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Determination of virulence of *L. monocytogenes* strains of environmental and food sources using an invertebrate model.

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ABSTRACT

The virulence potential of 9 *L. monocytogenes* strains isolated from food products at different stages of the fresh produce supply chain and a food processing facility was determined using *G. mellonella*. Strains were used to infect *G. mellonella larvae* through the haemocoel with a dose of 10^6 CFU, which were then compared to a *L. monocytogenes* reference EGD-e and also *L. ivanovii*. Virulence was assessed by evaluating mortality rates, larvae health index score (HIS), counting viable bacteria in larvae, and the larval immunological response to infection. Significant differences in ability to cause larval mortality were observed between strains. *L. monocytogenes* strains NLmo4 and NLmo5 caused the most mortality rates, 98.8% and 96.7%, respectively, at 7 d after infection, whilst NLmo20 induced a mortality rate of 65% at the same time point, significantly differing from NLmo4&5 ($p<0.5$). Six isolates that caused the least to most mortality rates we selected and tested for ability to replicate *in vitro, in vivo* and their effect on larvae haemocyte density. Growth rates *in vitro* were significantly not different amongst *L. monocytogenes* strains as well as when compared to *L. ivanovii.* However, whilst *L. monocytogenes* strains replicated and persisted in larvae for up to 7 d after infection *L. ivanovii* was gradually cleared from larvae decreasing by 5 Logs CFU at the end of the 7 d time course. The persistence of these strain in larvae caused damage to larval organs as seen by increasing melanisation and consequently larval death. Insignificant fluctuations in haemocyte density was observed after larvae infection, however, increased expressions of the antimicrobial peptides galiomycin and gallerimycin were noted upon infection with NLmo4, EGD-e and *L. ivanovii.* Taken together, results of this study suggest *L. monocytogenes* strains found in fresh produce products have different pathogenic potentials and are potential hazards for human health.

DECLARATION

I hereby confirm that the work contained in this thesis is my own and the use of material from other sources has been fully acknowledged and or properly cited.

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List of abbreviations

AMP	Antimicrobial peptide
BHI	Brain heart infusion
CFU	Colony forming unit
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EEA	European economic area
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substance
EU	European Union
EURL	European Union Reference Laboratory
FPLC	Fresh produce supply chain
FPSC	Fresh produce supply chain
IMPI	Insect metalloproteinase inhibitor
LB	Lauria Bertani
LIPI	*Listeria* pathogenic island
Min	minute
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MN	Maternal-neonatal
NHS	National health service
PBS	Phosphate buffered saline
PO	Phenoloxidase
QAC	Quaternary ammonium compound
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RTE	Ready-to-eat
RT-PCR	Real time quantitative polymerase chain reaction
sec	second
ST	Sequence type
STEC	Shiga toxin-producing *E. coli*
VBNC	viable but non-culturable
vgc	Virulence gene cluster
VP	Virulence profile
WGS	Whole genome sequencing
WHO	World Health Organisation

Chapter 1 - LITERATURE REVIEW

1.1 General introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, facultative intracellular anaerobe frequently implicated in foodborne disease outbreaks. *Listeria monocytogenes* (*L. monocytogenes*) is ubiquitous in nature and can persist in food processing environments, causing contaminations to ready-to-eat (RTE) food products and fresh agricultural produce. When introduced into a host, often through ingestion of contaminated food products, the organism causes the foodborne illness listeriosis. In mild cases the illness will lead to febrile gastroenteritis, but in invasive cases it traverses the intestines resulting in septicaemia, meningitis, and prenatal complications in expectant mothers (preterm birth, miscarriage or stillbirth) (Vázquez-boland et al., 2001). Survivors often end up with lasting neurological sequelae (cognitive impairment, hearing loss, focal neurological deficits and epilepsy) (Lucas et al., 2016). Infants, the elderly, immunocompromised, and pregnant individuals are particularly susceptible to listeriosis (Barocci et al., 2015; Okike et al., 2013).

In the European Union and European Economic Area (EU/EEA) *L. monocytogenes* infections are relatively rare with an incidence rate of 0.6 per 100,000 people (EFSA and ECDC, 2015). However, the number of reported listeriosis cases is steadily rising as 2,502 cases were reported in 2017, a 48.4% rise from total reported cases in 2010 (ECDC, 2017). Fatality rates from *L. monocytogenes* infections are also tremendously high (20-30%) relative to other bacterial and fungal infections (≤1%) making the bacterium an ever increasing public health concern (EFSA, 2015). In the USA, for instance, though *L. monocytogenes* is estimated to cause 1,591 foodborne ill health cases annually (0.02% of total cases) it accounts for 18.9% of all foodborne related deaths. By comparison, *Salmonella* accounts for 646 times as many cases but only causes 123 more deaths (0.5% mortality rate), and although *E. coli* causes 40 times as many cases it causes 92.2% less deaths annually as compared to *L. monocytogenes* (Scallan et al., 2011). These make listeriosis a notifiable disease of great concern and reporting it as mandatory in all EU member states, including the United Kingdom (EU Directive 2003/99/EC).

Furthermore, *L. monocytogenes* also poses significant economic concerns. The bacterium can contaminate fresh produce, processed RTE meats, and dairy products

(Amato et al., 2017; Ponniah et al., 2010; Ruiz-Cruz et al., 2007). Currently, all *L. monocytogenes* strains are treated the same for regulatory purposes, thus the presence of *L. monocytogenes* on foodstuffs is sufficient to result in product recall and this costs the UK economy an estimated £245 million annually (Rayner & Scarborough, 2005). However, *L. monocytogenes* is genetically diverse and *in silico* predictions suggest there are significant differences in the disease-causing potential of different strains of the bacterium. Therefore, it is important to determine the virulence potential of isolates of the bacterium in order to evaluate the human health risks they pose.

Mammals have been the first model animals of choice for virulence testing. However, increasing concerns around their use has led to the development of alternative insect model hosts, such as larvae of the greater wax moth (*Galleria mellonella*) (Mukherjee et al., 2015; Scully and Bidochka, 2006). *G. mellonella* larvae are inexpensive, readily available, and their use avoids the ethical issues and regulatory restrictions faced with the use of other animal models. Tests using *G. mellonella* has been carried out at 37^0C, which is significant in the study of human pathogens as these are often temperature-sensitive and preferentially express virulence factors at 37^0C. This is unlike in other invertebrate models, such as *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*D. melanogaster*), that do not facilitate testing at 37^0C (Joyce & Gahan, 2010). More so, *G. mellonella* shares commonalities with mammals(Browne et al., 2013; Kavanagh and Reeves, 2007), has been used to discriminate between pathogenic species of different virulence potential (Harding et al., 2012; Mukherjee et al., 2010), with virulence observed in the model also been found to positively correlate with other mammalian models (Brennan et al., 2002; Slater et al., 2011). Thus, research groups now routinely use this model for virulence testing of many human pathogens (Camejo et al., 2009; Cotter et al., 2000; Mylonakis et al., 2005). This model was therefore used in this study to test for differences in virulence of *L. monocytogenes* strains from different food and environmental sources.

1.2 *Listeria*

1.2.1 History and Nomenclature

Though the first complete description of *Listeria monocytogenes* only dates back to about a century ago, its isolation and partial characterisation dates as far back as the 1800s. The bacterium was first described by Murray et al. in 1926, who reported discovering a short, rod-shaped, non-sporing, Gram-positive bacillus in septicemic laboratory rabbits and guinea pigs at their laboratory in Cambridge, United Kingdom. The bacterium's salient character to increase mononuclear leucocytes (white blood cells) production (monocytosis) led the authors to name it *Bacterium monocytogenes.* In their publication, however, it was acknowledged that the newly discovered organism was identical to an isolate earlier been identified as the causative agent of disease outbreaks in South Africa (Murray et al., 1926).

Between 1916 and 1924 sporadic outbreaks of plague were documented in South Africa that resulted in 204 human deaths in its later years (Mitchell et al., 1927). The plague, reported by Harvey H. Pirie, was in wild gerbils (*Tatera lobengulae*) predominantly in the sandy stretches of the Tiger River (South Africa), which became known as the ''Tiger River disease'' (Pirie, 1927). Pirie named the etiological agent *Listeria hepatolytica,* in part, due to its distinctive character of causing severe necrosis to the livers of gerbils and also to honour British Surgeon Lord Joseph Lister 'father of antiseptic surgery' (Gibbons, 1972). Having determined that Murray's *Bacterium monocytogenes* and Pirie's *Listerella hepatolytica* were identical in every physiological character tested, the name *Listerella monocytogenes* was proposed. However, this name was rejected a decade later by the Judicial Commission on Bacterial Nomenclature and Taxonomy at the third summit of the International Congress for Microbiology that was held in New York in 1939. It emerged *Listerella* had prior been adopted for a mycetozoan (slime mould) discovered by Jahn in 1906 (Jahn, 1906). This prompted the submission of a proposal for a name change to '*Listeria monocytogenes'* in 1940 by Pirie (Pirie, 1940). In 1948 '*Listeria monocytogenes'* was included for the first time in the Sixth Edition of Bergey's manual of determinative bacteriology (Hulphers, 1911), and in 1954 the name *Listeria monocytogenes* was adopted by the Scientific community as it gained the Microbiology Commission's approval (Dumon and Cotoni, 1921).

Nonetheless, though there was no complete description nor classification of *L. monocytogenes* hitherto 1926, there are multiple reports of isolations of *L. monocytogenes* resembling organisms causing infections in humans, and animals alike that with furtherance to our understanding of the organism are now believed to be *Listeria monocytogenes.* These include reports of its isolation from patients' tissues-who in retrospect had died of listeriosis-by pathologists Hayem and Henle, in 1891 (France) and 1893 (Germany), respectively, who described in their reports isolating rod-shaped, Gram-positive bacteria (Gray and Killinger, 1966). There are also reports of *L. monocytogenes* isolation from necrotic foci of a rabbit's livers by Hulphers in Sweden in 1911-who named it *Bacillus hepatis* (Hulphers, 1911); from meningitis patients in France in 1917, 1920, and 1921 (Dumon and Cotoni, 1921; Miller et al., 1990); from pigs in Russia in 1924; and a flock of sheep in Germany in 1925 (Gray and Killinger, 1966). Though these and many other reports (FAO, 2004) indicate that *L. monocytogenes* was isolated from humans and animals even prior its description in 1926, it nonetheless continued to be unrecognised as a human pathogen for decades post 1926.

