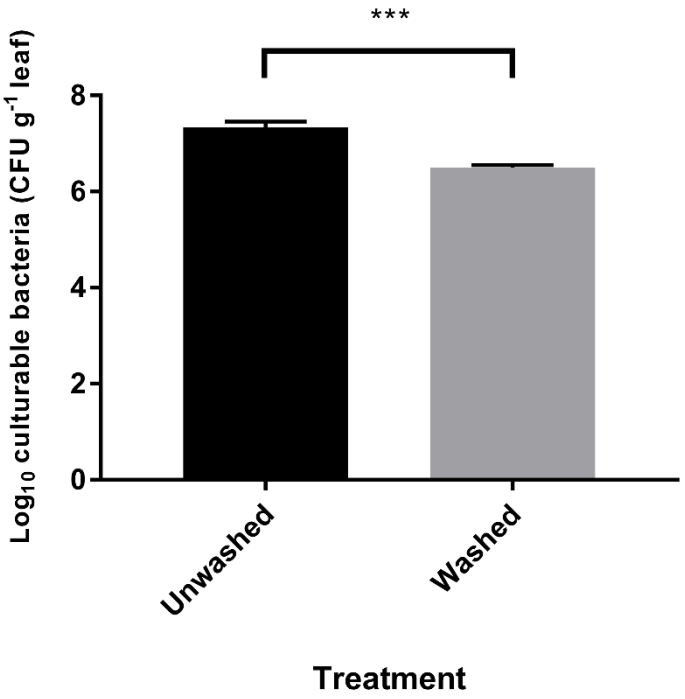

Figure 5.13. **Number of culturable bacteria on washed and unwashed spinach leaves. *** indicates significant difference according to T test. Error bars show the SEM of 3 independent replicates (n = 3).

Survival of *L. monocytogenes* populations from an initial number of ~10^6 CFU g^{-1} spinach leaf were monitored over 4 days on washed and unwashed spinach leaves. This process was repeated at two different temperatures. At 20°C (Figure 5.14.), the *L. monocytogenes* population on washed spinach leaves reached a maximum size of 7.6 Logs after 3 days before slightly reducing after on day 4. On unwashed spinach, the population grew steadily until 4 days, reaching a final population size of 6.8 Logs.

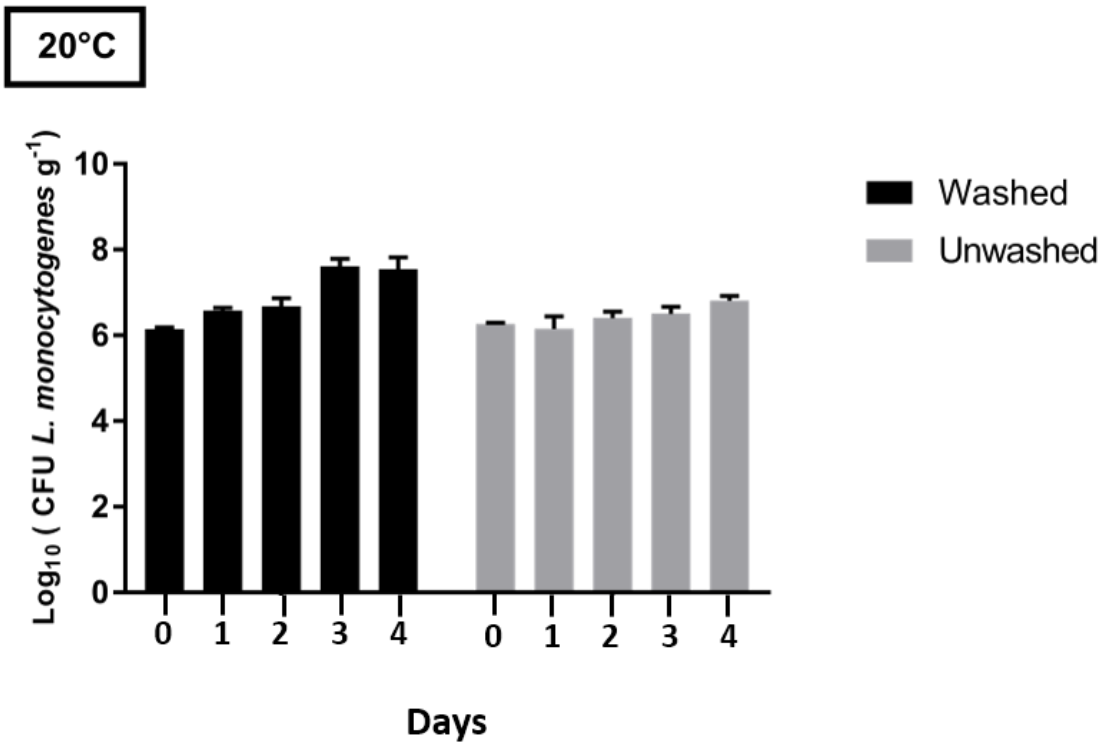

Figure 5.14. **Survival of *L. monocytogenes* on washed and unwashed spinach leaves (using industry standard practices) over 4 days at 20°C.** Error bars show the SEM of 3 independent replicates (n = 3).

At 4°C, the (Figure 5.15.), the *L. monocytogenes* population on unwashed spinach remained at the inoculum size until day 3 where the population dropped before increasing slightly on day 4. On washed spinach, the population maintained its size for the duration of the experiment.

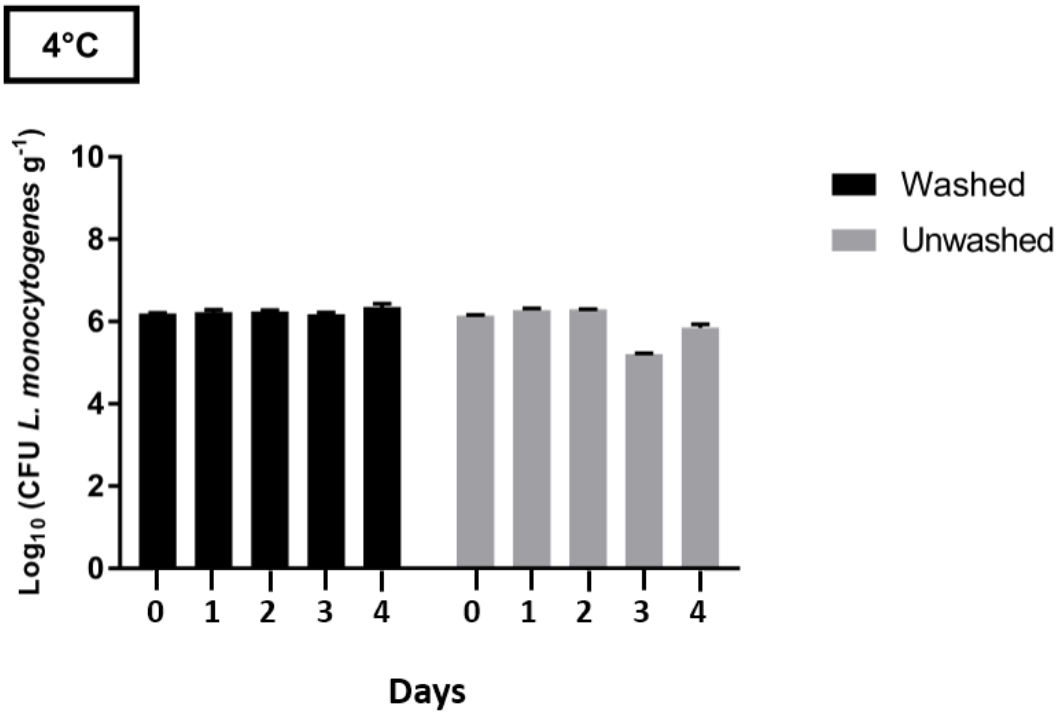

Figure 5.15. **Survival of *L. monocytogenes* on washed and unwashed spinach leaves (using industry standard practices) over 4 days at 4°C.** Error bars show the SEM of 3 independent replicates (n = 3).

5.4. Discussion

5.4.1. Factors affecting L. monocytogenes on spinach spoilt with contaminated soil

Results from this chapter suggest that soil contamination of spinach leaves is a viable route of contamination for *L. monocytogenes*. In these experiments, spinach leaves were artificially spoiled by mixing with contaminated soil but this process has been shown to occur in naturally in 'field' experiments through water/soil splash (Girardin et al. 2005). This experiment demonstrated that the factors of; inoculum size, level of soil contamination and time that inoculum has spent in soil all significantly affect the frequency of *L. monocytogenes* detection when using industry standard protocols for detection of *L. monocytogenes* on foodstuffs. As would be expected, a higher inoculum size and higher level of soil contamination resulted in more positive detections while the frequency of detections went down the longer the inoculum spent in the soil.

Although soil splash is a viable route of contamination for *L. monocytogenes* on to fresh leafy produce, the number of positive *L. monocytogenes* detections from this

type of contamination may be low due to the decline in *L. monocytogenes* populations in fresh soil (as seen in survival experiments in Chapter 4) and lack of global (e.g. field wide) contamination events. In previous research assessing the prevalence and number of *L. monocytogenes* across natural soil samples (Locatelli et al. 2013a), it was shown that *L. monocytogenes* was detected in 17% of soils by the selective enrichment method but not by qPCR (detection limit of 10^4 CFU g^{-1}) suggesting that the initial number of *L. monocytogenes* was low in soil. Although soil splash may be common in the field, this low incidence and number suggests that the *L. monocytogenes* contamination risk from this source is low. Moreover, the likelihood that a field-wide contamination event that could transfer a high inoculum to a large part of a production field (and subsequently contaminate a high volume of crop) is low too. In part, this is due to regulations discussed previously which, for example, prevent the watering of crops by irrigation and use of animal manure as fertiliser for 3-years prior to the growing of produce for human consumption. However, it is possible that contamination of the soil with a high inoculum could arise from sources such as wild animal droppings (Smith et al. 2018) and contaminate fresh produce sporadically. It is also true that even if a small number of *L. monocytogenes* are transferred through soil contamination, this population could potentially grow larger on fresh produce such as spinach during storage and transport.

5.4.2. The effect of chlorine wash and electrolysed water wash on *L. monocytogenes* populations on spinach leaves

Results from washing artificially contaminated spinach with different levels (0, 20, 40, & 60ppm) of free chlorine in tap water showed that only a 40 & 60ppm chlorine wash significantly reduced *L. monocytogenes* populations on leaves 24h post-contamination. 60ppm is the current acceptable highest concentration for free chlorine in wash water to avoid damage to more sensitive leaves (spinach, rocket, etc.) and excess chlorine residue. Whilst 60ppm chlorinated water was effective at reducing the *L. monocytogenes* population on the leaf compared to tap water (0ppm) after 24h post-contamination, results showed that the efficacy of this wash fell after 48h suggesting the importance washing fresh produce as soon as possible post-contamination. Tap water and 60ppm chlorinated water had the same sanitation efficacy after 1h post-contamination further demonstrating the importance of washing spinach quickly. In

summary, the faster produce is washed post contamination, the more effective the wash step is, regardless of whether tap water or chlorinated water is used.

Control of pathogenic bacteria including *L. monocytogenes*, on the leaf surface, is not the primary function of chlorinated water in the wash step. Instead this is a secondary effect, as the primary use of chlorinated water is to stop growth of pathogenic bacteria in wash water between changing water in the tanks (Gil et al. 2009). Based on its popularity, cost and effectiveness the importance of chlorine as a sanitiser is clear. However, some processors choose to use tap water instead of chlorinated water as part of their wash step for reasons including; concern over the chlorine residue on leaves, impact on the environment and cost. Processors which follow this procedure are giving up the clear advantages chlorinated water gives in terms of sanitation of fresh produce. Looking forward, the EU's project to phase out the use of chlorinated water (citing similar concerns to stakeholders in the UK FLPSC) will mean processors will have to find an alternative, effective sanitiser in the near future.

Electrolysed water is a promising alternative to chlorinated water due to its efficacy as a sanitiser and cost, as well as maintaining the sensory quality of fresh produce (Kaczmarek et al. 2019). However, as shown in this chapter, at accepted levels of free chlorine (60ppm) in the wash water combined with operational times for washing spinach, the electrolysed water wash was ineffective at reducing *L. monocytogenes* populations. Further research is needed to establish 'safe' levels of free chlorine in electrolysed water as well as the efficacy of higher strength (in terms of free chlorine content) electrolysed water on human pathogenic bacteria on food.

5.4.3. The effect of chlorine wash on *L. monocytogenes* survival in a 'post-wash' contamination scenario

In the pilot study monitoring populations of *L. monocytogenes* in a 'post-wash' contamination scenario, the number of culturable bacteria on RTE leaves was reduced compared to unwashed leaves and the lowest number of bacteria were cultured on leaves treated in EtOH. Interestingly, while EtOH treatment reduced the number of cultured bacteria from spinach leaves, RTE leaves showed the least diverse microbial communities (Figures 5.9. and 5.10.). Unwashed leaves both had the highest number of culturable bacteria and the most diverse microbial communities. While monitoring

L. monocytogenes populations on artificially inoculated spinach leaves at room temperature (20°C), *L. monocytogenes* populations on both unwashed and ready to eat leaves rose slightly to around the same level (from ~6 - ~8 Logs) while populations on EtOH treated leaves rose dramatically in comparison. This could be due to the damaging effect that the treatment had on spinach leaves meaning that more substrate (leaf exudates etc.) was available for the population to grow. These results demonstrate that regardless of treatment, if spinach is contaminated post-wash and kept at room temperature then the *L. monocytogenes* population grows to a higher level. These results demonstrate the importance of refrigerating produce in terms of reducing the risk of pathogenic bacteria to the consumer.