1.2.2 *Listeria* as the aetiological agent of human listeriosis

Despite numerous reports of human listeriois in the late 1800s to early 1900s it was not until 1929 that *L. monocytogenes* was first reported as the aetiological agent of the disease. In 1929 NYfeldt (1929) isolated the bacterium from three patients with grandular fever who presented with symptoms of a mononucleosis-like disease in Denmark. For the first time, Nyfeldt reported *L. monocytogenes* as the causative agent. Four years later, Burn (1936) also isolated the bacterium from heart blood, ileum and colon from post-mortem samples of a human infant. The following year he encountered two other identical cases from two female human infants, and a fourth case of isolation from the brain, liver, and kidneys of a 53 year old male. In the almost following two decades leading to 1949 few more cases of human listeriosis were reported (Fischer, 1941; Kapsenberg, 1941; Savino, 1940). However, during the same time period far more cases of listeriosis were reported in animals, including cattle (Graham et al., 1943), sheep (Poppensiek, 1944), goats (Olafson, 1940), pigs (Biester and Schwarte, 1940), foal (Krage, 1944), poultry (Hoffman and Lenarz, 1942), rabbits (Schoop, 1946), dog (Wramby, 1944), wood grouse (Lilleengen, 1942), and wild rat

(Macchiavello, 1942). This enforced an earlier held believe that *L. monocytogenes* is only pathogenic in non-human subjects. In addition, the assertion of *L. monocytogenes* being the aetiological agent of human listeriosis was hardly accepted in the scientific community due to several unsuccessful attempts at the time by many research groups to culture the organism. But as routine bacterial isolation procedures at the time mostly involved directly culturing samples on simple agar media, reasons for the failed attempt to culture the bacterium are now implicit; components present in food and tissue samples can impair *L. monocytogenes* growth on unenriched nonselective media (Beumer and Hazeleger, 2003), and *L. monocytogenes* can reach a viable but non-culturable (VBNC) state making it undetectable by conventional culturing techniques (Giao and Keevil, 2014; Lindbäck et al., 2010).

It was not until the late 1940s the consensus started to emerge as links were established associating listeriosis to consumption of contaminated food products. An outbreak of listeriosis in Eastern Germany and Czech Republic between 1949 and 1957 saw increased research efforts in listeriosis. During the outbreak, at the Institute of Pathology of the University of Halle, Germany, Potel (Potel, 1951) isolated *L. monocytogenes* from two new-borns who suffered acute septicaemia. This was linked to the consumption of contaminated milk by the mother. In the following years Potel et al. (Reiss et al., 1951a, 1951b) also documented 83 more cases around Halle and Jena (Germany), and at the same time other authors (Benda, 1953, Patocka et al., 1956; Suchanova et al., 1958) reported cases totalling 53 in Prague, Czech Republic. Leading to 1960 over 472 cases of human listeriosis had been reported in Europe and the USA (as reported by (Gray and Killinger, 1966). These in conjunction with H.P.R. Seeliger's publication of his monograph 'Listeriosis' in 1955 (reviewed in 1957), which reviewed *Listeria* literature in the preceding almost 50 years, saw sustained research effort in *Listeria* and acknowledgment of it being the causative agent of human listeriosis. Ascribing a source to human listeriosis however remained contentious and this was evidence by failure by the World Health Organisation to recognise the bacterium as a foodborne pathogen, and *L. monocytogenes* been listed under the section of ''Bacteria Not Conclusively Proved to Be Foodborne'' in the Second edition of '*Foodborne Infections and Intoxications*' by Riemann and Bryan (Riemann, 1979) in 1979. However, a 1981 *Listeria* outbreak in the Maritime Provinces of Canada provided conclusive evidence on the long contended problem. The epidemic strain (4b) was isolated from a coleslaw sample obtained from the freezer of one of the

patients. The product, it was discovered, was manufactured by a producer who obtained cabbage from a farm that had a flock of sheep. Compost and raw manure from the flock were been used to fertilise the vegetables and it occurred that two sheep had died of literiosis at the farm in the previous three years. Publication of these findings by Schlech et al. (1983) followed by several similar cases in the subsequent years (Fleming et al., 1985; Ho et al., 1986; Linnan et al., 1988) saw *Listeria* been listed as a 'priority foodborne pathogen' by the WHO in 1986 (as reported by Pal et al. (2017). Though this spurred regulations and directives in the EU, for instance, that prioritised all members of the genus *Listeria* it is now implicit that not only is over four-fifth of the members of the genus non-pathogenic, strains within the pathogenic species also exhibit varied pathogenic potentials, as discussed later.

1.2.3 *Listeria spp.* and discovery

The first species of the genus *Listeria* to be identified was that described by E.G.D. Murray, R.A. Webb and M.B.R. Swann in 1926 (Murray et al., 1926). The strain, EGD (later named EGD-e with 'e' denoting 'European'), belongs to the first species of the genus to be discovered (*Listeria monocytogenes*). Until 1948 *L. monocytogenes* remained the only species comprising the genus *Listeria.* Consequently that year, Sohier et al. (1948) isolated *Listeria denitrificans* from boiled ox blood in Germany, which was later transferred to a new genus, *Jonesia* (Rocourt et al., 1987). In 1955 Ivanov (Ivanov, 1962) isolated the now known second member of the genus *Listeria ivanovii,* formerly *Listeria monocytogenes* serotype 5' and *Listeria bulgarica* (Heinz et al., 1984), from lambs with congenital listeriosis in Bulgaria. As at the moment of writing, nineteen *Listeria* species have been discovered, most of which in recent years (Table 1. 1).

Expansion of the genus in recent years has been credited to the rapid development of whole genome sequencing technologies with the genus now divided into two groups: (i) *Listeria sensu stricto*, which constitutes six species that share common phenotypic characteristics (e.g. flagella motility, growth in low temperatures) and consists of *L. monocytogenes, L. ivanovii, L. marthii, L. seeligeri, L. welshimeri,* and *L. innocua*; and (ii) *Listeria sensu lato,* which consists of the remaining 13 members of the genus, generally regarded as non-pathogenic, non-motile (except *L. grayi* and *L. costaricensis* (Larsen et al., 2018), and nitrate reducing (except *Listeria floridensis* and *L. goaensis* (den Bakker et al., 2014; Doijad et al., 2018). However, only two, *L. ivanovii* and *L.*

monocytogenes, are pathogenic to humans and animals. But due to the rarity of *L. ivanovii* infections in humans, nine documented cases since 1970 as at the moment of writing (Beye et al., 2016), only *L. monocytogenes* is regarded as a priority pathogen and of a global public health concern (Allerberger and Wagner, 2010). Thus, this report will be based on *L. monocytogenes* hereafter.

Table 1. 1 *Listeria spp*., discovery dates and sources of isolation. This table excludes any bacterial species that were prior identified as *Listeria* but later reclassified to other genera.

Listeria species	**Year Discovered**	**Sample isolated from (Country)**	**Reference**
L. monocytogenes	1926	Rabbits and Guinea-pigs	Murray et al. (1926)
L. ivanovii	1955	Lambs (Bulgaria)	Ivanov (1962)
L. grayi	1966	-	Larsen & Seeliger (1966)
L. innocua	1981	Soil (Germany)	Seeliger (1981)
L. seeligeri	1983	Soil (Germany)	Rocourt & Grimont (1983)
L. welshimeri	1983	Decaying vegetation (USA)	Rocourt & Grimont (1983)
L. marthii	2010	Forest Soil (New York, USA)	Graves et al. (2010)
L. rocourtiae	2010	Lettuce (Salzburg, Austria)	Leclercq et al. (2010)
L. fleischmannii	2013	Hard cheese (Switzerland)	Bertsch et al. (2013)
L. weihenstephanensis	2013	Freshwater pond (Germany)	Halter et al. (2013)
L. floridensis	2014	Running water (Florida, USA)	Bakker et al. (2014)
L. riparia	2014	Running water (Florida, USA)	Bakker et al. (2014)
L. cornellensis	2014	Water (Colorado, USA)	Bakker et al. (2014)
L. aquatic	2014	Running water (Florida, USA)	Bakker et al. (2014)
L. grandensis	2014	Water (Colorado, USA)	Bakker et al. (2014)
L. booriae	2015	Dairy processing plant (USA)	Weller et al. (2015)
L. newyorkensis	2015	Seafood processing plant (USA)	Weller et al. (2015)
L. costaricensis	2018	Food processing facility (Costa Rica)	Montero et al. (2018)
L. goaensis	2018	Mangrove swamps (Goa, India)	Doijad et al. (2018)

1.2.4 *L. monocytogenes* classification: lineages and serotypes

A combination of physiological and molecular based approaches have been used to group *L. monocytogenes* strains into four evolutionary groups, termed lineages I, II, III, and IV, and at least 13 serotypes. While lineage groupings use multigene phylogenetic to determine the relatedness of *L. monocytogenes* strains to one another, serotyping classify strains base on variations on the group-specific surface antigens somatic (O) and flagellar (H). Thus, each *L. monocytogenes* lineage comprises multiple serotypes with some serotypes overlapping lineages. Methods such as multilocus enzyme electrophoresis (MLEE), agglutination, Enzyme-linked immunosorbent assay (ELISA), ribotyping, and DNA-based techniques, such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and PCR-restriction fragment length polymorphism (PCR-RFLP) have been used to enable these classifications. In 1940 the first method used for *L. monocytogenes* subtype delineation was developed by Paterson (1940). As it had become apparent that *L. monocytogenes* organisms do not form a homogenous group, based on infection outcomes and physiological characteristics, Paterson collected and examined fifty-eight strains isolated from human and animal sources around Europe. Agglutination tests were performed for presence of the somatic (O) and flagellar (H) agents, and *L. monocytogenes* strains were assigned into four groups based on the correlation outcomes of the two tests, marking the first *L. monocytogenes* groupings recorded. With several modifications to this method (Orsi et al., 2011) and further propositions on what constitutes a better way to group strains in the following years, in 1989 Piffaretti et al. (1989) grouped *L. monocytogenes* strains into two distinct phylogenetic divisions or lineages (I & II) using MLEE typing. The technique which involved analysing 16 genetic loci that encode metabolic enzymes was employed to analyse one hundred and seventy-five *L. monocytogenes* strains of human, animal, food, and environmental origins. Similar results were obtained using PFGE by Brosch et al. (1994) on a similar sample size (176 isolates) but with better discriminatory capability, as was ribotyping of an even larger sample size (305 isolates) (Graves et al., 1994), all validating the earlier reported groupings.