Interestingly, on artificially contaminated leaves which were incubated at refrigeration temperature (4°C), populations of *L. monocytogenes* rose slightly (by ~1Log) on ready to eat leaves but dropped on unwashed leaves (by ~1Log). These data suggest that the indigenous leaf microbiota, which was more diverse and more numerous compared to leaves from other treatments, have a suppressive effect on *L. monocytogenes* populations on spinach leaves. Further, that RTE spinach has an innate microbial community which is less diverse and numerous than unwashed spinach due to the wash-step.

Finally, in this chapter, spinach was washed in house using industry standard methods, and results showed that chlorine wash significantly reduced the number of culturable bacteria on leaves. Results showed that the suppressive effect of the innate microbiota on unwashed leaves on *L. monocytogenes* survival was less pronounced than in the pilot study. However, at both room and refrigeration temperature, unwashed leaves yielded the lowest *L. monocytogenes* populations between 2-4 days compared to leaves washed in chlorinated water. Whilst these results don't completely coincide with the pilot study (*L. monocytogenes* population on unwashed leaves in the pilot study reduced more), it could be that specific inhibitory bacterial species are needed to suppress *L. monocytogenes* populations. In this respect, bacteria that outcompete *L. monocytogenes*, like the ones isolated from enrichment broths in earlier chapters may be important.

5.5. Conclusion

Experiments testing the effect of various factors on *L. monocytogenes* detection rates showed that soil is a potential source of *L. monocytogenes* and be subsequently detected on leaves. Chlorine was is an effective sanitiser, as shown in this chapter, and cost effective. However, there are still problems with using this step in the fresh produce supply chain. These include many environmental and public health concerns. In addition, new evidence has come to light which suggests that chlorine wash triggers pathogenic bacteria to enter a protective, VBNC state instead of destroying them (Highmore et al. 2018). This chapter has also shown that while the chlorine wash at operational levels for the UK FLPSC is an effective sanitiser, it can cause *L. monocytogenes* populations to grow to a higher level on spinach leaves possibly by reducing the number and diversity of the microbial community on the leaf. This raises the 'unlikely' idea that it may be better in terms of public health (and more cost effective) for processors to leave fresh produce products like spinach, unwashed, still containing an unaltered microbial community that is able to suppress subsequent *L. monocytogenes* growth on the leaf surface.

Overall, it is clear that research is urgently needed into cheap and effective sanitisers to replace the chlorine wash, which is likely to be phased out soon due to previously discussed concerns (European Commission 2014).

Chapter 6 General discussion

6.1. Introduction

Listeria monocytogenes remains a serious threat to public health in the UK and abroad. The bacterium is the causative agent of listeriosis, an intracellular disease which predominantly affects immunocompromised people such as the elderly and those undergoing treatment for terminal illnesses. Contaminated foodstuffs are the main cause of infection, as evidenced by many previous outbreaks (Salamina et al. 1996; Makino et al. 2005; Pichler et al. 2011; McCollum et al. 2013; Garner and Kathariou 2016). To emphasise the risk from this pathogen, as recently as August 2019, 6 people died from eating contaminated food in hospitals in England. The source of the contamination for this outbreak has since been identified as the production facility where the foods were processed. Outbreaks of disease occur despite legislation from governing bodies designed to minimise the risk of *L. monocytogenes* contaminating foods.

One food group that is particularly vulnerable to *L. monocytogenes* contamination is fresh produce. There are two main reasons for this, 1) this food group is not cooked before consumption, preventing destruction of the bacteria. 2) the UK supply chain for fresh produce is complex, involving many stakeholders including growers, processers and retailers which means that within the supply chain there are numerous potential sources of the bacterium (including soil, animal sources, the processing environment etc.) (Smith et al. 2018). Additionally, *L. monocytogenes* exhibits a complex stress response and mechanisms such as biofilm formation, cold tolerance, resistance to physiochemical stresses and the ability to turn VBNC ensure it has a more robust cross-stress tolerance compared to other foodborne pathogens. As a result, *L. monocytogenes* can survive in many environments associated with the fresh produce supply chain including soil, the product surface and the processing environment. Furthermore, among foodborne pathogens (such as *E. coli, Salmonella*), *L. monocytogenes* has a high rate of fatality from infection. For these reasons, *L. monocytogenes* should not be considered by regulatory bodies in the food industry in the same way as other food-borne pathogens with regards to contamination prevention and control. Instead, comparatively stronger measures relating to

contamination of food and food associated environments are employed to control its presence.

Due to the risk from *L. monocytogenes* and its ability to survive, the UK FLPSC is subject to heavy internal and external regulatory pressure to minimise contamination from *L. monocytogenes*. Steps such as washing produce before going out to retail and regular cleaning and sanitation of processing facilities are some examples of the methods that stakeholders in the FLPSC use to control *L. monocytogenes* (and other pathogenic bacteria) contamination. Not only is contamination of produce by *L. monocytogenes* a risk to public health, but stakeholders in the supply chain stand to lose business value if an outbreak occurs due to a drop in consumer confidence (McCollum et al. 2013). However, despite strict regulations, *L. monocytogenes* is sporadically detected through obligatory microbial monitoring schemes undertaken by growers and processors. This is unsurprising due to the number of potential sources of *L. monocytogenes* in the supply chain, but sampling regimes are often irregular, non-targeted and only characterise the bacteria down to the species level. Characterising the bacteria in this way gives little information and importantly, does not allow investigators in the supply chain to accurately assess the relatedness of isolates, an essential step in source tracking *L. monocytogenes* which has been employed, successfully, in outbreak situations. Moreover, the current detection methods give no indication of the risk different isolates pose (e.g. whether they are closely related to outbreak strains and have the necessary genetic components to cause disease).

Therefore, the first step of this study was to collect a suite of *L. monocytogenes* strains isolated from different locations in the UK FLPSC and confirm that they were *L. monocytogenes* using molecular methods. Next, these strains were subject to MLST to determine if the strains were different, or not, and this information was combined with information from selected phenotypic tests including monitoring the ability of isolates to form biofilm. Results from MLST showed a genetically diverse population exists in the UK FLPSC, but some isolates could not be distinguished by this method. Therefore, a more accurate method of characterising the strains was used (WGS), which also allowed for elucidation of pathogenic profiles of strains based on the presence of key virulence genes. Despite the relatively small number of strains used for this purpose (15 in this study), results showed indistinguishable isolates (indicative

of cross-contamination) and showed isolates had the necessary genetic components for disease. This information is valuable to stakeholders in the FLPSC and indicates the promising potential of the technology to be used in food supply chains. Following this, the survival of selected strains was assessed in commonly used horticultural soils to help inform growers of the risk of different soil types. Finally, the impact of the wash-step evaluated on *L. monocytogenes* populations on spinach leaves in pre and post-wash contamination scenarios. The following sections discuss these individual aspects in more detail.

6.2. The *L. monocytogenes* population in the UK fresh produce supply chain is genetically diverse and isolates exhibit variability in phenotypic traits

All strains in this work were identified as *L. monocytogenes* by a species-specific PCR. MLST analysis revealed a diverse population, spanning two lineages (I & II). This observation is in keeping with other research that has focussed on using MLST to determine genetic diversity of *L. monocytogenes* in the food supply chain and the environment (Wu et al. 2016; Jennison et al. 2017). Some identified sequence types have been associated with recent outbreaks (e.g. NLmo20, ST6) whilst others are not often associated with outbreaks (isolates from lineage II). This traditional method of MLST showed 4 sets of isolates which could not be distinguished and lacked the discriminatory power to accurately distinguish between closely related isolates due to the size of sequence being analysed.

Different strains exhibited differences in the phenotypic characteristics tested. Biofilm production is thought to contribute to the persistence and spread of *L. monocytogenes* in food supply chains (Colagiorgi et al. 2017) and isolates in this study varied in their ability to produce biofilm. In this respect it could be argued that isolates which produced more biofilm have an increased chance of persisting in the FLPSC. However, evidence from this work has shown that biofilm production is affected by environmental variables such as temperature meaning that a strong biofilm former in this study may not necessarily be a strong biofilm former under different conditions (such as those encountered in the FLPSC). Furthermore, there is conflicting evidence as to whether high biofilm production is an indicator of persistence (Borucki et al. 2003; Nowak et al. 2017). In biofilm production, isolates of the same sequence type did not exhibit similar phenotypic characteristics *per se*. The same is true for the other

phenotypic traits tested in this work. Interestingly, in growth rate experiments, some strains showed a high degree of variability between replicates, which could indicate phenotypic heterogeneity within the genetically homogeneous population. However, these results are preliminary and require further investigation.

Overall, phenotypic characterisation methods were satisfactory to determine differences between isolates and MLST provided a genetic context. However, as discussed there are difficulties relating phenotypic traits to survivability/persistence of *L. monocytogenes* in the context of the fresh produce supply chain.

6.3. Should whole genome sequencing be used to monitor *L. monocytogenes* contamination in the fresh produce supply chain?

MLST is a useful technique for determining the relatedness of *L. monocytogenes* strains in a broad sense but it lack the discriminatory power needed for source tracking the bacterium. Furthermore, MLST gives no information on the presence and intactness of virulence genes, which can be used to provide a pathogenic profile of strains. This information is important for inferring the potential risk that isolates pose to consumers and in highlighting persistent strains. Understanding these aspects of *L. monocytogenes* ecology in food supply chains can help regulators and operators to design more effective microbial surveillance and prevention strategies and inform risk assessments. WGS is now the gold standard technology for characterising bacteria genetically and provides accurate assessment of relatedness between different isolates and information on virulence genes.

While it is difficult to draw general conclusions about the whole UK fresh produce supply chain due to the limited number of strains used in this work, these data provide a preliminary insight into the diversity of the *L. monocytogenes* population and virulence potential of *L. monocytogenes* directly isolated from the UK fresh produce supply chain. This study showed possible evidence of cross contamination in the supply chain (indistinguishable strains) but recognises that the determination of a specific source of *L. monocytogenes* would require sampling over a longer time period, with a more targeted sampling plan. Such a plan may be currently beyond the scope (in terms of financial investment and technical expertise) of stakeholders in the UK FLPSC. Additionally, this study demonstrated the power of WGS in outlining the pathogenic

profile of isolates by showing the presence of key virulence factors required to cause disease (discussed in more detail in section 3.5.).

Overall, the *L. monocytogenes* population in the UK fresh produce supply chain is diverse, in line with food isolates other countries such as Ireland (Hilliard et al. 2018), France (Moura et al. 2017), and Australia (Kwong et al. 2015). While some isolates have the necessary genomic components to cause disease and are closely related to outbreak strains, others are distantly related and are of less concern. In terms of the fresh produce supply chain, a more targeted sampling plan is needed to determine whether potentially virulent strains are sporadic (i.e. infrequently isolated) or persistent in each environment. This is particularly important in environments that have the potential to contaminate produce on a broad scale (such as processing environments). Therefore, in terms of policy, fresh produce supply chains should begin to phase in sampling regimes that, implement WGS as standard. Implementing this technology may give customers (retail) and consumers added confidence that growers and processors are informed of the potential risk of *L. monocytogenes* contamination in their supply chain and demonstrates a precautionary, rather than reactionary approach to consumer safety. This is the first work of its kind in the UK and demonstrates the power of WGS as a subtyping tool for *L. monocytogenes* isolates. More efforts are needed to implement this technology as routine surveillance in the UK, these efforts should include designing appropriate sampling strategies and streamlining protocols for microbial testing of food products.

6.4. The risk from *L. monocytogenes* contaminated soil in the fresh produce supply chain

L. monocytogenes strains isolated from the UK FLPSC survived (from an initial inoculum of 10^8 CFU g^{-1}) in different soil types (obtained from farms used to grow leafy produce) for more than 70 days. In the short term (70 days), survival was best in clay loam soil compared to silty clay and sandy loam soils. This result is in agreement with other studies which have investigated *L. monocytogenes* survival in soils, that *L. monocytogenes* survives better in clay type soils compared to other soils (Dowe et al. 1997; Vivant et al. 2013). It has been proposed that this effect is due to clay type (finer) particles being able to harbour more negatively charged particles, meaning that soils of this type contain more base cations essential for bacterial life (Locatelli et al.

2013b). However, over a long time period (256 days), *L. monocytogenes* was detected at a higher level (relative abundance) in sandy loam soil than clay or silty clay loam soils. 16S rRNA analysis also revealed that sandy loam soil had a lower microbial diversity than the other soils tested which may be beneficial for *L. monocytogenes* survival in the long term. This is due to the suppressive effect that endogenous soil microbial populations have on *L. monocytogenes* survival, as shown in this work and in agreement with other authors (McLaughlin et al. 2011; Falardeau et al. 2018). On the other hand, it could also be argued that the lower microbial diversity in sandy loam soil meant that *L. monocytogenes* represented a higher proportion of the microbial population while in clay loam soil, *L. monocytogenes* represented a smaller proportion, simply because the indigenous microbial population was more diverse and abundant. Thus, whether *L. monocytogenes* survived at a higher rate or was simply present at a higher relative abundance in soil due to a less diverse microbial community remains an open question.

During attempts at quantification of *L. monocytogenes* by the MPN-enrichment of fresh soil, competitor organisms growing in enrichment broth meant that *L. monocytogenes* survival in soil could not be quantified in fresh soils after 70 days. This observation may suggest complications with the enrichment process during the normal sampling of fresh produce for *L. monocytogenes.* For example, if product that has been contaminated with soil is sampled, soil-borne bacterial competitors may outgrow *L. monocytogenes* in the enrichment broth, generating a false negative result. While *L. monocytogenes* in fresh (fresh) soil microcosms could not be quantified by this method, MPN-enrichment quantified *L. monocytogenes* in sterilised soil microcosms at a higher number than direct plate counts. This result suggests that long incubation in the soil environment may be causing a portion of the *L. monocytogenes* population to turn VBNC similar to incubation in manure microcosms (Desneux et al. 2016). However, this is a preliminary result and, needs to be confirmed. In general, the viable count of *L. monocytogenes* in soil microcosms declined quickly over 70 days and to a low (often undetectable) level after 256 days. So, while contamination via this route is possible, results suggest that contamination of fresh leafy produce by soil borne bacteria is not likely to be a high risk to consumers. However, it should be noted that individuals can become ill even after consuming low levels of the bacteria and while

infection from low levels of soil-borne bacteria is unlikely based on the low levels of *L. monoytogenes* that survive for extended periods, it is not possible to completely rule out infection via this route.

6.5. The impact of the wash step on *L. monocytogenes* populations on spinach leaves in pre- and post-wash contamination scenarios

In this work it was shown that *L. monocytogenes* can survive in different horticultural soil types for extended periods. This means that soil is a potential contamination route for *L. monocytogenes* onto fresh produce due to incidences of soils splash during the growing process. Factors that affect detections of *L. monocytogenes* via this route (from soil) were investigated and showed that a higher inoculum size and higher amount of soil increase detections but the longer the initial inoculum was in soil, the frequency of detection became less as *L. monocytogenes* numbers decreased. As previously discussed, the likelihood of a field-wide contamination event, which could transfer a high inoculum to a large part of a production field (and subsequently contaminate a high volume of crop) is low. This is, in part due to regulations concerning the growing of fresh produce in the UK. However, it is possible that contamination of the soil with a high inoculum could arise from sources like wild animal droppings (Inoue et al. 1992; Hellström et al. 2008) and contaminate fresh produce sporadically.

To combat this kind of contamination, and to help remove other dirt and debris, retailers can request that their produce is washed by a dedicated facility. The washing process also means that the product can be sold at a higher price. Commonly in the UK, chlorine is the sanitiser that is employed in the wash step. This work has shown that the sanitising effect of chlorine increased with increasing concentrations in wash water, up to the maximum accepted operating value for chlorine wash to be used with spinach leaves (60ppm) in a pre-wash contamination scenario. As time passed post-contamination, the chlorine wash was less effective. Based on EU directives, chlorine wash is due to be phased out of supply chains, which presents a challenge for processers as this sanitiser is cheap and effective and alternatives are not readily available. Electrolysed water is a promising alternative to chlorinated water due to its efficacy as a sanitiser and cost, as well as maintaining the sensory quality of fresh produce. Furthermore, electrolysed water supposedly leaves lower residues of

trihalomethanes (THMs) on leaves (Kaczmarek et al. 2019) (THMs have been associated with negative health effects such as cancer (Hood 2005). However, at accepted levels of free chlorine (60ppm) in the wash water combined with operational times for washing spinach, the electrolysed water wash was ineffective at reducing *L. monocytogenes* populations. Further research is needed to establish 'safe' levels of free chlorine in electrolysed water as well as the efficacy of higher strength (in terms of free chlorine content) electrolysed water on human pathogenic bacteria on food.

This work has shown that on ready to eat (washed) spinach, *L. monocytogenes* survives better (at a higher rate) than on leaves that have not been washed. Results showed that ready to eat spinach had a lower microbial diversity and number compared to unwashed spinach leaves and it could be argued that the more diverse leaf microbiota of unwashed leaves had a suppressive effect on *L. monocytogenes* populations added to the leaf compared to washed spinach. This effect was less pronounced when washing spinach using industry standard protocols. However, at both room and refrigeration temperature, unwashed leaves yielded the lowest *L. monocytogenes* populations between 2-4 days compared to leaves washed in chlorinated water. More work needs to be done to investigate the impact of the wash step on microbial populations on fresh produce and subsequently how this affects survival of *L. monocytogenes* over time.

6.6. Conclusions and future perspectives

In this work, 15 *L. monocytogenes* from the fresh leafy produce supply chain were characterised using selected phenotypic and genetic tests. Phenotypic tests showed variability between strains. Whole genome sequencing of *L. monocytogenes* isolates provided valuable information in terms of relatedness and potential pathogenicity which can be used to inform the UK fresh produce supply chain of the risk that the *L. monocytogenes* population present poses to consumers. Additionally, this information was used to infer incidences of cross contamination within the supply chain. To the best of my knowledge, this is the only study of its kind to do so in the UK and has shown that WGS technology may be a viable method for characterising pathogenic bacteria in the future due to the added information it provides over traditional methods. The WGS platform can be used as a source tracking tool in the UK FLPSC but would require substantial investment in terms of finance and time to implement.

However, as the cost of the NGS technology comes down, it will become more plausible for companies to do this. Moreover, use of the technology requires in depth knowledge of the supply chain being investigated and inferring the incidences of direction of cross contamination can be difficult.

Additionally, this work has shown that *L. monocytogenes* can survive for extended periods in horticultural soils commercially used to produce leafy produce, meaning that contamination from this source is possible. However, populations in soil decline quickly meaning that contamination from soil is likely low risk to consumers. On the other hand, *L. monocytogenes* can survive over the typical growing period demonstrating that this source of contamination is a risk within the fresh produce supply chain. The chlorine wash is an effective sanitiser for use in pre-wash contamination scenarios but may aid *L. monocytogenes* survival in post-wash contamination scenarios e.g. contamination from *L. monocytogenes* harbouring on equipment surfaces. Results from this work suggest this effect may be due to reduction in endogenous leaf microbial diversity and number after the chlorine wash. As chlorine is phased out (based on EU directives), more work is needed to look for effective alternatives. Furthermore research is needed to determine the impact of these sanitiser washes on indigenous leaf microbiota, and whether it may be more beneficial to public health and the environment (in terms of prevent growth of *L. monocytogenes*) to stop washing produce before it reaches the consumer, although this approach would currently be considered controversial.

6.7. Recommendations for future research

- A more rigorous and targeted sampling plan to test for *L. monocytogenes* should be applied to the UK fresh produce supply chain to prevent outbreaks
    - Sampling plans should focus on supply chain pinch points such as processing facilities

- WGS platforms should be developed to track *L. monocytogenes* in the UK fresh produce supply chain. The same method can be applied to tracking other important human pathogens in food supply chains

- Future research should focus on methods to increase microbial diversity and number in horticultural soils. Evidence from this, and other work has shown the suppressive effect of endogenous soil microbial populations on survival of *L. monocytogenes* and other pathogenic bacteria

- An understudied aspect of *L. monocytogenes* physiology is the VBNC state. This whole area is interesting and requires further research as the VBNC state could be aiding survival and persistence of *L. monocytogenes* in food supply chains.

- Research should investigate the role of leaf microbiota to reduce *L. monocytogenes* growth during processing e.g. add known (and safe for humans) *L. monocytogenes* competitors to leaves

References

Ackermann, M. (2015) A functional perspective on phenotypic heterogeneity in microorganisms. *Nat Rev Microbiol* **13**:497–508. doi: 10.1038/nrmicro3491

Agricultural Development and Advisory Service. (1986) Analysis of Agricultural Materials: manual of the analytical methods used by the Agricultural Development and Advisory Service., 3rd edn. London: HMSO

Allende, A., Artes, F. (2005) Processing lines and alternative preservation techniques to prolong the shelf- life of minimally fresh processed leafy vegetables. *Eur J Hortic Sci* **70**:4

Allende, A., Monaghan, J. (2015) Irrigation water quality for leafy crops: A perspective of risks and potential solutions. *Int J Environ Res Public Health* **12**:7457–7477. doi: 10.3390/ijerph120707457

Allende, A., Selma, M. V., López-Gálvez, F., *et al*. (2008) Role of commercial sanitizers and washing systems on epiphytic microorganisms and sensory quality of fresh-cut escarole and lettuce. *Postharvest Biol Technol* **49**:155–163. doi: 10.1016/j.postharvbio.2007.12.010

Allerberger, F., Wagner, M. (2010) Listeriosis: A resurgent foodborne infection. *Clin Microbiol Infect* **16**:16–23. doi: 10.1111/j.1469-0691.2009.03109.x

Alonzo, F., Xayarath, B., Whisstock, J.C., Freitag, N.E. (2011) Functional analysis of the *Listeria monocytogenes* secretion chaperone PrsA2 and its multiple contributions to bacterial virulence. *Mol Microbiol* **80**:1530–1548. doi: 10.1111/j.1365-2958.2011.07665.x

Altschul, S.F., Gish, W., Miller, W., *et al*. (1990) Basic Local Alignment Search Tool. *J Mol Biol* **215**:403–410. doi: 10.1016/S0022-2836(05)80360-2

Álvarez-Ordóñez, A., Leong, D., Hickey, B., *et al*. (2015) The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document. *Food Res Int* **75**:233–243. doi: 10.1016/j.foodres.2015.06.004

Aravena, A., Maza, F., Marquez-Fontt, B., *et al*. (2016) Different Transcriptional Responses from Slow and Fast Growth Rate Strains of *Listeria monocytogenes* Adapted to Low Temperature. *Front Microbiol* **7**:1–9. doi: 10.3389/fmicb.2016.00229

Asano, K., Nakane, A., Hu, D.-L., *et al*. (2013) Fibronectin-binding protein, FbpA, is the adhesin responsible for pathogenesis of *Listeria monocytogenes* infection. *Microbiol Immunol* **57**:253–262. doi: 10.1111/1348-0421.12030

Asano, K., Sashinami, H., Osanai, A., *et al*. (2011) Autolysin amidase of *Listeria monocytogenes* promotes efficient colonization of mouse hepatocytes and enhances host immune response. *Int J Med Microbiol* **301**:480–487. doi: 10.1016/j.ijmm.2011.01.002

Aubry, C., Goulard, C., Nahori, M.A., *et al*. (2011) OatA, a peptidoglycan O-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence. *J Infect Dis* **204**:731–740. doi: 10.1093/infdis/jir396

Ayrapetyan, M., Oliver, J.D. (2016) The viable but non-culturable state and its relevance in food safety. *Curr Opin Food Sci* **8**:127–133. doi: 10.1016/j.cofs.2016.04.010

Balestrino, D., Nahori, M.-A., Villiers, V., *et al*. (2010) The *Listeria monocytogenes* InlC protein interferes with innate immune responses by targeting the I B kinase subunit IKK. *Proc Natl Acad Sci* **107**:17333–17338. doi: 10.1073/pnas.1007765107

Barbosa, W.B., Cabedo, L., Wederquist, H.J., *et al*. (1994) Growth Variation Among Species and Strains of *Listeria* in Culture Broth. *J Food Prot* **57**:765–769. doi: 10.4315/0362-028x-57.9.765

Berche, P., Escuyer, V., Nguyen, L., *et al*. (1999) ClpE, a novel member of the HSP100 family, is involved in cell division and virulence of *Listeria monocytogenes*. *Mol Microbiol* **31**:185–196. doi: 10.1046/j.1365-2958.1999.01159.x

Berrang, M.E., Brackett, R.E., Beuchat, L.R. (1989) Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. *J Food Prot* **52**:702–705

Beuchat, L.R., Brackett, R.E. (1990) Survival and Growth of *Listeria monocytogenes* on

Lettuce as Influenced by Shredding, Chlorine Treatment, Modified Atmosphere Packaging and Temperature. *J Food Sci* **55**:755–758. doi: 10.1111/j.1365-2621.1990.tb05222.x

Bierne, H., Cossart, P. (2002) InlB, a surface protein of *Listeria monocytogenes* that behaves as an invasin and a growth factor. *J Cell Sci* **115**:3357–3367

Borezée, E., Pellegrini, E., Beretti, J.L., Berche, P. (2001) SvpA, a novel surface virulence-associated protein required for intracellular survival of *Listeria monocytogenes*. *Microbiology* **147**:2913–2923. doi: 10.1099/00221287-147-11-2913

Borucki, M.K., Call, D.R. (2003) *Listeria monocytogenes* Serotype Identification by PCR. **41**:5537–5540. doi: 10.1128/JCM.41.12.5537