Lineage III was added to the list following analysis of DNA sequences of three virulence-associated genes, flagellin (*flaA*), the invasive associated protein (*iap*), and listeriolysin 0 (hly), in 1995, which revealed that *L. monocytogenes* strains are more

diverse than earlier indications suggest (Rasmussen et al., 1995). An analysis of 46 lineage III isolates and earlier reported lineages I & II strains (Roberts et al., 2006) indicated that *L. monocytogenes* strains belonging to lineage III could further be divided into three groups (IIIA, IIIB, and IIIC). This observation was based on differences found in the virulence-related gene *actA* (encoding proteins needed for cytoplasmic movement) and stress-response gene *sigB*. In 2008 lineage IIIB was reported as been phylogenetically divergent enough from lineages IIIA & IIIC and thus should be considered as lineage IV (Ward et al., 2008).

L. monocytogenes lineages contain disproportionate numbers of serotypes with some serotypes overlapping lineages. The *L. monocytogenes* surface antigen somatic (O) consists of 15 subtypes (I-XV) and the flagellar (H) comprises four subtypes (A-D). Using unique combinations of O & H from serological reactions between the two, Seeliger & Höhne (1979) identified at least thirteen *L. monocytogenes* serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7). Lineages I and II contain a higher proportion of serotypes, and lineage IV with fewer and less characterised serotypes (**Table 1. 2**).

Table 1. 2: *L. monocytogenes* lineages and serotypes groupings based on pooled data from sequencing and serotyping reports by various authors.

L. monocytogenes lineage	Serotypes within lineage	Reference
I	1/2b, 3b, 3c, 4b, 4d, 4e, 7	(Borucki & Call, 2003; Kuenne et al., 2013; Piffaretti et al., 1989)
II	1/2a, 1/2c, 3a, 3c	(Borucki & Call, 2003; Ward et al., 2008, 2004)
III	4a, 4b, 4c,4d	(Borucki & Call, 2003; Roberts et al., 2006; Ward et al., 2008)
IV	4a, 4b, 4c	(Roberts et al., 2006; Ward et al., 2008)

L. monocytogenes sequencing and serotyping has proved to be useful tools in surveillance of listeriosis outbreaks in both human and animal cases (CDC, 2011; FDA, 2014; Schlech et al., 1983). More importantly, in the food industry where *L. monocytogenes* contaminations remain a serious concern, it has been used to track contaminating strains at various stages of the food chain, and to identify strains that are persistent in the food processing environment to help mitigate contamination incidences. However, given that at least 95% of all human listeriosis cases and incidences of food contaminations are caused by only three serotypes (as discussed in the following section) the value of serological data is also rather limited.

1.2.5 Prevalence of *L. monocytogenes* lineages and serotypes

L. monocytogenes lineages have different, but overlapping, ecological niches with serotypes of given lineages being more prevalent in animals and humans. Lineage IV, the least described, comprises only three serotypes (4a, 4b, 4c) that have been predominantly isolate from animal sources. Cases of human infections with lineage IV isolates and their isolation from food substances remain rare. Unsurprising, as a lineage consisting of serotypes until fairly recently grouped as lineage III, it has similar prevalence rates to lineage III serotypes. For example, of the 112 food isolates characterised by Ward et al. (2004) only 2% were of serotypes now known to belong to lineages III and IV. Also, in a far more larger study consisting of 994 *L. monocytogenes* isolates from food products and clinical human samples by Gray et al. (2004), prevalence rates of lineages III and IV isolates were 0.4% in food products, and 2.24% in clinical human samples. More studies have found *L. monocytogenes* prevalence rates of <1% in food products (Norton et al., 2001) and as ranging from 0% to 1.8% in clinical human cases (Jeffers et al., 2001; Norton et al., 2001; Wiedmann et al., 1997). However, prevalence rates of these lineages are higher in animals with studies showing incidence rates of 10-11% (Jeffers et al., 2001; Wiedmann et al., 1997).

In contrast, lineage I and II serotypes dominate listeriosis incidence rates in humans, animals, and food products, with lineage I been more prevalent in clinical human cases, and lineage II slightly more prevalent in animal and food products. For instance, a surveillance study by Norton et al. (2001) that characterised 117 food isolates found prevalence rates of 36.8% and 63.2% for lineages I and II, respectively. Also, studies on animal isolates have shown prevalence rates of 42.1% and 47.4% for lineage I and

II serotypes, respectively (Jeffers et al., 2001; Wiedmann et al., 1997), all indicating a higher prevalence of lineages II in food products and animals as compared to lineage I. Conversely, comparative studies have shown lineage I serotypes to have higher prevalence in clinical human cases as compared to lineage II. These studies show a range from 65% to 69.1% for lineage I, and 29.1% to 35% for lineages II serotypes prevalence in clinical human samples (Jeffers et al., 2001; Norton et al., 2001; Wiedmann et al., 1997).

Taken into context, more than 95% of the clinical human cases are caused by only three serotypes (1/2a, 1/2b, and 4b) belonging to lineages I and II (Kathariou, 2002). This has led some to hypothesize that lineages I and II isolates have increased pathogenic potential in humans in comparison to lineages III and IV (Jeffers et al., 2001; Wiedmann et al., 1997). However, as lineage I and II serotypes also predominate in food isolations this has led many to suggest that pathogenicity in humans is due to the higher exposure rates to these serotypes as compared to lineages III and IV serotypes that are rarely isolated from food products (Roberts et al., 2006). Yet, the latter view did not support the fact that whilst lineage II serotypes are over represented in food contaminations, lineage I serotypes predominate human clinical infections. Hence, pathogenic potential could be as a result of underlying genotypic differences in serotypes which enable them to colonise and adapt to different host and ecological environments, as discussed in the subsequent sections.

1.3 *L. monocytogenes*: a priority foodborne pathogenic bacteria

1.3.1 Natural niches and prevalence

1.3.1.1 *L. monocytogenes* prevalence in soil and water

L. monocytogenes is ubiquitous in nature and has been isolated from various sources including soil, decaying vegetation, water courses, and animals (livestock and wildlife) which are believed to serve as the natural reservoirs of the bacterium. Evidence of *L. monocytogenes* survival in soils was first reported by Welshimer (1960) who tested the bacterium's survival in clay and fertile garden soil. The bacterium was found to survive for over 28 weeks in both soil types without nutrient supplementation. Similar findings showing *L. monocytogenes* ability to survive in sterile and natural sandy, clay

loam, and sandy loam soils (Dowe et al., 1997). While *L. monocytogenes* numbers in soils are mostly low, prevalence rates are relatively high. For example, a 2013 nationwide study of *L. monocytogenes* incidences in French soils that examined 1315 soil samples found a prevalence rate of 17% but only with a bacterial density of 2.88 x 10^4/g of dry soil (Locatelli et al., (2013). An earlier investigation (Welshimer & Donker-Voet, 1971) also reported a prevalence rate of 33% in samples collected from 12 farms during three seasons. Variations in prevalence rates in soil of 9% to 51.4% have also been reported by other authors (Strawn et al., 2013; Weis & Seeliger, 1975), with studies of its isolation from vegetation and different natural soil environments including forests, meadows, creeks, pastures, and mountainous regions (Linke et al., 2014; Sauders et al., 2012; Weis & Seeliger, 1975; Welshimer, 1968) also been reported. These demonstrate soil is an environmental *L. monocytogenes* niche but also show the bacterium's ability to adapt to diverse soil environments.

Water is also an ecological niche for *L. monocytogenes*. A study (Watkins & Sleath, 1981) examining nine surface river waters reported isolating the bacterium from all the samples tested. Also, a study (Stea et al., 2015) that examined *L. monocytogenes* prevalence in urban and rural watersheds around Nova Scotia, Canada, reported a detection frequency of 17.5%. In another two year longitudinal study of waters around five locations in New York, USA (Strawn et al., 2013), a 28% prevalence rate for *L. monocytogenes* was reported; higher than any of the other bacterial species tested for, *Salmonella* (9%), and Shiga toxin-producing *E. coli* (STEC) (2%). Other studies have reported *L. monocytogenes* prevalence rates of 8.6% to 67% in water courses (Colburn et al., 1990; Dijkstra, 1982; Lyautey et al., 2012; Rodas-Suárez et al., 2006) with reported loads of up to 1.8x10^2 counts/l (Watkins & Sleath, 1981). However, in almost all these studies, the authors suggested or affirmed outflow of agricultural effluents into water courses, runoffs, or contaminations with sewage sludge for the high prevalence rates of *L. monocytogenes*. This, it is believed, is due to numerous other studies that have reported livestock as being reservoirs of the bacterium.

1.3.1.2 *L. monocytogenes* in livestock and livestock feed.

A study by Strawn et al. (2013) analysing faecal samples of cattle at farms around the New York metropole reported *L. monocytogenes* prevalence rate of 15%. Prevalence rates were even higher in earlier findings by a previous study (Watkins & Sleath, 1981) who reported *L. monocytogenes* incidences in all livestock sewage and sewage sludge

samples that were examined. This study found *L. monocytogenes* concentrations of up to 1.8 x 10^4 counts/l in the sewage and sludge samples. In a more recent study (Boscher et al., 2012) *L. monocytogenes* was detected in 14% of the 172 pig faecal samples that were analysed with *L. monocytogenes* incidences in 25% of the 43 farms investigated. Conversely, a 2007-2008 exploratory study on 14 pig farms in France (Fosse et al., 2011) reported no *L. monocytogenes* incidences. The direct streaking method used in this study was however cited by other authors as a potential reason for the no detection, due to the low sensitivity of the technique.