Borucki, M.K., Peppin, J.D., White, D., *et al*. (2003) Variation in biofilm formation among strains of *Listeria monocytogenes*. *Appl Environ Microbiol* **69**:7336–7342. doi: 10.1128/AEM.69.12.7336

Braid, M.D., Daniels, L.M., Kitts, C.L. (2003) Removal of PCR inhibitors from soil DNA by chemical flocculation. *J Microbiol Methods* **52**:389–393. doi: 10.1016/S0167-7012(02)00210-5

Bregenholt, S., Jaubert, F., Gaillot, O., *et al*. (2001) Stress-Induced ClpP Serine Protease of *Listeria monocytogenes* Is Essential for Induction of Listeriolysin O-Dependent Protective Immunity. *Infect Immun* **69**:4938–4943. doi: 10.1128/iai.69.8.4938-4943.2001

Bruce, J.B., West, S.A., Griffin, A.S. (2017) Bacteriocins and the assembly of natural *Pseudomonas fluorescens* populations. *J Evol Biol* **30**:352–360. doi: 10.1111/jeb.13010

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., *et al*. (2017) A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control* **75**:1–13. doi: 10.1016/j.foodcont.2016.12.016

Burkholder, K.M., Bhunia, A.K. (2010) *Listeria monocytogenes* uses Listeria adhesion protein (LAP) to promote bacterial transepithelial translocation and induces

expression of LAP receptor Hsp60. *Infect Immun* **78**:5062–5073. doi: 10.1128/IAI.00516-10

Busch, S. V., Donnelly, C.W. (1992) Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and Listeria innocua. *Appl Environ Microbiol* **58**:14–20

Cabanes, D., Dussurget, O., Dehoux, P., Cossart, P. (2004) Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. *Mol Microbiol* **51**:1601–1614. doi: 10.1111/j.1365-2958.2003.03945.x

Cabanes, D., Sousa, S., Cebriá, A., *et al*. (2005) Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein. *EMBO J* **24**:2827–2838. doi: 10.1038/sj.emboj.7600750

Calderon-Gonzalez, R., Teran-Navarro, H., Marimon, J.M., *et al*. (2016) Biomarker tools to design clinical vaccines determined from a study of annual listeriosis incidence in northern Spain. *Front Immunol* **7**:1–9. doi: 10.3389/fimmu.2016.00541

Carpentier, B., Cerf, O. (2011) Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int J Food Microbiol* **145**:1–8. doi: 10.1016/j.ijfoodmicro.2011.01.005

Cartwright, E.J., Jackson, K.A., Johnson, S.D., *et al*. (2013) Listeriosis outbreaks and associated food vehicles, United States, 1998-2008. *Emerg Infect Dis* **19**:1–9. doi: 10.3201/eid1901.120393

Carvalho, F., Sousa, S., Cabanes, D. (2014) How *Listeria monocytogenes* organizes its surface for virulence. *Front Cell Infect Microbiol* **4**:1–22. doi: 10.3389/fcimb.2014.00048

Casey, A., Jordan, K., Coffey, A., *et al*. (2016) Comparative Genomic Analysis of Two Serotype 1/2b *Listeria monocytogenes* Isolates from Analogous Environmental Niches Demonstrates the Influence of Hypervariable Hotspots in Defining Pathogenesis. *Front Nutr* **3**:54. doi: 10.3389/fnut.2016.00054

CDC Centers for Disease Control and Prevention. (2016) FOOD Tool | CDC. In: CDC.gov

Chan, Y.C., Wiedmann, M. (2009) Physiology and genetics of *Listeria monocytogenes*

survival and growth at cold temperatures. *Crit Rev Food Sci Nutr* **49**:237–253. doi: 10.1080/10408390701856272

Chen, L., Zheng, D., Liu, B., *et al*. (2016a) VFDB 2016: Hierarchical and refined dataset for big data analysis - 10 years on. *Nucleic Acids Res* **44**:D694–D697. doi: 10.1093/nar/gkv1239

Chen, Y., Gonzalez-Escalona, N., Hammack, T.S., *et al*. (2016b) Core Genome Multilocus Sequence Typing for Identification of Globally Distributed Clonal Groups and Differentiation of Outbreak Strains of *Listeria monocytogenes*. *Appl Environ Microbiol* **82**:6258–6272. doi: 10.1128/AEM.01532-16

Cheng, C., Yang, Y., Dong, Z., *et al*. (2015) *Listeria monocytogenes* varies among strains to maintain intracellular pH homeostasis under stresses by different acids as analyzed by a high-throughput microplate-based fluorometry MATERIALS AND METHODS. **6**:1–10. doi: 10.3389/fmicb.2015.00015

Cheng, Y., Promadej, N., Kim, J.W., Kathariou, S. (2008) Teichoic acid glycosylation mediated by gtcA is required for phage adsorption and susceptibility of *Listeria monocytogenes* serotype 4b. *Appl Environ Microbiol* **74**:1653–1655. doi: 10.1128/AEM.01773-07

Chignard, M., Bouriotis, V., Cossart, P., *et al*. (2007) A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system . *Proc Natl Acad Sci* **104**:997–1002. doi: 10.1073/pnas.0609672104

Colagiorgi, A., Bruini, I., Di Ciccio, P.A., *et al*. (2017) *Listeria monocytogenes* Biofilms in the Wonderland of Food Industry. *Pathogens* **6**:41. doi: 10.3390/pathogens6030041

Colagiorgi, A., Di Ciccio, P., Zanardi, E., *et al*. (2016) A Look inside the *Listeria monocytogenes* Biofilms Extracellular Matrix. *Microorganisms* **4**:22. doi: 10.3390/microorganisms4030022

Cossart, P., Vicente, M.F., Mengaud, J., *et al*. (1989) Listeriolysin O is essential for virulence of *Listeria monocytogenes*: Direct evidence obtained by gene complementation. *Infect Immun* **57**:3629–3636

Dahl, V., Sundqvist, L., Hedenström, I., *et al*. (2017) A nationwide outbreak of listeriosis associated with cold-cuts, Sweden 2013-2014. *Infect Ecol Epidemiol* **7**:1324232. doi: 10.1080/20008686.2017.1324232

Dailey, R.C., Martin, K.G., Smiley, R.D. (2016) The effects of competition from non-patogenic foodborne bacteria during the selective enrichment of *Listeria monocytogenes* using buffered *Listeria* enrichment broth. *Food Microbiol* **44**:173–179. doi: 10.1016/j.fm.2014.05.004.The

den Bakker, H.C., Desjardins, C.A., Griggs, A.D., *et al*. (2013) Evolutionary Dynamics of the Accessory Genome of *Listeria monocytogenes*. *PLoS One* **8**:. doi: 10.1371/journal.pone.0067511

Desneux, J., Biscuit, A., Picard, S., Pourcher, A.M. (2016) Fate of viable but non-culturable *Listeria monocytogenes* in pig manure microcosms. *Front Microbiol* **7**:1–13. doi: 10.3389/fmicb.2016.00245

Dhariwal, A., Chong, J., Habib, S., *et al*. (2017) MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* **45**:W180–W188. doi: 10.1093/nar/gkx295

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

Doijad, S.P., Barbuddhe, S.B., Garg, S., *et al*. (2015) Biofilm-forming abilities of listeria monocytogenes serotypes isolated from different sources. *PLoS One* **10**:. doi: 10.1371/journal.pone.0137046

Doumith, M., Buchrieser, C., Glaser, P., *et al*. (2004) Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. *J Clin Microbiol* **42**:3819–3822. doi: 10.1128/JCM.42.8.3819

Dowe, M., Jackson, E., Mori, J., Colin, B. (1997) *Listeria monocytogenes* Survival in Soil and Incidence in Agricultural Soils. *J Food Prot* **10**:1158–1286. doi: 10.4315/0362-028X-60.10.1201

Dreux, N., Albagnac, C., Federighi, M., *et al*. (2007) Viable but non-culturable *Listeria monocytogenes* on parsley leaves and absence of recovery to a culturable state. *J Appl Microbiol* **103**:1272–1281. doi: 10.1111/j.1365-2672.2007.03351.x

Drevets, D.A., Bronze, M.S. (2008) *Listeria monocytogenes*: Epidemiology, human disease, and mechanisms of brain invasion. *FEMS Immunol Med Microbiol* **53**:151–165. doi: 10.1111/j.1574-695X.2008.00404.x

Dreyer, M., Aguilar-Bultet, L., Rupp, S., *et al*. (2016) *Listeria monocytogenes* sequence type 1 is predominant in ruminant rhombencephalitis. *Sci Rep* **6**:1–11. doi: 10.1038/srep36419

European Commission. (1999) Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on *Listeria monocytogenes.* Health & Consumer Protection Directorate-General. Directorate B - Scientific Health Opinions. Unit B3 - Management of scientific committees II

European Commission. (2014) Final Report Summary (SUSCLEAN). https://cordis.europa.eu/project/id/287514

European Food Safety Authority. (2018) Multi-country outbreak of *Listeria monocytogenes* serogroup IVb, multi-locus sequence type 6, infections linked to frozen corn and possibly to other frozen vegetables – first update. *EFSA Support Publ* **15**:. doi: 10.2903/sp.efsa.2018.EN-1448

Fagerlund, A., Langsrud, S., Schirmer, B.C.T., *et al*. (2016) Genome analysis of *Listeria monocytogenes* sequence type 8 strains persisting in salmon and poultry processing environments and comparison with related strains. *PLoS One* **11**:1–22. doi: 10.1371/journal.pone.0151117

Falardeau, J., Walji, K., Haure, M., *et al*. (2018) Native bacterial communities and *Listeria monocytogenes* survival in soils collected from the Lower Mainland of British Columbia, Canada. *Can J Microbiol* **64**:659–705

Faralla, C., Bakardjiev, A.I., Shiow, L.R., *et al*. (2016) InlP, a New Virulence Factor with Strong Placental Tropism. *Infect Immun* **84**:3584–3596. doi: 10.1128/iai.00625-16

Farber, J.M., Ross, W.H., Harwig, J. (1996) Health risk assessment of *Listeria*

monocytogenes in Canada. *Int J Food Microbiol* **30**:145–156. doi: 10.1016/0168-1605(96)01107-5

Fenlon, D.R. (1985) Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. *J Appl Bacteriol* **59**:537–543. doi: 10.1111/j.1365-2672.1985.tb03357.x

Ferreira, A., O'Byrne, C.P., Boor, K.J. (2001) Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. *Appl Environ Microbiol* **67**:4454–4457. doi: 10.1128/AEM.67.10.4454

Ferreira, A., Sue, D., O'Byrne, C.P., Boor, K.J. (2003) Role of *Listeria monocytogenes* sigma(B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Appl Env Microbiol* **69**:2692–2698. doi: 10.1128/AEM.69.5.2692

Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J. (2014) *Listeria monocytogenes* Persistence in Food-Associated Environments: Epidemiology, Strain Characteristics, and Implications for Public Health. *J Food Prot* **77**:150–70. doi: 10.4315/0362-028X.JFP-13-150

Fox, E.M., Allnutt, T., Bradbury, M.I., *et al*. (2016) Comparative genomics of the *Listeria monocytogenes* ST204 subgroup. *Front Microbiol* **7**:1–12. doi: 10.3389/fmicb.2016.02057

Fravalo, P., Cherifi, T., Feliciano, K.D.N., *et al*. (2017) Characterisation of *InlA* truncation in *Listeria monocytogenes* isolates from farm animals and human cases in the province of Quebec. *Vet Rec Open* **4**:1–6. doi: 10.1136/vetreco-2016-000199

Freitag, N.E., Port, G.C., Miner, M.D. (2009) *Listeria monocytogenes* — from saprophyte to intracellular pathogen. *Nat Rev Microbiol* **7**:623–628. doi: 10.1038/nrmicro2171

FSA. (2007) Consumer Attitudes to Food Standards. *Food Standards Agency* **7**:1–124

Fugett, E.B., Schoonmaker-Bopp, D., Dumas, N.B., *et al*. (2007) Pulsed-Field Gel Electrophoresis (PFGE) Analysis of Temporally Matched *Listeria monocytogenes* Isolates from Human Clinical Cases, Foods, Ruminant Farms, and Urban and

Natural Environments Reveals Source-Associated as Well as Widely Distributed PFGE. *J Clin Microbiol* **45**:865–873. doi: 10.1128/JCM.01285-06

García-Gimeno, R.M., Zurera-Cosano, G., Amaro-Lopez, M. (1996) Incidence, survival and growth of *Listeria monocytogenes* in ready-to- use mixed vegetable salads in Spain. *J Food Saf* **16**:75–86

Garner, D., Kathariou, S. (2016) Fresh Produce–Associated Listeriosis Outbreaks, Sources of Concern, Teachable Moments, and Insights. *J Food Prot* **79**:337–344. doi: 10.4315/0362-028X.JFP-15-387

Gaul, L.K., Farag, N.H., Shim, T., *et al*. (2013) Hospital-acquired listeriosis outbreak caused by contaminated diced celery-texas, 2010. *Clin Infect Dis* **56**:20–26. doi: 10.1093/cid/cis817

Gelbíčová, T., Koláčková, I., Pantůček, R., Karpíšková, R. (2015) A novel mutation leading to a premature stop codon in *inlA* of *Listeria monocytogenes* isolated from neonatal listeriosis. *New Microbiol* **38**:293–296

Ghosh, P., Halvorsen, E.M., Ammendolia, D.A., *et al*. (2018) Invasion of the brain by *Listeria monocytogenes* is mediated by InlF and host cell vimentin. *MBio* **9**:1–11. doi: 10.1128/mBio.00160-18

Gil, M.I., Selma, M. V., López-Gálvez, F., Allende, A. (2009) Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *Int J Food Microbiol* **134**:37–45. doi: 10.1016/j.ijfoodmicro.2009.05.021

Girardin, H., Morris, C.E., Albagnac, C., *et al*. (2005) Behaviour of the pathogen surrogates *Listeria innocua* and *Clostridium sporogenes* during production of parsley in fields fertilized with contaminated amendments. *FEMS Microbiol Ecol* **54**:287–295. doi: 10.1016/j.femsec.2005.04.003

Giulieri, S.G., Baines, S.L., Guerillot, R., *et al*. (2018) Genomic exploration of sequential clinical isolates reveals a distinctive molecular signature of persistent *Staphylococcus aureus* bacteraemia. *Genome Med* **10**:1–17. doi: 10.1186/s13073-018-0574-x

Glaser, P., Buchrieser, C., Lecuit, M., *et al*. (2003) *Listeria monocytogenes* bile salt

hydrolase is a virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol Microbiol* **46**:903–903. doi: 10.1046/j.1365-2958.2002.03230.x

Gray, M.J., Boor, K.J. (2006) How the Bacterial Pathogen *Listeria monocytogenes* Mediates the Switch from Environmental Dr. Jekyll to Pathogenic Mr. Hyde. *Society* **74**:2505–2512. doi: 10.1128/IAI.74.5.2505

Grote, J., Krysciak, D., Streit, W.R. (2015) Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. *Appl Environ Microbiol* **81**:5280–5289. doi: 10.1128/AEM.00900-15

Guindon, S., Dufayard, J.F., Lefort, V., *et al*. (2010) New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Syst Biol* **59**:307–321. doi: 10.1093/sysbio/syq010

Haase, J.K., Didelot, X., Lecuit, M., *et al*. (2014) The ubiquitous nature of *Listeria monocytogenes* clones: A large-scale Multilocus Sequence Typing study. *Environ Microbiol* **16**:405–416. doi: 10.1111/1462-2920.12342

Harter, E., Wagner, E.M., Zaiser, A., *et al*. (2017) Stress survival islet 2, predominantly present in *Listeria monocytogenes* strains of sequence type 121, is involved in the alkaline and oxidative stress responses. *Appl Environ Microbiol* **83**:. doi: 10.1128/AEM.00827-17

Heaton, J.C., Jones, K. (2008) Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: A review. *J Appl Microbiol* **104**:613–626. doi: 10.1111/j.1365-2672.2007.03587.x

Heinz, D.W., Wehland, J., Beier, V., *et al*. (2002) Structure of Internalin, a Major Invasion Protein of *Listeria monocytogenes*, in Complex with Its Human Receptor E-Cadherin. *Cell* **111**:825–836. doi: 10.1016/s0092-8674(02)01136-4

Hellström, S. (2011) Contamination routes and control of *Listeria monocytogenes* in food production. University of Helsinki

Hellström, S., Kiviniemi, K., Autio, T., Korkeala, H. (2008) *Listeria monocytogenes* is common in wild birds in Helsinki region and genotypes are frequently similar with those found along the food chain. *J Appl Microbiol* **104**:883–888. doi:

10.1111/j.1365-2672.2007.03604.x

Henri, C., Félix, B., Guillier, L., *et al*. (2016) Population genetic structure of *Listeria monocytogenes* strains determined by pulsed-field gel electrophoresis and multilocus sequence typing. *Appl Environ Microbiol* **82**:AEM.00583-16. doi: 10.1128/AEM.00583-16

Heras, A. De., Cain, R.J., Bielecka, M.K. (2011) Regulation of *Listeria* virulence : PrfA master and commander. *Curr Opin Microbiol* 118–127. doi: 10.1016/j.mib.2011.01.005

Highmore, C.J., Warner, J.C., Rothwell, S.D., *et al*. (2018) Viable-but-Nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson Induced by Chlorine Stress Remain Infectious. *MBio* **9**:1–12. doi: 10.1128/mBio.00540-18

Hill, C., Guinane, C.M., Ross, P.R., *et al*. (2014) Atypical *Listeria innocua* strains possess an intact LIPI-3. *BMC Microbiol* **14**:58. doi: 10.1186/1471-2180-14-58

Hilliard, A., Leong, D., Callaghan, A.O., *et al*. (2018) Genomic Characterization of *Listeria monocytogenes* Isolates Associated with Clinical Listeriosis and the Food Production Environment in Ireland. Genes (Basel). doi: 10.3390/genes9030171

Hoang, D.T., Chernomor, O., von Haeseler, A., *et al*. (2017) Supplementary Data: Ufboot2: Improving The Ultrafast Bootstrap Approximation. **35**:518–522. doi: 10.5281/zenodo.854445

Hoelzer, K., Pouillot, R., Van Doren, J.M., Dennis, S. (2014) Reduction of *Listeria monocytogenes* contamination on produce - A quantitative analysis of common liquid fresh produce wash compounds. *Food Control* **46**:430–440. doi: 10.1016/j.foodcont.2014.06.011

Hood, E. (2005) Tap Water and Trihalmethanes. *Environ Heal Concerns* **113**:472–474

Inoue, S., Tanikawa, T., Kawaguchi, J., *et al*. (1992) Prevalence of *Listeria* (spp.) in Wild Rats Captured in the Kanto Area of Japan. *J Vetinary Med Sci* **54**:461–463

Jami, M., Ghanbari, M., Zunabovic, M., *et al*. (2014) *Listeria monocytogenes* in aquatic food products-A review. *Compr Rev Food Sci Food Saf* **13**:798–813. doi: 10.1111/1541-4337.12092

Jennison, A. V., Masson, J.J., Fang, N.X., *et al*. (2017) Analysis of the *Listeria monocytogenes* population structure among isolates from 1931 to 2015 in Australia. *Front Microbiol* **8**:1–13. doi: 10.3389/fmicb.2017.00603

Jordan, K., Leong, D., Alvarez-Ordonez, A. (2015) *Listeria monocytogenes* in the Food Processing Environment

Kaczmarek, M., Avery, S. V., Singleton, I. (2019) Microbes associated with fresh produce: Sources, types and methods to reduce spoilage and contamination. Adv Appl Microbiol. doi: 10.1016/bs.aambs.2019.02.001

Kagkli, D.M., Iliopoulos, V., Stergiou, V., *et al*. (2009) Differential *Listeria monocytogenes* strain survival and growth in Katiki, a traditional Greek soft cheese, at different storage temperatures. *Appl Environ Microbiol* **75**:3621–3626. doi: 10.1128/AEM.01571-08

Kaiser, D. (2007) Bacterial Swarming: A Re-examination of Cell-Movement Patterns. *Curr Biol* **17**:561–570. doi: 10.1016/j.cub.2007.04.050

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., *et al*. (2017) ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat Methods* **14**:587–589. doi: 10.1038/nmeth.4285

Kämpfer, P., Falsen, E., Lodders, N., Schumann, P. (2010) *Sporosarcina contaminans* sp. nov. and *Sporosarcina thermotolerans* sp. nov., two endospore-forming species. *Int J Syst Evol Microbiol* **60**:1353–1357. doi: 10.1099/ijs.0.014423-0

Kazmierczak, M.J., Mithoe, S.C., Boor, K.J., Wiedmann, M. (2003) *Listeria monocytogenes* σ B Regulates Stress Response and Virulence Functions. *J Bacteriol* **185**:5722–34. doi: 10.1128/JB.185.19.5722

Kearns, D.B. (2010) A field guide to bacterial swarming motility. **8**:634–644. doi: 10.1038/nrmicro2405.A

Keynote. (2015) Fruit & Vegetables Market Reports 2015

Khan, I., Khan, J., Miskeen, S., *et al*. (2016) Prevalence and control of *Listeria monocytogenes* in the food industry – a review. *Czech J Food Sci* **34**:469–487. doi: 10.17221/21/2016-CJFS

Kim, S.W., Haendiges, J., Keller, E.N., *et al*. (2018) Genetic diversity and virulence profiles of *Listeria monocytogenes* recovered from bulk tank milk, milk filters, and milking equipment from dairies in the United States (2002 to 2014). *PLoS One* **13**:1–17. doi: 10.1371/journal.pone.0197053

Kimura, B. (2006) Recent Advances in the Study of the Genotypic Diversity and Ecology of *Listeria monocytogenes*. *Microbes Environ* **21**:69–77. doi: 10.1264/jsme2.21.69

Kirchner, M., Higgins, D.E. (2008) Inhibition of ROCK activity allows InlF-mediated invasion and increased virulence of *Listeria monocytogenes*. *Mol Microbiol* **68**:749–767. doi: 10.1111/j.1365-2958.2008.06188.x

Koukkidis, G., Haigh, R., Allcock, N., *et al*. (2016) Salad leaf juices enhance *Salmonella* growth, fresh produce colonisation and virulence. *Appl Environ Microbiol* AEM.02416-16. doi: 10.1128/AEM.02416-16

Kremer, P.H.C., Lees, J.A., Koopmans, M.M., *et al*. (2017) Benzalkonium tolerance genes and outcome in Listeria monocytogenes meningitis. *Clin Microbiol Infect* **23**:265.e1-265.e7. doi: 10.1016/j.cmi.2016.12.008

Kwong, J.C., Chow, E.P.F., Stevens, K., *et al*. (2018) Whole-genome sequencing reveals transmission of gonococcal antibiotic resistance among men who have sex with men: An observational study. *Sex Transm Infect* **94**:151–157. doi: 10.1136/sextrans-2017-053287

Kwong, J.C., Mccallum, N., Sintchenko, V., Howden, B.P. (2015) Whole genome sequencing in clinical and public health microbiology. *Pathology* **47**:199–210. doi: 10.1097/PAT.0000000000000235

Kwong, J.C., Mercoulia, K., Tomita, T., *et al*. (2016) Prospective Whole-Genome Sequencing Enhances National Surveillance of *Listeria monocytogenes* . *J Clin Microbiol* **54**:333–42. doi: 10.1128/JCM.02344-15

Laksanalamai, P., Joseph, L.A., Silk, B.J., *et al*. (2012) Genomic Characterization of *Listeria monocytogenes* Strains Involved in a Multistate Listeriosis Outbreak Associated with Cantaloupe in US. *PLoS One* **7**:. doi: 10.1371/journal.pone.0042448

Lecuit, M., Gouin, E., Cossart, P., *et al*. (2008) The *Listeria monocytogenes* Virulence Factor InlJ Is Specifically Expressed In Vivo and Behaves as an Adhesin. *Infect Immun* **76**:1368–1378. doi: 10.1128/iai.01519-07

Lee, B., Hébraud, M., Bernardi, T., Lee, B. (2017a) Increased Adhesion of *Listeria monocytogenes* Strains to Abiotic Surfaces under Cold Stress. *Front Microbiol* **8**:1–10. doi: 10.3389/fmicb.2017.02221

Lee, C., Kim, J.Y., Song, H.S., *et al*. (2017b) Genomic Analysis of *Bacillus licheniformis* CBA7126 Isolated from a Human Fecal Sample. *Front Pharmacol* **8**:1–5. doi: 10.3389/fphar.2017.00724

Leong, D., Alvarez-Ordóez, A., Guillas, F., Jordan, K. (2013) Determination of *Listeria monocytogenes* Growth during Mushroom Production and Distribution. *Foods* **2**:544–553. doi: 10.3390/foods2040544

Leong, D., Alvarez-Ordonez, A., Jordan, K. (2014) Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. *Front Microbiol* **5**:1–8. doi: 10.3389/fmicb.2014.00436

Leong, D., NicAogáin, K., Luque-Sastre, L., *et al*. (2017) A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland. *Int J Food Microbiol* **249**:18–26. doi: 10.1016/j.ijfoodmicro.2017.02.015

Li, L., Mendis, N., Trigui, H., *et al*. (2014) The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* **5**:1–1. doi: 10.3389/fmicb.2014.00258

Li, Z., Pérez-Osorio, A., Wang, Y., *et al*. (2017) Whole genome sequencing analyses of *Listeria monocytogenes* that persisted in a milkshake machine for a year and caused illnesses in Washington State. *BMC Microbiol* **17**:134. doi: 10.1186/s12866-017-1043-1

Linke, K., Rückerl, I., Brugger, K., *et al*. (2014b) Reservoirs of *Listeria* species in three environmental ecosystems. *Appl Environ Microbiol* **80**:5583–5592. doi: 10.1128/AEM.01018-14

Little, C.L., Sagoo, S.K., Gillespie, I.A., *et al*. (2009) Prevalence and Level of *Listeria*

monocytogenes and Other *Listeria* Species in Selected Retail Ready-to-Eat Foods in the United Kingdom. *J Food Prot* **72**:1869–1877. doi: 10.4315/0362-028X-72.9.1869