An earlier study (Fenlon et al., 1996) of farms around Aberdeenshire, Scotland, suggested that the incidences of *L. monocytogenes* in the faeces of livestock is linked to contaminated animal feeds, such as spoiled silage been fed to livestock. However, this cannot explain the numerous cases of *L. monocytogenes* isolations from other animals, including zoo animals (Bauwens et al., 2003; Kalorey et al., 2006), companion animals such as cats, dogs (Wramby, 1944), and wildlife; rabbits (Schoop, 1946), wood grouse (Lilleengen, 1942), wild rat (Macchiavello, 1942), racoon, otter, moose, and deer (Lyautey et al., 2007). In addition, clinically healthy humans reportedly carry the bacterium in their guts. A study of 92 clinically healthy office personnel (Kampemacher & Jansen, 1969) reported a *L. monocytogenes* recovery rate of 11.9% from faecal samples of these individuals. An even higher rate of 13.3-29.1% was reported for abattoir workers at different sampling points over a two year period in the same study. These indicate that, though animal feeding regiments may increase faecal shedding of *L. monocytogenes* in ruminants, clinically healthy animals can serve as reservoirs of the bacterium. Nevertheless, silage (fermented grass or green fodder commonly used to feed ruminants) is reportedly *L. monocytogenes* niche. Various studies (Fenlon et al., 1996; Grant et al., 1995; Sharifzadeh et al., 2015; Tasci et al., 2010) have reported isolating *L. monocytogenes* from silage with concentrations of up to 1.1 x 10^6 CFU/g (Fenlon et al., 1996). This, however, is reportedly due to contaminations from soil sources during ensiling, and also the poor quality of the silage which is often caused by ensiling plant matter with inadequate moisture content, or poorly fermenting silage, caused by loosely packing silos which boost aerobic conditions (Fentahun & Fresebehat, 2012; Perry & Donnelly, 1990). Hence, these facilitate growth of this bacterium and when fed to ruminants help exacerbate its dissemination back into the environment through direct faecal shedding or via spreading of slurry on farmlands, but also to consumers through animal products

such as meat, cheese, and milk. Nevertheless, the contaminated environment, the variety of the contaminated food product(s) and the genotype of the contaminating *L. monocytogenes spp.* are pivotal in the transmission of the bacterium, as discussed in the subsequent sections.

1.3.2 *L. monocytogenes* persistence in the fresh produce food chain

L. monocytogenes contaminations of fresh produce mainly emanate during growth at the fields or at the food processing facility (Smith et al., 2018). The ability of *L. monocytogenes* to persist in these environments enables it to contaminate fresh agricultural produce, at both the pre and post-harvest stages, all facilitating its dissemination. Persistence refers to the consistent isolation of a bacterial strain from the same location over a period of several months or years. Various hypotheses have been proposed for *L. monocytogenes*' persistence in various environments including; high prevalence and ability to withstand severe conditions in outdoor environments; enhanced tolerance to cleaning agents and disinfectants in processing facilities; and enhanced adherence to the surfaces of both food processing environments and the food products themselves (Bae et al., 2013; Cox et al., 1989; Lundén et al., 2002). The persistence of *L. monocytogenes* in farmlands, which can cause contaminations during plant growth, and at the processing environment, which poses a serious challenge for contamination post-harvesting, will be discussed.

1.3.2.1 *L. monocytogenes* persistence in fresh produce farmlands

Though soil is a natural ecological niche for *L. monocytogenes*, as discussed above, the practice of applying animal manure, and fresh sewage sludge to farmlands as fertilisers, or irrigating with livestock wastewater, is also responsible for increasing bacterial numbers and in the dissemination of the bacterium in the environment (Ivanek et al., 2006; Schlech et al., 1983). It is common practice to apply sewage sludge and other livestock manures to agricultural lands by many fresh produce farmers. Yet, for every 1-2 tonnes of sludge applied a *L. monocytogenes* load of 10^6-10^8 CFU is introduced per hectare of farmland per year (Garrec et al., 2003). It was reported by Nicholson et al. (2005) who examined survival of four faecal pathogens in livestock manure that *Listeria* can survive in dairy slurry for up to 6 months; twice that observed for any of the other pathogens investigated in the study. Also, it was seen in (Welshimer, 1960) demonstration earlier that *L. monocytogenes* can survival for up to

295 days in non-sterile soils. A more recent study by Locatelli et al. (2013) of 100 soil samples (representative of all soil types around France) also found that *L. monocytogenes* can survive up to 84 days in 71% of the tested soil types following inoculation with an initial dose of $1x10^6$ CFU/gram of dry soil. Thus, the further introduction of *L. monocytogenes* to farmland could result in its persistence in the soil long enough to cause contaminations to growing crops. In fact, Watkins & Sleath (1981) isolated $1.6x10^2$ CFU/100g of soil at 8 weeks post-sewage sludge application to a farmland, with no indications of a significant decline in bacterial numbers anytime soon post that time point; which is about the length of time it can take for spinach to grow to a harvestable stage in the field (Conte et al., 2008). However, reasons for *L. monocytogenes* persistence in soil environments is only partially understood.

As both biotic and abiotic factors, such as soil pH, moisture content (Dowe et al., 1997), soil type (Dowe et al., 1997; Welshimer, 1960), clay content, and soil microflora have been evidenced as pivotal in *L. monocytogenes* persistence in soil (Locatelli et al., 2013; McLaughlin et al., 2011; Vivant et al., 2013), studies have also explored the role of intrinsic bacterial factors in *L. monocytogenes*' persistence in soil environment. While experimental data on the mechanisms of *L. monocytogenes* survival and persistence in soil is still limited, there has been a few documenting these processes in recent years. A study of *L. monocytogenes* strains reported a correlation between strain motility and soil survival (McLaughlin et al., 2011). The study found that the typically motile *L. monocytogenes* strain EGD-e and hyper-motile strain CD83 had increased soil survival when compared to other two *L. monocytogenes* EGD-e mutants (Δ*fliP* and Δ*fla*) that lacked motility.

Bacterial metabolism is also vital for *L. monocytogenes* survival in soil as this regulates nutrients acquisition, transport, and expulsion of toxic substances, among others, enabling adaptation to various environments. In *L. monocytogenes* the alternative stress factor SigmaB regulates expression of numerous genes involved in such processes that enable its adaptation (Becker et al., 2000; Ferreira et al., 2001). While this is only partially understood in *in vitro* conditions, Piveteau et al. (2011) reported on expressions of these genes in soil environments. In this study soil extracts (40ml) and sterilised soil (35g) were inoculated with the *L. monocytogenes* strain EGD-e at an initial dose of $1x10^5$ CFU/ml and $1x10^5$ CFU/g, respectively, and monitored over a year period. EGD-e populations were enumerated periodically with a whole-genome arrays analysis also conducted. Bacterial populations of 10^6 cfu/ml and 10^3 cfu/g were

reported at the end of the one-year period, signifying *L. monocytogenes*' persistence in various soil conditions. Also, in the whole-genome arrays analysis SigmaB regulated genes expressions were prevalent, such as the ABC transporters and PTS used in nutrient acquisition, and catabolising enzymes. This study provided insights into a transcriptional re-profiling in *L. monocytogenes* in soil environments, which implicated stress regulators in its persistence in soil.

In a further study SigmaB was found to be a determinant is *L. monocytogenes* survival in soil (Gorski et al., 2011). In this study, contaminated soil samples were seeded with either of a SigmaB wild-type or SigmaB mutant of the *L. monocytogenes* serotype 4b. Over the course of the four weeks of this investigation a 3-5 log units differences in survival was reported in the soil samples between the wild-type and the mutant, with the lesser survival being in the SigmaB mutant. However, as *L. monocytogenes* was still recovered throughout the course of this investigation it indicates that other factors are also used by the bacterium to persist in soil. Evidently, this was demonstrated in a latter study by Vivant et al. (2015). In this study, an *L. monocytogenes* response regulator AgrA was investigated in 10 soil microcosms. *agrA* regulates genes encoding proteins, such as those involved in cell envelope and cellular processes, the ABC transporters, and antimicrobial resistance peptides, among others. They later observed that the mutant EGD-e strain (Δ*agrA*) had significantly reduced survival in soil when compared to the parental strain.

Other bacterial intrinsic factors such as the possession of plasmids-which can enable resistance to heavy metals, oxidative stress, multidrug efflux-are also probable soil persistence enablers (Harvey & Gilmour, 2001; Lebrun et al., 1992; McLauchlin et al., 1997). Though a plasmid carried by a virulent *L. monocytogenes* lineage IV isolate (FSL J1-208) was found to have no role in resistance to antibiotics nor heavy metals by den Bakker et al. (2012), with the vast array of plasmids in environmental *L. monocytogenes* isolates (Harvey & Gilmour, 2001) the possibility of their roles in soil persistence cannot be ruled out. Nonetheless, as the interplay of these numerous factors enable the bacterium's persistence in soil they in effect enable it to contaminate fresh produce during growth, and agricultural machinery during harvesting, all resulting in its transmission to food processing facilities where the cycle of transmission and persistence can continue.

1.3.2.2 *L. monocytogenes* persistence in food-processing environments

During fresh produce harvesting contamination to harvesting equipment and personnel can be caused by *L. monocytogenes* contaminated soils, but also by contaminated fresh produce (Lianou and Sofos, 2007). As is often the case, fresh produce are sent to a dedicated processing facility post-harvest to be cut, washed and packaged for consumption. Despite strict sanitary and hygiene measures implemented at many food processing facilities *L. monocytogenes* have been known to be introduced into the processing environment through field-contaminated machinery used to transport fresh produce, the harvesting personnel's clothing and footwear, and raw materials (Camargo et al., 2017; CDC, 2011). At the processing facility control of *L. monocytogenes* is a challenge as it can persist for years.

Once introduced into the processing environment *L. monocytogenes* can colonise various sections of the plant including work benches, sinks, grinders, dicers, floor surfaces, walls, standing water, interior of drainages, niches (hard to clean sites) and fresh produce washing tanks, among others (Cox et al., 1989; Lundén et al., 2002). It is common practice in many facilities to disinfect the processing environment and equipment with quaternary ammonium compounds (QACs) such as sodium dichloroisocyanurate, sodium hypochloride, and peracetic acid (Camargo et al., 2017). As reviewed by Camargo et al. (2017), the use of alternative means such as bacteriophages, ultraviolet radiation, and ultrasonic treatments, among others, have also been trialled with some gaining industry approval.

However, *L. monocytogenes* is capable of growth at a broad range of conditions that are often observed at processing facilities. Hudson et al. (1994) reported that temperatures as low as -1.5^0C can initiate *L. monocytogenes* growth and that it can also survive in temperatures as high as 45^0C, making it a psychrotroph and a mesophile. Upregulation of *L. monocytogenes* genes that cause increased metabolism and adaptation when exposed to low temperatures was reviewed recently by Saldivar et al. (2018). Protein transporters such BetL, Gbu, and OpuC which facilitate osmolytes uptake; HisJ, TrpG, CysS, and AroA which are needed for amino acids metabolism at low temperatures; and stress response genes such as *trxB, flp,* and *rpoN* were all reported to be upregulated at low temperatures. Additionally, *L. monocytogenes* is also reported to grow at broad pH ranges of 4.3 to 9.4 (Giffel & Zwietering, 1999), which can enable the bacterium to survive washes by many acid

based disinfectants. Even so, of major concern is the ability of *L. monocytogenes* to form biofilms which enables it to persist.

Biofilms are a collection of microbial cells that are embedded in a layer of extracellular polymeric substances (EPSs). When *L. monocytogenes* colonies are deposited on a surface they attach, grow and multiply to form a monolayer consisting of a colony of fewer cells, then they produce EPSs to entrap organic and inorganic debris and other microorganisms, they continue to multiply forming a larger mass of multi-layered micro-colonies known as the *L. monocytogenes* biofilm (Di Ciccio et al., 2012). *L. monocytogenes* is able to form biofilms as early as 4 hours when deposited on a surface and can do so in a wide range of temperatures (4^0C-37^0C) (Giao & Keevil, 2014). Biofilm formation enables the bacterium to adhere to various materials in the processing environment, such as stainless steel surfaces, floors, wastewater pipes, seals, interior of drainages, bends in pipes, and conveyor belts, among others (Beresford et al., 2001; Colagiorgi et al., 2017). The formation of biofilms can enhance protection of the bacterium from biocides, detergents or sanitisers (Klæboe et al., 2006), and other cleaning agents that may be used at the facility. Though the use of disinfectants and frequent cleaning routines may be effective in clearing most of the formed biofilms (Chaitiemwong et al., 2014), the last stage of the biofilms formation process complicates its eradication from the processing environment. As part of the last stage, cells get detached from the mature biofilm and are released into the environment resulting in colonisation of new niches and a repeat of the cycle, thus resulting in persistence. Though conditions such as temperature, exposure to sanitisers or detergents, among others, are all known to induce the formation of biofilms (Jefferson, 2004), the mechanisms of its formation in *L. monocytogenes* is still partially understood.

Flagella motility was reported as essential for biofilm formation by Lemon et al. (2007). In this study, a wild-type environmental strain of a *L. monocytogenes* serovar commonly associated with human literiosis (1/2a) and non-motile mutants of the parent strain were used. The mutant strains were observed to have defective attachment to glass and steel, and were unable to form mature biofilms. However, the inability to form mature biofilms could be attributed to the reported defective attachment to these surfaces as attachment is essential for biofilms formation. The *L. monocytogenes agr* operon has also been implicated in biofilm formation. The operon, which consists of four genes (*agrB, agrD, agrC,* and *agrA*), is required in bacteria

species for cell-to-cell communication during biofilm formation and maturation. Rieu et al. (2007) carried out an in-frame deletion of *agrA* and *agrD*, a transcriptional regulator and a propeptide encoding gene, respectively, in the *L. monocytogenes* strain EGD-e. The authors observed that the mutants had significantly reduced adherence to glass and polystyrene surfaces. Also, biofilm formation within the first 24 hours of the experiment was impaired when compared to the parent strain. Similarly, a EGD-e *agrD* mutant (Δ*agrD*) was found to have impaired biofilm formation on polystyrene in another study by Riedel et al. (2009). The authors also reported reduced invasion in Caco-2 intestinal epithelial cells and attenuated virulence in mice by the mutant strain. In contrast, enhanced adherence to polystyrene was observed by Vuong et al. (2000) in *S. aureus* when a mutant (Δ*agr*) was tested against a wild-type; suggesting the role of the operon in biofilm formation is species-dependent.

1.3.3 *L. monocytogenes* contaminations and prevalence in RTE fresh produce

Food contamination by *L. monocytogenes* can occur at any point in the fresh produce food chain, from the pre-harvest to post-harvest stage (farm-to fork), through direct or indirect sources including water, soil, dirty equipment, animals (wild and domesticated), and human handling, as summarised in ***Figure 1.1***. Early reports of *L. monocytogenes* isolation from food products dates as far back as 1949 (Potel, 1951), and thereafter numerous listeriosis outbreaks have been linked to fresh produce and other RTE foods such as soft cheese, milk, frozen corn, and cabbage, among others (Dalton et al., 1997; Fleming et al., 1985; Ho et al., 1986; Linnan et al., 1988; Schlech et al., 1983). Although *L. monocytogenes* contamination has also been documented in other food products such as beef, pork, and poultry products (Gómez et al., 2015; Ingham et al., 2004), this section will only assess prevalence of the bacterium in fresh produce.

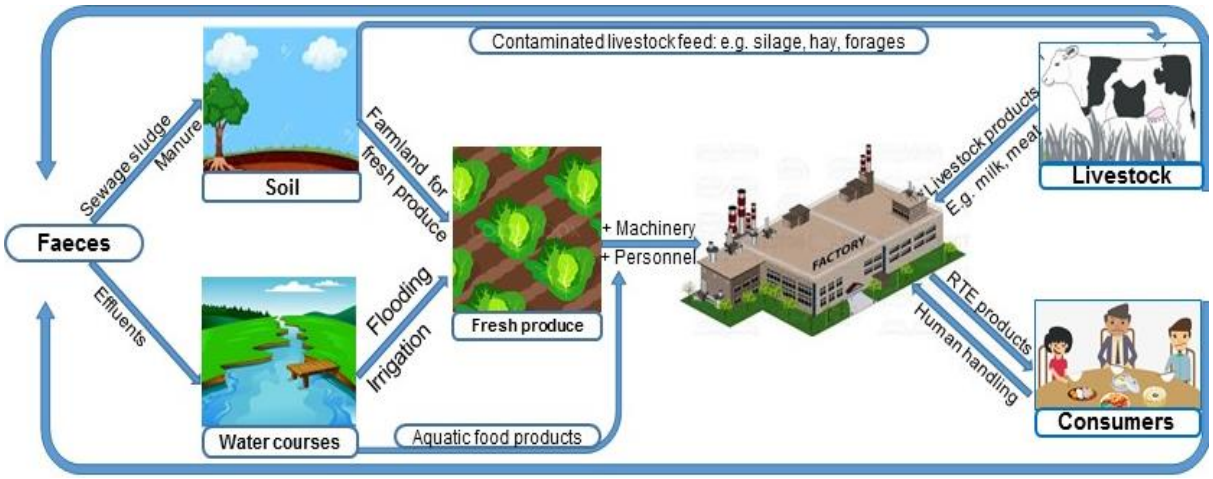


Figure 1.1*:* Potential sources of contamination, and transmission of *L. monocytogenes* in the fresh produce food chain.

L. monocytogenes has been found to survive in a wide variety of fresh produce products. However, prevalence rates have been found to vary among various products. A 2010 study by Ponniah et al. (2010) of randomly selected commercial vegetables at four markets in Selangor, Malaysia, discovered *L. monocytogenes* in all 10 vegetable types tested, 100% prevalence. However, *L. monocytogenes* was only Isolated from 69 of 306 (22.5%) samples that were investigated. Higher prevalence rates were found in yardlong bean (31.3%) and Japanese parsley (27.2%), with lower incidence rates reported in Winged bean and Indian pennyworth (15.6%). Other vegetables that tested positive for *L. monocytogenes* include cabbage, cucumber, and sweet potatoes. Lower prevalence rates were reported in a later study by Althaus et al. (2012). The authors who investigated bacterial burden in RTE fresh produce in Swiss markets reported a prevalence rate of 3.5% (5 of 142 samples collected). However, all *L. monocytogenes* isolations were from RTE lettuce samples with no reported incidences in the sprouts and fresh-cut fruits samples investigated. Similar observations were reported by Hossein et al. (2013) in a survey of markets around Northern and Eastern Tehran, Iran, where a prevalence rate of 7% was reported. Interestingly, whilst the previous study by Althaus et al. found only one *L. monocytogenes* serotype (1/2a) in all *L. monocytogenes* positive samples, four serotypes (1/2a, 1/2b, 3b, and 4b) were found contaminating vegetables in this study. However, most of the serotypes were isolated from RTE mayonnaise salads (3 serovars: 1/2a, 1/2b, and 4b), and cucumber (4 serovars), whilst only two serotypes

were found on lettuce and one in cabbage, 1/2a and 1/2b, and 1/2a, respectively. Several other studies demonstrating the prevalence of *L. monocytogenes* in fresh produce in recent years are summarised in Table 1.3:

Table 1.3: Incidences and prevalence of *L. monocytogenes* in selected fresh produce products from selected studies between 2010 and 2017.

Produce	Country	Prevalence[a]	Reference
Beans	Malaysia	64 (24, 37.5%)[c]	Ponniah et al. (2010)
	Chicago, USA	-	FDA (2014)
Cabbage	Malaysia	32 (12, 37.5%)	Ponniah et al. (2010)
	Iran	50 (3, 6%)	Hossein et al. (2013)
	Brazil	11 (2, 18.2%)	Sant'Ana et al. (2012)
Carrot	Malaysia	33 (8, 42.9%)[b]	Ponniah et al. (2010)
	India	60 (1, 1.7%)	Mritunjay & Kumar (2017)
Cucumber	Malaysia	32 (14, 43.8%)	Ponniah et al. (2010)
	Iran	50 (7, 14%)	Hossein et al. (2013)
	Greece	-	Nastou et al. (2012)
	India	60 (3, 5%)	Mritunjay & Kumar (2017)
Collard greens	Brazil	30 (1, 3.3%)	Oliveira et al. (2010)
	Brazil	24 (1, 4.2%)	Sant'Ana et al. (2012)
Lettuce	Iran	50 (5, 10%)	Hossein et al. (2013)
	Switzerland	142 (5, 3.5%)[d]	Althaus et al. (2012)
	Greece	-	Nastou et al. (2012)
	Brazil	152 (3, 2.0%)	Sant'Ana et al. (2012)
Parsley	Malaysia	49 (21, 24.2%)	Ponniah et al. (2010)
	Poland	30 (3, 10.0%)	Szymczak et al. (2014)
	Greece	-	Nastou et al. (2012)
	Brazil	22(1, 4.5%)	Oliveira et al. (2010)
Spinach	Brazil	11 (1, 9.1%)	Sant'Ana et al. (2012)
	India	60 (8, 13.3%)	Mritunjay & Kumar (2017)
	Ireland	-	FSAI (2018)
Sweet potato	Malaysia	32 (9, 28.1%)	Ponniah et al. (2010)
	Poland	30 (9, 30%)	Szymczak et al. (2014)
Tomato	Malaysia	32 (7, 21.9%)	Ponniah et al. (2010)
	India	60 (4, 6.7%)	Mritunjay & Kumar (2017)

[a] Number of total analysed samples (number, and percent of positive sample for *L. monocytogenes)*, [b] Includes Wild and Japanese Parsley, [c] include Winged and Yardlong beans, and [d] RTE lettuce, **-** (prevalence rates not reported for product).