Liu, D. (2008) Handbook of *Listeria Monocytogenes.* CRC Press

Liu, D. (2006) Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *J Med Microbiol* **55**:645–659. doi: 10.1099/jmm.0.46495-0

Liu, D., Lawrence, M.L., Ainsworth, A.J., Austin, F.W. (2005) Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiol Lett* **243**:373–378. doi: 10.1016/j.femsle.2004.12.025

Locatelli, A., Depret, G., Jolivet, C., *et al*. (2013a) Nation-wide study of the occurrence of *Listeria monocytogenes* in French soils using culture-based and molecular detection methods. *J Microbiol Methods* **93**:242–250. doi: 10.1016/j.mimet.2013.03.017

Locatelli, A., Spor, A., Jolivet, C., *et al*. (2013b) Biotic and Abiotic Soil Properties Influence Survival of *Listeria monocytogenes* in Soil. *PLoS One* **8**:1–8. doi: 10.1371/journal.pone.0075969

Lomonaco, S., Nucera, D. (2012) Molecular Subtyping Methods for *Listeria monocytogenes*: Tools for Tracking and Control. In: DNA Methods in Food Safety: Molecular Typing of Foodborne and Waterborne Bacterial Pathogens

Lomonaco, S., Verghese, B., Gerner-Schmit, P., *et al*. (2013) Novel Epidemic Clones of *Listeria monocytogenes* United States, 2011. **19**:2011–2014

Lundén, J.M., Miettinen, M.K., Autio, T.J., Korkeala, H.J. (2000) Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *J Food Prot* **63**:1204–1207. doi: 10.4315/0362-028X-63.9.1204

Magalhães, R., Ferreira, V., Biscottini, G., *et al*. (2017) Biofilm formation by persistent and non-persistent *Listeria monocytogenes* strains on abiotic surfaces. *Acta Aliment* **46**:43–50. doi: 10.1556/066.2017.46.1.6

Makino, S.I., Kawamoto, K., Takeshi, K., *et al*. (2005) An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. *Int J Food Microbiol* **104**:189–196. doi: 10.1016/j.ijfoodmicro.2005.02.009

Marquis, H., Goldfine, H., Portnoy, D.A. (1997) Proteolytic pathways of activation and degradation of a bacterial phospholipase C during intracellular infection by *Listeria monocytogenes*. *J Cell Biol* **137**:1381–1392. doi: 10.1083/jcb.137.6.1381

Maury, M.M., Tsai, Y., Charlier, C., Touchon, M. (2016) Uncovering Listeria monocytogenes hypervirulence by harnessing its biodiversity. *Nat Genet* **48**:308–313. doi: 10.1038/ng.3501.Uncovering

Maynaud, G., Pourcher, A.M., Ziebal, C., *et al*. (2016) Persistence and potential viable but non-culturable state of pathogenic bacteria during storage of digestates from agricultural biogas plants. *Front Microbiol* **7**:. doi: 10.3389/fmicb.2016.01469

McCollum, J.T., Cronquist, A.B., Silk, B.J., *et al*. (2013) Multistate outbreak of listeriosis associated with cantaloupe. *N Engl J Med* **369**:944–53. doi: 10.1056/NEJMoa1215837

McLaughlin, H.P., Casey, P.G., Cotter, J., *et al*. (2011) Factors affecting survival of *Listeria monocytogenes* and *Listeria innocua* in soil samples. *Arch Microbiol* **193**:775–785. doi: 10.1007/s00203-011-0716-7

Monaghan, J., Adams, H., Hutchison, M. (2009) Monitoring microbial food safety of fresh produce. FSA Factsheet.

Monaghan, J., Beacham, A. (2017) Salad Vegetable Crops. Encyclopedia of Applied Plant Sciences, Vol 3, Waltham, MA: Academic Press, 2017, pp. 262–267

Monaghan, J.M., Hutchison, M.L. (2012) Distribution and decline of human pathogenic bacteria in soil after application in irrigation water and the potential for soil-splash-mediated dispersal onto fresh produce. *J Appl Microbiol* **112**:1007–1019. doi: 10.1111/j.1365-2672.2012.05269.x

Moura, A., Tourdjman, M., Leclercq, A., *et al*. (2017) Real-Time Whole-Genome Sequencing for Surveillance of *Listeria monocytogenes* , France. *Emerg Infect Dis* **23**:. doi: 10.3201/eid2309.170336

Moynihan, E.L., Richards, K.G., Brennan, F.P., *et al*. (2015) Enteropathogen survival in soil from different land-uses is predominantly regulated by microbial community composition. *Appl Soil Ecol* **89**:76–84. doi: 10.1016/j.apsoil.2015.01.011

Nair, S., Milohanic, E., Berche, P. (2000) ClpC ATPase is required for cell adhesion and invasion of *Listeria monocytogenes*. *Infect Immun* **68**:7061–7068. doi: 10.1128/IAI.68.12.7061-7068.2000

Nastasijevic, I., Milanov, D., Velebit, B., *et al*. (2017) Tracking of *Listeria monocytogenes* in meat establishment using Whole Genome Sequencing as a food safety management tool: A proof of concept. *Int J Food Microbiol* **257**:157–164. doi: 10.1016/j.ijfoodmicro.2017.06.015

National Listeria Incident Management Team. (2018) Situation Report. Health department, Republic of South Africa

Nemoy, L.L., Kotetishvili, M., Tigno, J., *et al*. (2005) Multilocus Sequence Typing versus Pulsed-Field Gel Electrophoresis for Characterization of Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolates. *Society* **43**:1776–1781. doi: 10.1128/JCM.43.4.1776

Nguyen, L.-T., Schimidt, H.A., Haeseler, A. von., Minh, B.Q. (2015) IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol Biol Evol* **32**:268–274. doi: 10.1093/molbev/msu300

Nightingale, K.K., Nightingale, K.K., Windham, K., *et al*. (2005) Select *Listeria monocytogenes* Subtypes Commonly Found in Foods Carry Disctinct Donsense Mutations in *inlA*, Leading to Expression of Truncated and Secreted Internalin A, and Are Associated with a Reduced Invasion Phenotype for Human Intestina. *Appl Environ Microbiol* **71**:8764–8772. doi: 10.1128/AEM.71.12.8764

Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., *et al*. (2004a) Ecology and Transmission of *Listeria monocytogenes* infecting ruminants in the farm environment. *Society* **70**:4458–4467. doi: 10.1128/AEM.70.8.4458

Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., *et al*. (2004b) Ecology and Transmission of *Listeria monocytogenes* Infecting Ruminants and in the Farm Environment. *Society* **70**:4458–4467. doi: 10.1128/AEM.70.8.4458

Nowak, J., Cruz, C.D., Tempelaars, M., *et al*. (2017) Persistent *Listeria monocytogenes* strains isolated from mussel production facilities form more biofilm but are not linked to specific genetic markers. *Int J Food Microbiol* **256**:45–53. doi: 10.1016/j.ijfoodmicro.2017.05.024

O'Riordan, M., Moors, M., Portnoy, D. (2003) *Listeria* Intracellular Growth and Virulence Require Host-Derived Lipoic Acid. *Science (80-)* **302**:462–464. doi: 10.1126/science.1088170

Oliveira, M., Usall, J., Villas, I., *et al*. (2011) Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves. *Food Microbiol* **28**:590–596. doi: 10.1016/j.fm.2010.11.004

Oliver, J.D. (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev* **34**:415–425. doi: 10.1111/j.1574-6976.2009.00200.x

Ölmez, H., Kretzschmar, U. (2009) Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *LWT - Food Sci Technol* **42**:686–693. doi: 10.1016/j.lwt.2008.08.001

Ölmez, H., Temur, S.D. (2010) Effects of different sanitizing treatments on biofilms and attachment of *Escherichia coli* and *Listeria monocytogenes* on green leaf lettuce. *LWT - Food Sci Technol* **43**:964–970. doi: 10.1016/j.lwt.2010.02.005

Orsi, R.H., Bakker, H.C. den., Wiedmann, M. (2011) *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *Int J Med Microbiol* **301**:79–96. doi: 10.1016/j.ijmm.2010.05.002

Overney, A., Jacques-André-Coquin, J., Ng, P., *et al*. (2017) Impact of environmental factors on the culturability and viability of *Listeria monocytogenes* under conditions encountered in food processing plants. *Int J Food Microbiol* **244**:74–81. doi: 10.1016/j.ijfoodmicro.2016.12.012

Palumbo, J.D., Borucki, M.K., Mandrell, R.E., Gorski, L. (2003) Serotyping of *Listeria monocytogenes* by Enzyme-Linked Immunosorbent Assay and Identification of Mixed serotype Cultures by Colony Immunoblotting. *Mol Cells* **41**:564–571. doi: 10.1128/JCM.41.2.564

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

Pellegrini, E., Charbit, A., Berche, P., Reglier-Poupet, H. (2003) Identification of LpeA, a PsaA-Like Membrane Protein That Promotes Cell Entry by *Listeria monocytogenes*. *Society* **71**:474–482. doi: 10.1128/IAI.71.1.474

Pichler, J.G., Appl, G., Pietzka, a., Allerberger, F. (2011) Lessons to be Learned from an Outbreak of Foodborne Listeriosis, Austria 2009–2010. *Food Prot Trends* **31**:268–273

Piveteau, P., Depret, G., Pivato, B., *et al*. (2011) Changes in gene expression during adaptation of *Listeria monocytogenes* to the soil environment. *PLoS One* **6**:. doi: 10.1371/journal.pone.0024881

Portnoy, D.A., Auerbuch, V., Glomski, I.J. (2002) The cell biology of *Listeria monocytogenes* infection: The intersection of bacterial pathogenesis and cell-mediated immunity. *J Cell Biol* **158**:409–414. doi: 10.1083/jcb.200205009

Pouillot, R., Klontz, K.C., Chen, Y., *et al*. (2016) Infectious Dose of *Listeria monocytogenes* in Outbreak Linked to Ice Cream, United States, 2015. *Emerg Infect Dis* **22**:2113–2119. doi: 10.3201/eid2212.160165

Pulsenet. (2013) Standard Operating Procedure for Pulsenet PFGE of *Listeria monocytogenes* . https://www.cdc.gov/pulsenet/PDF/listeria-pfge-protocol-508c.pdf

Quereda, J.J., Meza-Torres, J., Cossart, P., Pizarro-Cerdá, J. (2017) Listeriolysin S: A bacteriocin from epidemic *Listeria monocytogenes* strains that targets the gut microbiota. *Gut Microbes* **8**:1–8. doi: 10.1080/19490976.2017.1290759

Rahman, S.M.E., Khan, I., Oh, D.H. (2016) Electrolyzed Water as a Novel Sanitizer in the Food Industry: Current Trends and Future Perspectives. *Compr Rev Food Sci Food Saf* **15**:471–490. doi: 10.1111/1541-4337.12200

Ramamurthy, T., Ghosh, A., Pazhani, G.P., Shinoda, S. (2014) Current Perspectives on Viable but Non-Culturable (VBNC) Pathogenic Bacteria. *Front Public Heal* **2**:1–9.

doi: 10.3389/fpubh.2014.00103

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., *et al*. (2007) *Listeria* — review of epidemiology and pathogenesis. 4–13

Réglier-Poupet, H., Frehel, C., Dubail, I., *et al*. (2003) Maturation of Lipoproteins by Type II Signal Peptidase is Required for Phagosomal Escape of *Listeria monocytogenes*. *J Biol Chem* **278**:49469–49477. doi: 10.1074/jbc.M307953200

Rieu, A., Weidmann, S., Garmyn, D., *et al*. (2007) agr system of *Listeria monocytogenes* EGD-e: Role in adherence and differential expression pattern. *Appl Environ Microbiol* **73**:6125–6133. doi: 10.1128/AEM.00608-07

Roberts, A.J., Williams, S.K., Wiedmann, M., Nightingale, K.K. (2009) Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, inlA transcript levels, and swarming motility in vitro. *Appl Environ Microbiol* **75**:5647–5658. doi: 10.1128/AEM.00367-09

Ruppitsch, W., Pietzka, A., Prior, K., *et al*. (2015) Defining and evaluating a core genome multilocus sequence typing scheme for whole-genome sequence-based typing of *Listeria monocytogenes*. *J Clin Microbiol* **53**:2869–2876. doi: 10.1128/JCM.01193-15

Ryan, E.M., Gahan, C.G.M., Hill, C. (2008) A significant role for Sigma B in the detergent stress response of *Listeria monocytogenes*. *Lett Appl Microbiol* **46**:148–154. doi: 10.1111/j.1472-765X.2007.02280.x

Salamina, G., Dalle Donne, E., Niccolini, A., *et al*. (1996) A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiol Infect* **117**:429–436. doi: 10.1017/S0950268800059082

Salazar, J.K., Sahu, S.N., Hildebrandt, I.M., *et al*. (2017) Growth Kinetics of *Listeria monocytogenes* in Cut Produce. *J Food Prot* **80**:1328–1336