1.3.4 EU regulations on *L. monocytogenes* in RTE food products

Within the European Union (EU) every level of the food chain is controlled by food safety regulations. As EU regulations, these are binding and affect all member countries, and any foreign food supplier that exports food into the EU. As per 2005 regulations, effective January 1st 2006, and amended in 2007, the European Commission regulation (EU Commission, 2007) categorises RTE food products into 3 categories of 2 food groups (Table 1.4).

Table 1. 4: Microbiological criteria for *L. monocytogenes* in food products as per European Commission regulation (EU Commission, 2007). The critical limits as indicated are the limits during the shelf-life of the product, except where stated in the food notes.

RTE-food category	Critical limit	Comment on criteria
Support growth	None in 25g [(a)]	For foods intended for infants and individuals with special medical needs
Support growth	100 cfu/g [(b)]	For foods not intended for infants and individuals with special medical needs.
Unable to support growth - pH ≤ 4,4 or aw ≤ 0,92, -pH ≤ 5,0 and aw ≤ 0,94, -shelf-life < days	100 cfu/g	For foods not intended for infants and individuals with special medical needs.

(a) If food product is intended for persons other than infants and ones with special medical needs, before dispatch none must be detected in 25g but can be detected during shelf-life of the product.

(b) If the manufacturer is not able to demonstrate the product will not exceed 100 cfu/g lower limits must be set.

RTE foods are grouped into two; ones that support *L. monocytogenes* growth and ones that do not. Criteria for foods not supporting *L. monocytogenes* growth are based on pH, water activity and shelf-life of the product, and challenge studies can be conducted as per the European Union Reference Laboratory (EURL) Technical guidelines of June 2014 (Beaufort et al., 2014) to ensue this determination. In addition to the two food groups, the intended consumer of any of the food products was used to categorise products into three; (a) foods that support *L. monocytogenes* growth that are intended for infants and person(s) with special medical needs (i.e. immunocompromised, organ transplants, etc.), (b) foods that support growth but not

intended for the former two groups of people, (c (i)) food that cannot support growth and intended for any group of consumers, and (c (ii)) food that cannot support growth and intended for any consumer. In all categories, foods intended for infants and persons with special needs must have less than 25 cfu/g during the shelf-life of the product. Foods intended for individuals, other than infants and persons with special medical needs, that support *L. monocytogenes* growth can have up to 100 cfu/g during the shelf-life of the product. However, it must be demonstrated by the manufacturer that the product will not exceed the limit of 100 cfu/g throughout the shelf-life of the product. If this cannot be demonstrated, no *L. monocytogenes* should be detectable in 25g of the product at the time it is to be dispatched by the manufacturer. For food products that require the absence of *L. monocytogenes* in 25g and intended for infants and persons with special medical needs, the manufacturer must test ten samples to ascertain this and five samples if not intended for these groups of people. As demonstrated by Hunt et al. (2018) using the EURL guidelines, examples of RTE products that do not support *L. monocytogenes* growth include coleslaw and cheese and ones that do support growth include smoked salmon and pork.

1.4 An overview of *L. monocytogenes* virulence factors

Though *L. monocytogenes* lives as a saprophyte in the environment it can transition into a disease causing organism (pathogen) when it enters into a human or animal (host), all due to the possession of a range of molecules (virulence factors) that enable it to replicate and disseminate intracellularly (virulence). Virulence factors refer to any secreted bacterial gene products that may contribute in the bacterium establishing itself on or within its host. In *L. monocytogenes* these factors are encoded by genes that cluster in groups known as virulence gene clusters (*vgc*) within chromosomal loci referred to as *Listeria* pathogenic islands (LIPIs). To date, all, except one (Bigot et al., 2009), of the identified *L. monocytogenes* virulence factors are chromosomally encoded. In *L. monocytogenes* three pathogenic islands have been identified, LIPI-1, LIPI-3, and LIPI-4. LIPI-1 'the central virulence gene cluster' is a 9kb chromosomal region of six genes (*prfA, plcA, plcB, hly, mpl, actA*) and a potential 7th (*orfX*) encoding the proteins PrfA-a virulence genes transcriptional regulator, PlcA & B-two phospholipases, LLO-hemolysin, Mpl-zinc metalloproteinase, ActA-actin assembly inducing protein, and OrfX-a secreted microphages' oxidation response dampener, respectively (Figure 1.2). LIPI-1 encode proteins necessary for the intracellular growth

and motility of *L. monocytogenes* and is found on all sequenced *L. monocytogenes* strains (Vázquez-boland et al., 2001).

LIPI-3 is the second *L. monocytogenes* vgc to be identified and it encodes a second hemolysin/cytolysin, listeriolysin S (LLS). LLS is vital in *L. monocytogenes* survival in human polymorphonuclear neutrophils (PMNs) and escape from the phagosomes of macrophages in murine models, thus contributing to the bacterium's virulence (Cotter et al., 2008). LIPI-3 consists of a vgc of 8 genes (*llsA, llsG, llsH, llsX, llsB, llsY, llsD,* and *llsP*) (Figure 1.3) encoding yet to be fully annotated proteins, and the only understanding held about them are by comparisons to other known proteins with closest identity. Presumptive functional roles include a peptide toxin (LlsA), components of an ABC transporter (LlsG, and LlsH), post-translational modification enzymes (LlsB, LlsY, and LlsD), a protease (LlsP) and a *L. monocytogenes* protein of undetermined function (LlsX) (Quereda et al., 2017). The island has been identified exclusively in a subset of *L. monocytogenes* lineage I strains, and only recently on atypical *L. innocua* strains (Clayton et al., 2014). Among *L. monocytogenes* lineage I strains LIPI-3 has been identified in only 52-72% of all sequenced strains (Clayton et al., 2014; Cotter et al., 2008; Kim et al., 2018), most of which have been strains frequently associated with sporadic and epidemic listeriosis outbreaks, such as serotypes 1/2b and 4b (EFSA & ECDC, 2018; Ho et al., 1986; Pérez-trallero et al., 2014). This has led many to postulate that LIPI-3 may after all provide answers to the whys and wherefores of the enhanced virulence of lineage I isolates.

However, the precise mechanisms through which LIPI-3 encoded proteins contributes to virulence is still barely understood.

Listeria pathogenic island (LIPI)-4 was only identified recently following analysis of 6,633 *L. monocytogenes* strains of food and clinical origin by Maury et al. (2016). Reports on *L. monocytogenes* isolates found to contain LIPI-4 has been mainly from lineages I and II isolates. Among these the serotype 4b isolates, which predominates in clinical human cases and also frequently implicated in listeriosis outbreaks, are the most LIPI-4 bearers (Kim et al., 2018). The island consists of six genes (Figure 1.4), most of which uncharacterised, and include the cellobiose-family phosphotransferase system (PTS). The PTS permeases are systems used by bacteria to transport carbohydrates and often consist of three domains, seldomly four (EIIA, EIIB, EIIC, and EIID), that enable the utilisation of carbon sources. In microbes PTSs have been grouped into seven (Barabote & Saier, 2005), and one of these is PTS^{Lac} which in turn

is divided into six families. Of the six PTS^{Lac} families PTS^{Lac}-4 is reported to be the main transporter of the disaccharide cellobiose (Stoll & Goebel, 2010), and this cellobiose-family PTS is the main constituent of LIPI-4 (Maury et al., 2016). Therefore, as carbon metabolism has been reported to modulate virulence (Eisenreich et al., 2010), the discovery of the *L. monocytogenes* LIPI-4 formed the basis for investigating its virulence potential.

The virulence of this gene cluster was evaluated by Maury et al. (2016) in a mouse model whereby a mutant lacking the entire cluster (∆PTS) was created. Bacterial growth prior to mouse infection was determined in culture media and ∆PTS was reported to have no impaired growth when compared to EGD-e. In the mouse model the parent strain and ∆PTS strain were tested against EGD-e on their ability to invade the central nervous system (CNS) and other organs (spleen, liver). Interestingly, a considerably reduced ability to infect the CNS was observed in ∆PTS whilst the parent strain showed better fit than EGD-e, but no differences in ability to colonise other tissues was observed among the three strains. The tests were also carried out in a mouse maternal-neonatal (MN) infection model with similar results only this time it was impaired infection in the placentas and foetuses of pregnant mice. Competitive index experiments using ∆PTS and an isogeneic PTS complemented strain were also carried out. In summation, the results thus show the PTS locus of LIPI-4 strains enhances CNS and MN tropism and contribute to strain virulence. However, as not all epidemic strains contain this pathogenic island it indicates the involvement of other factors in CNS and MN listeriosis.

Nevertheless, not all *L. monocytogenes* genes encoding virulence factors are within the above mentioned classical virulence gene clusters as several others lie outwith these regions on the bacterium's chromosome. These include the internalin genes (*inlA, inlB, inlC*, *inlF, inlJ,* and *inlK*), *aip* (invasion associated protein), *hpt* (hexose phosphate transporter), and transcriptional regulators such as *virR* (virulence regulator), *sigAB* (stress response factors), and *mogR* (motility gene repressor) that all enhance *L. monocytogenes* virulence. Though there exists overlapping functionalities of these vast arrays of *L. monocytogenes* virulence factors and with certain factors of distinct yet virulence roles, these can be generally categorised into four groups based on their roles in infections: adhesion to cells; cell invasion or internalisation; nutrient mobilisation and intracellular growth; escape from vacuoles and immune cells; and cell-to-cell spread.

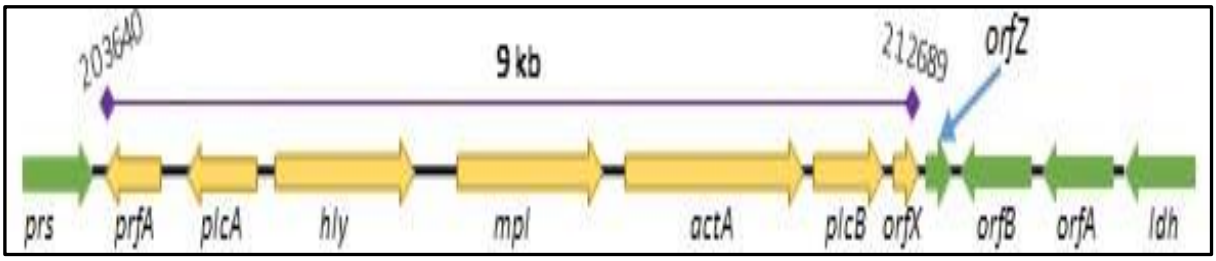


FIG 1.2: Transcriptional organisation of the chromosomal region of the virulence gene cluster LIPI-1 of *L. monocytogenes.* Yellow arrows show LIPI-1 genes and green arrows show flanking loci. *orfX,* only added to LIPI-1 recently, is only partially characterised and is therefore coloured green. Figure is as adapted from (Vázquez-boland et al., 2001).

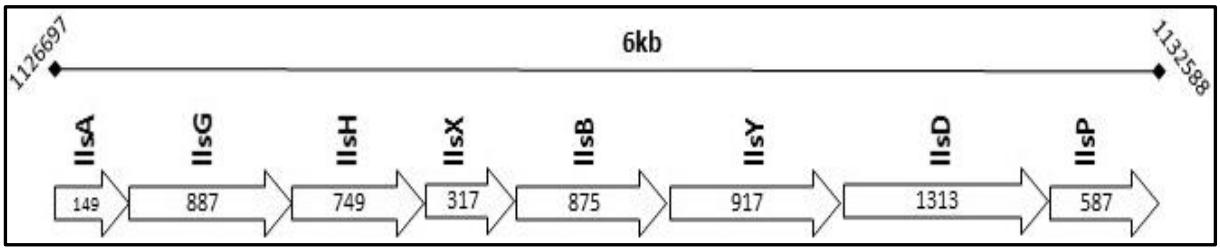


FIG 1.3 Arrangement of the haemolysin S (LLS) gene cluster (LIPI-3). The size of the genes, in base pairs, are enclosed within the arrow. Indicated at the cross line at the top is the size of the cluster and its position on the chromosome of *L. monocytogenes* serotype 4b strain F2365. Figure is adapted from (Cotter et al., 2008).

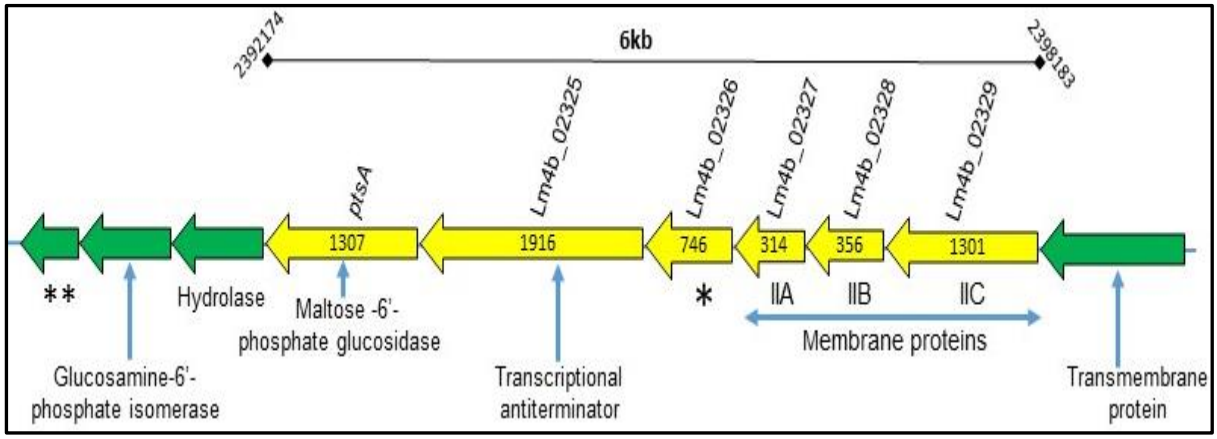


FIG 1.4: Chromosomal arrangement of LIPI-4 of *L. monocytogenes* serotype 4b. Yellow arrows show genes encoded within the cluster and green arrows show flanking loci. The encoded gene products are shown below the genes, and the size of the genes, in base pairs, are shown within the arrows. Indicated at the cross line at the top is the size of the cluster and its position on the bacterial chromosome. *, a PTS associated protein of undetermined characteristic; **, a hypothetical protein. Figure is adapted from (Maury et al., 2016).

1.5 *G. mellonella* as a model for studying *L. monocytogenes* virulence.

Mammalian models have been the gold standard for *in vivo* experimentation, however there are now increasing social and ethical concerns which has subjected their use to strict laws and regulations (Trevijano-Contador & Zaragoza, 2014). This is evidenced by the introduction and expansions of the 3Rs that now underpins the use of animals in scientific research, which enforces refinement of conventional scientific procedures around animal experimentation and a need for reducing the number of test animals in research if they cannot be entirely replaced (ASPA, 2012). Consequently, there is a desperate need for alternative, yet suitable, host models that approximate mammalian systems, and this has led to a surge in the development of insect models. As essential features of the innate immunity of insects share commonalities with mammalian systems (Browne et al., 2013; Kavanagh & Reeves, 2007; Muller et al., 2008) this has enabled researchers to extrapolate human-pathogen interactions from these insect infection models. This in addition to the readily availability of insects, rapid growth rates, ease of handling, low rearing costs, and ethical acceptance of their use in scientific research has modelled insects as the potential vanguards of scientific research (Mukherjee et al., 2015; Scully & Bidochka, 2006; Vilcinskas, 2011).

Larvae of the greater wax moth (Lepidoptera: Pyralidae [*Galleria mellonella*]) is an emerging and a useful model host now widely used as an alternative experimental model. *Galleria mellonella* (*G. mellonella*) larvae (Figure 1.5) at the sixth instar stage are used in experimental studies (Tsai et al., 2016). Tests using this model can be conducted at the physiological temperature of humans (37^0C), unlike other invertebrate models such as *C. elegans* and *D. melanogaster* (fruit fly), which is significant as it enables studying human pathogens at conditions mimicking their natural hosts environments. Compared to *C. elegans*, fruit flies and many other invertebrates, *G. mellonella* has a relatively more complex innate immune system akin to mammals that makes it more suitable for modelling human infections (Ramarao et al., 2012). Evidently, it is now recurrently used as a viable alternative to animal models for antimicrobials testing (Aperis et al., 2007; Betts et al., 2014; Cools et al., 2019; Dean et al., 2011; Krezdorn, Adams, & Coote, 2014; Luther et al., 2014; Polenogova et al., 2019; Thomas et al., 2013), disease modelling (Bohovych et al., 2016; Li et al., 2018; Loh et al., 2013; Mukherjee et al., 2013), and virulence determination of

numerous human pathogens (Camejo et al., 2009; Cotter et al., 2000; Harding et al., 2012; Mukherjee et al., 2010; Mylonakis et al., 2005; Sousa et al., 2018).

G. mellonella lacks specialised phagocytic cells and an adaptive immune system (Fallon et al., 2011), as can be found in mammals, however, it has features analogous to mammals both in structure and functions that enables it to effectively prevent an/or clear microbial infections. Such includes a physical first line of defence larval cuticle akin to a mammalian skin, haemolymph found in the larval body cavity or haemocoel which is analogous to mammalian blood in that it transports nutrients, metabolites, waste products, and the immune response haemocyte cells that are also comparable to mammalian neutrophils (Bergin et al., 2003; Browne & Kavanagh, 2013; Fuchs et al., 2010; Pereira et al., 2018). The *G. mellonella* haemocytes, a significant number of which are also found associated with the larval fat body-an organ functionally homologous to a mammalian liver (Lemaitre & Hoffmann, 2007; Meister et al., 1997), consists of six identified classes, including coagulocytes, granulocytes, oenocytoids, plasmatocytes, prohemocytes, and spherulocytes (Boman & Hultmark, 1987). These, haemocytes, recognise and phagocytose pathogens and initiate microbial killing through proteolytic enzymes activation, serving functionally mediating roles also similar to mammalian leukocytes (Jones, 1962; Siddiqui & Al-Khalifa, 2014).

Similarly, the mediating role of haemocytes is complemented by antimicrobial peptides (AMPs) synthesised by the larval fat body through haemocyte activation signalling pathways (Ferrandon et al., 2007; Marmaras & Lampropoulou, 2009; Park et al., 2005) that include insect metalloproteinase inhibitors (IMPI), cecropin-like peptides, and peptides also analogous to human defensins such as galiomycin and gallerimycin (defensin 1 and 2, respectively) (Mak et al., 2010; Mukherjee et al., 2011). Notwithstanding, microbial killing is initiated by an immunological response that has also been likened to the induction of the complement cascade in mammalian systems (Gillespie et al., 1997; Pereira et al., 2018), in that bacterial or fungal polysaccharides recognition by haemolymph proteins such as cecropins, GmCP8 and lectins (Kim et al., 2010; Klunner et al., 1994; Zdybicka-Barabas et al., 2019) opsonize pathogens forming nodulations that physically restrict growth and lead to pathogen elimination. These have also been as observed in virulence and disease modelling studies of many human pathogens that reported correlation between *G. mellonella* and mammalian models such as mice and rats (Table 1.5). These in addition to delivery routes options for microbes and or pharmaceuticals at variable doses, such as through oral

(Dubovskiy et al., 2016; Lange et al., 2018), injection (Harding et al., 2013), and topical applications (Scully & Bidochka, 2005; Wrońska et al., 2018) has modelled *G. mellonella* as a viable alternative to mammalian models.

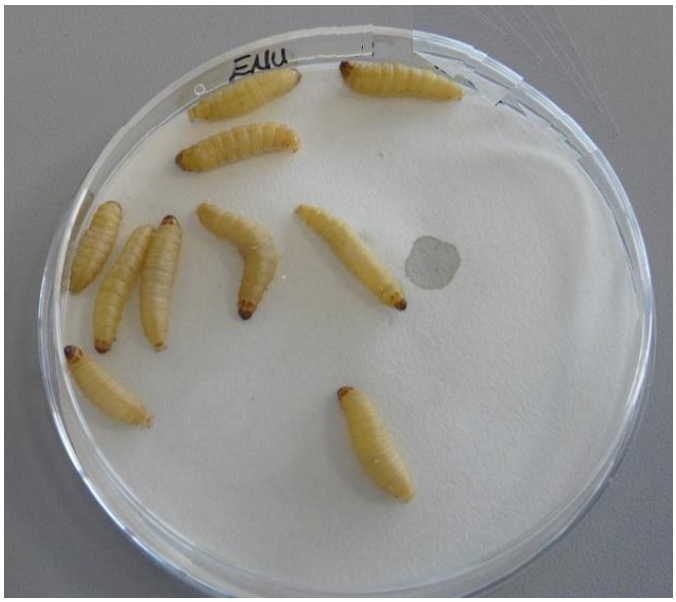


FIG. 1.5:
Galleria mellonella larvae (Lepidoptera: Pyralidae) at sixth instar stage.