Salazar, J.K., Wu, Z., Yang, W., *et al*. (2013) Roles of a Novel Crp/Fnr Family Transcription Factor Lmo0753 in Soil Survival, Biofilm Production and Surface Attachment to Fresh Produce of *Listeria monocytogenes*. *PLoS One* **8**:. doi: 10.1371/journal.pone.0075736

Salcado, C., Arreaza, L., Alcala, B., *et al*. (2003) Development of a Multilocus Sequence Typing Method for Analysis of *Listeria monocytogenes* Clones. *J Clin Microbiol* **41**:757–762. doi: 10.1099/mic.0.28482-0

Sauders, B.D., Overdevest, J., Fortes, E., *et al*. (2012) Diversity of *Listeria* species in urban and natural environments. *Appl Environ Microbiol* **78**:4420–4433. doi: 10.1128/AEM.00282-12

Sauders, B.D., Sanchez, M.D., Rice, D.H., *et al*. (2009) Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. *J Food Prot* **72**:2337–49

Scallan, E., Hoekstra, R.M., Angulo, F.J., *et al*. (2011) Foodborne illness acquired in the United States-Major pathogens. *Emerg Infect Dis* **17**:7–15. doi: 10.3201/eid1701.P11101

Schloter, M., Nannipieri, P., Sørensen, S.J., van Elsas, J.D. (2018) Microbial indicators for soil quality. *Biol Fertil Soils* **54**:1–10. doi: 10.1007/s00374-017-1248-3

Seeliger, H.P., Hohne, K. (1979) Serotyping of *Listeria monocytogenes* and Related Species. In: Methods in Microbiology. pp 3–7

Seemann, T., Concalves, da S.A., Bulach, D.M., *et al*. (2019) Nullarbor. In: Github. https://github.com/tseemann/nullarbor. Accessed 6 Mar 2019

Shabala, L., Lee, S.H., Cannesson, P., Ross, T. (2008) Acid and NaCl Limits to Growth of *Listeria monocytogenes* and Influence of Sequence of Inimical Acid and NaCl Levels on Inactivation Kinetics. *J Food Prot* **71**:1169–1177. doi: 10.4315/0362-028X-71.6.1169

Sheng, L., Edwards, K., Tsai, H.C., *et al*. (2017) Fate of *Listeria monocytogenes* on fresh apples under different storage temperatures. *Front Microbiol* **8**:1–8. doi: 10.3389/fmicb.2017.01396

Smith, A., Hearn, J., Taylor, C., *et al*. (2019) *Listeria monocytogenes* isolates from ready to eat plant produce are diverse and have virulence potential. *Int J Food Microbiol* **299**:23–32. doi: 10.1016/j.ijfoodmicro.2019.03.013

Smith, A., Moorhouse, E., Monaghan, J., *et al*. (2018) Sources and survival of *Listeria*

monocytogenes on fresh, leafy produce. *J Appl Microbiol* 1–13. doi: 10.1111/jam.14025

Sousa, S., Camejo, A., Cossart, P., *et al*. (2010) LapB, a Novel *Listeria monocytogenes* LPXTG Surface Adhesin, Required for Entry into Eukaryotic Cells and Virulence. *J Infect Dis* **202**:551–562. doi: 10.1086/654880

Standing, T.A., Du Plessis, E., Duvenage, S., Korsten, L. (2013) Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants. *J Water Health* **11**:210–223. doi: 10.2166/wh.2013.164

Stasiewicz, M.J., Oliver, H.F., Wiedmann, M., den Bakker, H.C. (2015) Whole-genome sequencing allows for improved identification of persistent *Listeria monocytogenes* in food-associated environments. *Appl Environ Microbiol* **81**:6024–6037. doi: 10.1128/AEM.01049-15

Stopforth, J.D., Mai, T., Kottapalli, B., Samadpour, M. (2008) Effect of Acidified Sodium Chlorite, Chlorine, and Acidic Electrolyzed Water on Escherichia coli O157:H7, Salmonella, and Listeria monocytogenes Inoculated onto Leafy Greens. *J Food Prot* **71**:625–628. doi: 10.4315/0362-028x-71.3.625

Suarez, M., Scortti, M., Gonzalez-Zorn, B., *et al*. (2002) Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. *Proc Natl Acad Sci* **99**:431–436. doi: 10.1073/pnas.012363899

Tao, T., Chen, Q., Bie, X., *et al*. (2015) Mining of novel species-specific primers for PCR detection of *Listeria monocytogenes* based on genomic approach. *World J Microbiol Biotechnol* **31**:1955–1966. doi: 10.1007/s11274-015-1942-y

Tasara, T., Stephan, R. (2006) Cold stress tolerance of *Listeria monocytogenes*: A review of molecular adaptive mechanisms and food safety implications. *J Food Prot* **69**:1473–84. doi: 10.4315/0362-028X-69.6.1473

Tham, T.N., Lebreton, A., Dessen, A., *et al*. (2011) A Bacterial Protein Targets the BAHD1 Chromatin Complex to Stimulate Type III Interferon Response. *Science (80-)* **331**:1319–1321. doi: 10.1126/science.1200120

Tienungoon, S., Ratkowsky, D. a., McMeekin, T. a., Ross, T. (2000) Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. *Appl Environ Microbiol* **66**:4979–4987. doi: 10.1128/AEM.66.11.4979-4987.2000

Tilney, L.G., Portnoy, D.A. (1989) Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J Cell Biol* **109**:1597–1608. doi: 10.1083/jcb.109.4.1597

Toledo, V., Den Bakker, H.C., Hormazábal, J.C., *et al*. (2018) Genomic Diversity of *Listeria monocytogenes* Isolated from Clinical and Non-Clinical Samples in Chile. *Genes (Basel)* **9**:1–12. doi: 10.3390/genes9080396

Van Der Veen, S., Abee, T. (2010) Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. *Appl Environ Microbiol* **76**:7854–7860. doi: 10.1128/AEM.01519-10

Vazquez-Boland, J.A., Kuhn, M., Berche, P., *et al*. (2001) *Listeria* Pathogenesis and Molecular Virulence Determinants. **14**:584–640. doi: 10.1128/CMR.14.3.584

Velge, P., Roche, S.M. (2010) Variability of *Listeria monocytogenes* virulence: a result of the evolution between saprophytism and virulence? *Future Microbiol* **5**:1799–1821

Vivant, A.-L., Garmyn, D., Piveteau, P. (2013) *Listeria monocytogenes*, a down-to-earth pathogen. *Front Cell Infect Microbiol* **3**:87. doi: 10.3389/fcimb.2013.00087

Vivant, A., Desneux, J., Pourcher, A., Piveteau, P. (2017) Transcriptomic Analysis of the Adaptation of *Listeria monocytogenes* to Lagoon and Soil Matrices Associated with a Piggery Environment : Comparison of Expression Profiles. *Front Microbiol* **8**:1–16. doi: 10.3389/fmicb.2017.01811

Vivant, A.L., Garmyn, D., Gal, L., *et al*. (2015) Survival of *Listeria monocytogenes* in soil requires AgrA-mediated regulation. *Appl Environ Microbiol* **81**:5073–5084. doi: 10.1128/AEM.04134-14

Wang, Y., Jiao, Y., Lan, R., *et al*. (2015) Characterization of *Listeria monocytogenes* isolated from human Listeriosis cases in China. *Emerg Microbes Infect* **4**:e50. doi: 10.1038/emi.2015.50

Wei, Z., Knabel, S.J. (2005) Multiplex PCR Assay Simplifies Serotyping and Sequence Typing of *Listeria monocytogenes* Associated with Human Outbreaks. *J Food Prot* **68**:68 (9) 1907-1910. doi: 10.4315/0362-028X-68.9.1907

Weis, J., Seeliger, H.P. (1975) Incidence of *Listeria monocytogenes* in nature. *Appl Microbiol* **30**:29–32

Weller, D., Wiedmann, M., Strawn, L.K. (2015) Spatial and temporal factors associated with an increased prevalence of *Listeria monocytogenes* in spinach fields in New York State. *Appl Environ Microbiol* **81**:6059–6069. doi: 10.1128/AEM.01286-15

Welshimer, H.J. (1968) Isolation of *Listeria monocytogenes* from vegetation. *J Bacteriol* **95**:300–303

Whittaker, R.H. (1972) Evolution and Measurement of Species Diversity. *Taxon* **21**:213–251

Wiemer, E.A.C., Gouin, E., Cossart, P., *et al*. (2011) Recruitment of the Major Vault Protein by InlK: A *Listeria monocytogenes* Strategy to Avoid Autophagy. *PLoS Pathog* **7**:e1002168. doi: 10.1371/journal.ppat.1002168

Wright, K.M., Chapman, S., McGeachy, K., *et al*. (2013) The endophytic lifestyle of Escherichia coli O157:H7: quantification and internal localization in roots. *Phytopathology* **103**:333–40. doi: 10.1094/PHYTO-08-12-0209-FI

Wu, S., Wu, Q., Zhang, J., *et al*. (2016) Analysis of multilocus sequence typing and virulence characterization of *Listeria monocytogenes* isolates from chinese retail ready-to-eat food. *Front Microbiol* **7**:1–11. doi: 10.3389/fmicb.2016.00168

Yoshida, M., Sztul, E., Mimuro, H., *et al*. (2009) *Listeria monocytogenes* ActA-mediated escape from autophagic recognition. *Nat Cell Biol* **11**:1233–1240. doi: 10.1038/ncb1967

Zhu, Q., Gooneratne, R., Hussain, M. (2017) *Listeria monocytogenes* in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. *Foods* **6**:21. doi: 10.3390/foods6030021

Zoz, F., Grandvalet, C., Lang, E., *et al*. (2017) *Listeria monocytogenes* ability to survive desiccation: Influence of serotype, origin, virulence, and genotype. *Int J Food*

Microbiol **248**:82–89. doi: 10.1016/j.ijfoodmicro.2017.02.010

Appendix A Haemolysis of horse RBCs by *L. monocytogenes* supernatant

Table A.1. **Layout of 96-well plate used for testing the haemolysis capacity of supernatant from *L. monocytogenes* strains.** Strain designation (i.e. 1 (NLmo1) is indicated in each well followed by how supernatant was diluted. Blank wells contained nothing.

1 (neat)	1 (1/10)	1 (1/100)	1 (1/1000)	8 (neat)	8 (1/10)	8 (1/100)	8 (1/1000)	18 (neat)	18 (1/10)	18 (1/100)	18 (1/1000)
2 (neat)	2 (1/10)	2 (1/100)	2 (1/1000)	9 (neat)	9 (1/10)	9 (1/100)	9 (1/1000)	20 (neat)	16 (1/10)	16 (1/100)	16 (1/1000)
3 (neat)	3 (1/10)	3 (1/100)	3 (1/1000)	10 (neat)	10 (1/10)	10 (1/100)	10 (1/1000)	Con (neat)	16 (1/10)	16 (1/100)	16 (1/1000)
4 (neat)	4 (1/10)	4 (1/100)	4 (1/1000)	13 (neat)	13 (1/10)	13 (1/100)	13 (1/1000)				
5 (neat)	5 (1/10)	5 (1/100)	5 (1/1000)	14 (neat)	14 (1/10)	14 (1/100)	14 (1/1000)				
6 (neat)	6 (1/10)	6 (1/100)	6 (1/1000)	15 (neat)	15 (1/10)	15 (1/100)	15 (1/1000)				
7 (neat)	7 (1/10)	7 (1/100)	7 (1/1000)	16 (neat)	16 (1/10)	16 (1/100)	16 (1/1000)				

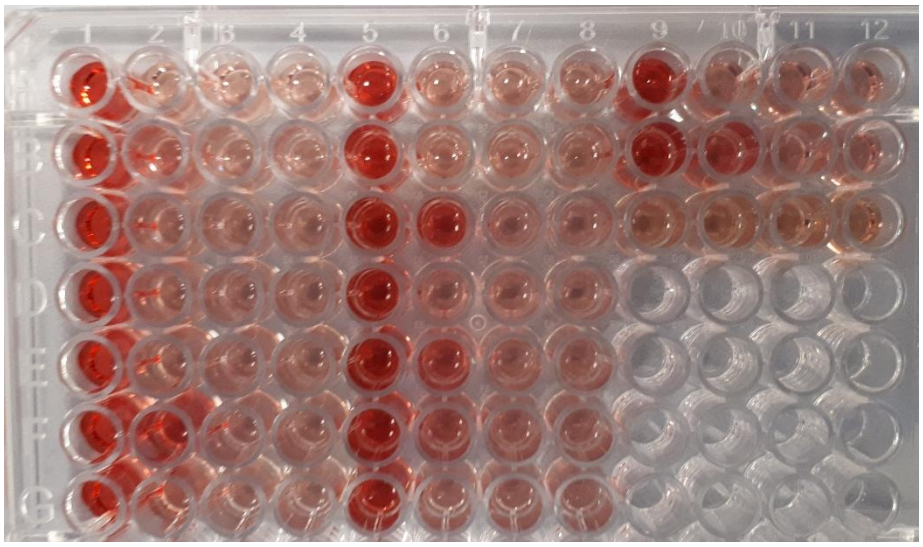

Figure A.1. **96-well plate used for testing the haemolysis capacity of supernatant from *L. monocytogenes* strains.** Absorbance (OD, 540nm) was subsequently measured using a FLUOstar OMEGA (Table A.2.)

Table A.2. **Absorbance (OD, 540nm) readings for horse RBCs that have been previously lysed using supernatant from different *L. monocytogenes* strains.** See Table A.1. for details.

0.96	0.20	0.20	0.19	0.96	0.25	0.22	0.22	0.99	0.24	0.25	0.24
1.09	0.33	0.20	0.19	0.94	0.23	0.23	0.22	0.95	0.53	0.26	0.26
0.97	0.20	0.20	0.19	0.98	0.76	0.22	0.22	0.24	0.24	0.24	0.24
0.95	0.21	0.20	0.19	0.98	0.22	0.23	0.22				
0.91	0.20	0.20	0.19	0.99	0.50	0.23	0.25				
0.98	0.76	0.20	0.20	0.98	0.38	0.26	0.25				
0.97	0.34	0.22	0.20	0.96	0.24	0.25	0.24				

Appendix B Results associated with the processing of whole genome sequencing data (Chapter 3)

Appendix Table B.1 **Assembly and annotation data associated with L. monocytogenes whole genomes from Illumina paired-end sequencing data**

Isolate	No. of reads	Depth of coverage	No. of contigs	Genome size (Mb)	GC content (%)	#1 Identified match (Kraken 8GB database)	% Similarity to #1 match	N50	rRNA copy number	tRNA
NLmo2	1871906	119	16	3022108	38.5	*Listeria monocytogenes*	89.62	537374	4	54
NLmo3	864832	59	19	3021207	38.4	*Listeria monocytogenes*	92.34	537190	4	63
NLmo4	909848	65	19	3065932	38.4	*Listeria monocytogenes*	91.76	432370	3	58
NLmo5	1508824	101	18	3065467	37.9	*Listeria monocytogenes*	89.49	432886	4	49
NLmo6	4673156	308	13	2878779	38.8	*Listeria monocytogenes*	92.08	477702	3	50
NLmo7	3859388	262	9	2919242	38.6	*Listeria monocytogenes*	91.87	596199	5	50
NLmo8	705258	48	16	2920871	38.4	*Listeria monocytogenes*	93.06	477606	6	49
NLmo9	612548	44	18	2920275	38.4	*Listeria monocytogenes*	94.64	302065	2	54

NLmo10	1941944	129	11	2917149	38.6	*Listeria monocytogenes*	91.47	476852	5	50
NLmo13	1000374	67	13	2929305	38.4	*Listeria monocytogenes*	92.86	1497074	3	63
NLmo14	1530718	103	9	2918988	38.3	*Listeria monocytogenes*	92.05	596189	5	50
NLmo15	2202970	158	16	2968413	37.9	*Listeria monocytogenes*	94.53	398348	2	61
NLmo16	850148	57	13	2927284	38.4	*Listeria monocytogenes*	93.48	1497064	3	54
NLmo18	488048	31	21	2968827	38.4	*Listeria monocytogenes*	88.87	455612	4	49
NLmo20	2052146	138	10	2915683	38.9	*Listeria monocytogenes*	91.8	556332	3	48

Appendix Table B.2 **Pairwise differences in number of single nucleotide polymorphisms (SNPs) between L. monocytogenes isolates from the UK fresh produce supply chain**.

	NLmo10	**NLmo13**	**NLmo14**	**NLmo15**	**NLmo16**	**NLmo18**	**NLmo2**	**NLmo20**	**NLmo3**	**NLmo4**	**NLmo5**	**NLmo6**	**NLmo7**	**NLmo8**	**NLmo9**	**Ref.**
NLmo10	0	39261	56	39512	39262	39965	3301	2738	3300	40017	40017	2687	56	2425	2708	39752
NLmo13	39261	0	39260	8008	1	12519	39294	39353	39294	8999	8999	39280	39260	39334	39272	7744

NLmo14	56	39260	0	39513	39261	39965	3305	2742	3304	40018	40018	2677	0	2415	2698	39753
NLmo15	39512	8008	39513	0	8009	12344	39571	39630	39571	8513	8513	39508	39513	39598	39496	3541
NLmo16	39262	1	39261	8009	0	12520	39295	39354	39295	9000	9000	39281	39261	39335	39273	7745
NLmo18	39965	12519	39965	12344	12520	0	40016	40066	40017	12815	12815	39985	39965	40038	39973	12125
NLmo2	3301	39294	3305	39571	39295	40016	0	3405	1	40069	40069	3176	3305	3218	3191	39831
NLmo20	2738	39353	2742	39630	39354	40066	3405	0	3404	40133	40133	2927	2742	3032	2948	39872
NLmo3	3300	39294	3304	39571	39295	40017	1	3404	0	40070	40070	3176	3304	3218	3191	39832
NLmo4	40017	8999	40018	8513	9000	12815	40069	40133	40070	0	2	40042	40018	40078	40030	8323
NLmo5	40017	8999	40018	8513	9000	12815	40069	40133	40070	2	0	40042	40018	40078	40030	8323
NLmo6	2687	39280	2677	39508	39281	39985	3176	2927	3176	40042	40042	0	2677	1672	59	39781
NLmo7	56	39260	0	39513	39261	39965	3305	2742	3304	40018	40018	2677	0	2415	2698	39753
NLmo8	2425	39334	2415	39598	39335	40038	3218	3032	3218	40078	40078	1672	2415	0	1693	39825
NLmo9	2708	39272	2698	39496	39273	39973	3191	2948	3191	40030	40030	59	2698	1693	0	39769
Ref.	39752	7744	39753	3541	7745	12125	39831	39872	39832	8323	8323	39781	39753	39825	39769	0

Appendix Table B.3. **Presence/absence of key virulence factors in *L. monocytogenes* strains isolated from the fresh produce supply chain as determined by Abricate.** The accession number for each gene is indicated below gene name. Legend Yes = ≥95% coverage & >75% identity, probable = ≥ 0% coverage & >75% identity - = absent.

Isolate	NLmo2	NLmo3	NLmo4	NLmo5	NLmo6	NLmo7	NLmo8	NLmo9	NLmo10	NLmo13	NLmo14	NLmo15	NLmo16	NLmo18	NLmo20
no. of virulence factors	34	34	31	31	41	41	41	41	41	32	41	32	32	33	41
***actA* NP_463735**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***ami* NP_466081**	Yes	Probable	Yes	Probable	-	-	-	-	-	Yes	-	Yes	Yes	Yes	-
***aut* NP_466081**	Yes	Yes	Yes	Yes	-	-	-	-	-	Yes	-	Yes	Yes	Yes	-
***bsh* NP_465591**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***clpC* NP_463763**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***clpE* NP_464522**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***clpP* NP_465991**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***cdsA* NP_464841.1**	Yes	Yes	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	Yes	Yes
***essC* NP_645079**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***fbpA* NP_465354**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***gtcA* NP_466072**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***hly* NP_463733**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***hpt* NP_464364**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***iap/cwhA* NP_464110**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***inlA* NP_463962**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
***inlB* NP_463963**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

inlC **NP_465311**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
inlF **NP_463939**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes
inlJ **NP_466343**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
inlK **NP_464815**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
lap **NP_465159**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
lapB **NP_465191**	Yes	Yes	-	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
llsA **AHK25016**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
llsB **AHK25020**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
llsD **AHK25022**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
llsG **AHK25017**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
llsH **AHK25018**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
llsP **AHK25023**	-	-	-	-	Yes	Yes	Yes	Probable	Yes	-	Yes	-	-	-	Yes
llsX **AHK25019**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
llsY **AHK25021**	-	-	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	-	Yes
lntA **NP_463967**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
lpeA **NP_465372**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
lplA1 **NP_464456**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
lspA **NP_465369**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

mpl **NP_463734**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
oatA **NP_464816**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
pdgA **NP_463944**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
plcA **NP_463732**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
plcB **NP_463736**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
prfA **NP_463731**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
prsA2 **NP_465743**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
vip **NP_463850**	Yes	Yes	-	-	Yes	Yes	Yes	Yes	Yes	-	Yes	-	-	Yes	Yes

Appendix Table B.4 **Presence/absence of key resistance genes in L. monocytogenes strains isolated from the fresh produce supply chain.** The accession number for each gene is indicated below gene name. Legend Yes = ≥95% coverage & >75% identity, - = absent.

Isolate	**NLmo2**	**NLmo3**	**NLmo4**	**NLmo5**	**NLmo6**	**NLmo7**	**NLmo8**	**NLmo9**	**NLmo10**	**NLmo13**	**NLmo14**	**NLmo15**	**NLmo16**	**NLmo18**	**NLmo20**
bcrB **A7J11_05169**	-	-	Yes	Yes	-	-	-	-	-	-	-	-	-	-	-
bcrC **A7J11_05170**	-	-	Yes	Yes	-	-	-	-	-	-	-	-	-	-	-
fosX **A7J11_01023**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

lin **A7J11_00340**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Appendix C Sources and survival of *Listeria monocytogenes* on fresh leafy produce.

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Sources and survival of *Listeria monocytogenes* on fresh, leafy produce

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Keywords
food processing, food safety, listeria, microbial contamination, monocytogenes, soil.

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2018/0678: received 4 April 2018, revised 31 May 2018 and accepted 18 June 2018

doi:10.1111/jam.14025

Summary

Listeria monocytogenes is 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 (FLPSC) 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 FLPSC 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.

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 United States advise that *L. monocytogenes* should not be present (<1 CFU 25 g^{-1}) in ready to eat (RTE) foods that support the growth of *L. monocytogenes* and should not be equal to or above 100 CFU per g 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 per g 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 food-borne 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 *Escherichia coli*.

Appendix D *Listeria monocytogenes* from ready to eat plant produce are diverse and have virulence potential

International Journal of Food Microbiology 299 (2019) 23–32

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Listeria monocytogenes isolates from ready to eat plant produce are diverse and have virulence potential

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ARTICLE INFO

Keywords:
Listeria
Contamination
Fresh produce
Whole genome sequencing
Virulence
Food microbiology

ABSTRACT

Listeria monocytogenes is sporadically detected on a range of ready to eat fresh produce lines, such as spinach and rocket, and is a threat to public health. However, little is known about the diversity of *L. monocytogenes* present on fresh produce and their potential pathogenicity. In this work, fifteen *Listeria monocytogenes* isolates from the UK fresh produce supply chain were characterised using whole genome sequencing (WGS). Additionally, isolates were characterised based on their ability to form biofilm. Whole genome sequencing data was used to determine the sequence type of isolates based on multi-locus sequence typing (MLST), construct a core single nucleotide polymorphism (SNP) phylogeny and determine the presence of virulence and resistance associated genes. MLST revealed 9 distinct sequence types (STs) spanning 2 lineages (I & II) with one isolate belonging to the ST6 subtype, strains from which have been recently implicated in two large, food-associated *L. monocytogenes* outbreaks in South Africa and across Europe. Although most of the 15 isolates were different, comparison of core genome SNPs showed 4 pairs of 'indistinguishable' strains (< 5 SNPs difference). Virulence profiling revealed that some isolates completely lacked the Listeria pathogenicity island-3 (LIPI-3) amongst other virulence factors. Investigation of the *inlA* gene showed that no strains in this study contained a premature stop codon (PMSC), an indicator of attenuated virulence. Assessment of biofilm production showed that isolates found in the fresh produce supply chain differ in their ability to form biofilm. This trait is considered important for *L. monocytogenes* to persist in environments associated with food production and processing. Overall the work indicates that a genetically diverse range of *L. monocytogenes* strains is present in the UK fresh produce supply chain and the virulence profiles found suggests that at least some of the strains are capable of causing human illness. Interestingly, the presence of some genetically indistinguishable isolates within the 15 isolates examined suggests that cross-contamination in the fresh produce environment does occur. These findings have useful implications in terms of food safety and for informing microbial surveillance programmes in the UK fresh produce supply chain.

1. Introduction

Listeria monocytogenes is a Gram-positive, facultative anaerobic bacterial pathogen that is ubiquitous in the environment. It is the causative agent of listeriosis, an intracellular disease which predominantly affects the elderly, immunosuppressed and pregnant women along with their unborn or new-born babies. Although incidence of the disease is low compared to other foodborne pathogens (Hernandez-Milian and Payeras-Cifre, 2014), the disease outcome is often more serious, making it a priority pathogen in many countries. Foods which have been previously implicated in *L. monocytogenes* outbreaks include milk, soft cheeses, delicatessen meats and fresh produce (both fruit and vegetables). Fresh fruit and vegetables account for a significant proportion of the UK market and consumer attitudes are increasingly leaning towards healthy, convenient options such as ready to eat bagged salads and prepared fruit and vegetables (Keynote, 2015). The UK fresh produce supply chain is complex and there are many potential sources of *L. monocytogenes* contamination. These include; soil splash, contaminated irrigation water, wild animal faecal contamination and cross contamination from surfaces and personnel in the processing environment (Smith et al., 2018). Microbial testing for *L. monocytogenes* and other microbial pathogens in the supply chain of a

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https://doi.org/10.1016/j.ijfoodmicro.2019.03.013
Received 8 January 2019; Received in revised form 19 March 2019; Accepted 21 March 2019
Available online 28 March 2019