Table 1.5: A selection of studies demonstrating correlation between *G. mellonella* and mammalian models using various.

Microbial spp. investigated	**Reference**
Aspergilus fumigatus	Slater et al. (2011)
Burkholderia cepacia	Seed & Dennis (2008)
Burkholderia pseudomallei, Burkholderia oklahomensis, Burkholderia thailandensis	Wand et al. (2011)
Candida albicans	Brennan et al. (2002)
Candida krusei	Scorzoni et al. (2013)
Escherichia coli	Ignasiak & Maxwell (2017)
Legionella pneumophila	Harding et al. (2012)
Listeria monocytogenes	Mukherjee et al. (2013)
Mycobacterium smegmatis	Ignasiak & Maxwell (2017)
Mycobacterium tuberculosis	Li et al. (2018)
Pseudomonas aeruginosa	George et al. (2000), Jander et al. (2000), Ignasiak & Maxwell (2017)
Streptococcus pneumoniae	Evans & Rozen (2012), Ignasiak & Maxwell (2017), Cools et al. (2019)
Yeast species	Cotter et al. (2000)
Yersinia pseudotuberculosis	Champion et al. (2009)

1.6 Rationale and objectives for this study.

Currently, all *L. monocytogenes* strains are treated the same for regulation purposes as potentially virulent isolates. However, *L. monocytogenes* is genetically diverse and *in silico* predictions suggest there are significant differences in the disease causing potential of different strains. In addition, the body of knowledge available that informed these regulations are mainly based on *L. monocytogenes* strains originating from clinical samples, whilst the virulence potential of *L. monocytogenes* strains isolated from ready-to-eat (RTE) foods and from the environment remains to be determined. Nonetheless, as agricultural activities increase leading to more interactions with the environment and the demand for RTE foods been on a daily rise, the possibility of people consuming *L. monocytogenes* contaminated foods are higher. Therefore, it is important to determine the virulence potential of food and environmental *L. monocytogenes* isolates in order to evaluate the risks they pose to human health. In this project, the differences in virulence potential of nine *L. monocytogenes* isolates collected from RTE fresh agricultural produce and from the environment were thus assessed in an invertebrate model with the following objectives;

1) Validate a model of *L. monocytogenes* virulence in larvae of the Great wax moth (*Galleria mellonella*)
2) Determine virulence of the environmental and food strains of *L. monocytogenes* in this model through survival assays.
3) Assess the immunological response of *G. mellonella* to infections with the various *L. monocytogenes* strains.

Chapter 2 - VALIDATION OF A MODEL OF *LISTERIA MONOCYTOGENES* INFECTION IN *GALLERIA MELLONELLA*

2.1 Introduction

In order to determine the virulence of different *L. monocytogenes* strains, infection models that approximate listeriosis pathogenesis in mammalian systems are required. The larvae of the greater wax moth *Galleria mellonella* have been successfully used to study virulence of a number of *Listeria* species including clinically derived *L. monocytogenes* strains. However, there is a lack of standardisation using this model which often results in variability of reported outcomes by research groups despite using similar methodologies; such is as seen with these group A streptococcus (GAS) studies (Bohovych et al., 2016; Patterson et al., 2013) that reported a 0% and 20% survival for the same strains at identical doses. This is known to be largely due to the source of larvae, their handling, and inocula preparations for larval infections (O. Champion, Titball, & Bates, 2018). Furthermore, the model has not been used to determine virulence of *L. monocytogenes* strains from food and environmental sources. Therefore, it was of importance to firstly validate *L. monocytogenes* infection in this model before it could be used to determine virulence of the *L. monocytogenes* strains investigated in this project.

The *L. monocytogenes* strain EGD-e (NCTC7973, serotype 1/2a) is the best characterised of the *L. monocytogenes* strains and the most cited in the literature. It is a laboratory strain derived from the isolate that was originally isolated from guinea pigs by E.G.D. Murray et al. (1926). We therefore selected EGD-e as our reference strain for this investigation. In this chapter we validated a model of *L. monocytogenes* infection in *Galleria mellonella* by firstly evaluating EGD-e growth *in vitro* to enable standardisation of bacterial inocula, and then evaluated the sensitivity of *G. mellonella* larvae to EGD-e infections. Also, the work adapted the *Galleria mellonella* health index scoring system (HISS) to assess larvae health post infection.

2.2 Methodology

2.2.1 Bacterial growth rate and inoculum size determination.

The *L. monocytogenes* strain EGD-e (reference strain, NCTC7973) was grown overnight in a shaken incubator (200rpm, 37ºC) in broth culture of brain heart infusion (BHI, Oxoid) media (calf brain 2.5g/L, disodium hydrogen phosphate 2.5g/L, D^+ glucose 2g/L, Nacl 5g/L), made up with distilled water and sterilised by autoclaving at 120ºC for 15 minutes. The optical density (OD_{600} nm) was recorded and the planktonic cultures were diluted to a starting OD_{600} of 0.05. The cultures were again incubated under the same experimental conditions and the OD_{600} nm recorded at hourly intervals for 7 hours. To enumerate the viable bacterial colony forming units (CFU) at each recorded OD_{600}, 100ul of culture was collected at hourly intervals, following OD_{600} nm determination, and serially diluted and plated on Oxford agar base (Oxoid Ltd) supplemented with *Listeria* selective antibiotics amphotericin B (10ug/ml), colistin sulphate (20ul/ml), acriflavin (5ul/ml), ceotetan (2ul/ml), and fosfomycin (10ul/ml). The agar plates were incubated at 37ºC and CFU determined after 24 hours. A calibration curve of OD_{600} and Log CFU was generated to determine the viable bacterial CFU ml^{-1} over time.

2.2.2 Bacterial culture preparations and *Galleria mellonella* larvae.

The *L. monocytogenes* strain EGD-e cultures were prepared by inoculating 10ml BHI broth with a colony of EGD-e and incubating at 37ºC in a shaking incubator (200rpm, aerobic conditions). The planktonic cultures, in stationary phase, were diluted in fresh BHI to an OD_{600} of 0.42 which corresponds to 10^9 CFU ml^{-1}, as determined from the growth curve (section 2.2.1). Cells were harvested by centrifugation at 12,000 x g for 10 min at 22ºC. Prior to inoculation the growth media was removed by washing cells twice and then re-suspended in PBS to the required concentrations.

Galleria mellonella larvae were purchased from UK Waxworms Ltd, Sheffield, UK. Larvae were in their final instar stage and were stored at 20ºC until used for bacterial challenge. Initial experiments were conducted on *G. mellonella* larvae 7 days after their arrival from the producers. This model was modified (for reasons explained in section 2.3.3) and all subsequent experiments were carried out on larvae a day post-delivery by the producer.

2.2.3 *Galleria mellonella* larvae infection.

Only *G. mellonella* larvae weighing 0.25-0.35g were used in all experiments. Experiments were repeated three times using larvae from a different batch each time. To assess dose-depended survival of *G. mellonella* larvae to EGD-e infections, larvae were separated into three treatment groups, using n = 30 per group. EGD-e planktonic culture concentrations of 10^9 CFU ml^{-1} 10^8 CFU ml^{-1} and 10^7 CFU ml^{-1} were prepared, and for each treatment group larvae were inoculated with 20µl corresponding to a dose of 10^7 CFU, 10^6 CFU and 10^5 CFU, respectively, per treatment group. The infecting dose was determined from OD_{600} values (2.2.1) and were confirmed by serial dilution and plating prior larval infection. *G. mellonella* larvae were inoculated with 20µl of the relevant culture into the last right proleg using an insulin syringe as described previously (Joyce & Gahan, 2010), and control larvae were inoculated with 20ul of PBS. Larvae were incubated in the dark at 37ºC in 9cm petri dishes lined with Whatman paper for the duration of the experiment.

2.2.4 Monitoring of *Galleria mellonella* larvae post bacterial infection.

Post infection larvae were examined individually on a daily basis. To check mortality larvae were turned over to look for movement of legs, healthy larvae up righted themselves and dead larvae showed no movement. To evaluate the overall health index of the larvae the following attributes were examined daily as described in previous studies (Loh et al., 2013): activity, cocoon formation, cuticle melanisation, and survival. For each attribute a score was assigned and scores were totalled to give an overall score for the health index of each larva. A healthy uninfected larva will usually be assigned a total score of 8 to 9, with infected dead larva commonly scoring 0. This scoring system was adapted from (Loh et al., 2013) and is as summarised in Figure 2.4.

2.3 Results and discussion

2.3.1 *L. monocytogenes* growth rate *in vitro*.

The *L. monocytogenes* strain EGD-e (NCTC7973) is the most characterised of the known strains of the species. We therefore used it as a reference strain to establish the virulence of *L. monocytogenes* strains in *G. mellonella* larvae. We began by characterising EGD-e growth parameters in order to determine the bacterial inocula that can be used to infect *G. mellonella* larvae. Figure 2.1 shows that the OD_{600} nm threshold value of 0.05 was approximately equivalent to 8.0 log CFU ml^{-1}, and a linear relation between the initial inoculum and the optical density was observed after this point. After 4 hours of incubation an OD_{600} nm of ~1.2 was observed, the rapid bacterial growth observed prior to this point slowed down for the remainder of the investigation; and this was also true for viable EGD-e CFU ml^{-1}. Bacterial growth peaked at ~$2.8x10^9$ CFU ml^{-1} after 7h incubation.

EGD-e growth in these experimental conditions was enabled and this was consistent with findings by other research groups (Rea et al., 2004; Schär et al., 2010). It was determined that after 7 h incubation EGD-e growth had reach stationary phase, as seen in both plots of optical density and CFU ml^{-1}. Earlier reports by Schär et al. (2010) also observed EGD-e can reach stationary growth after 6 h incubation at 37ºC, with similar findings also reported by Muchaamba et al. (2019) for EGD-e growth in BHI at 37^0C. It was significant to make this determinations due to reported differences in virulence profiles of *L. monocytogenes* in logarithmic and stationary growth phases (Hain et al., 2008; Riedel et al., 2009), which can have varied characteristics in virulence testing (Bortolussi et al., 1987). To ensure consistency and reproducibility of our results, we therefore used stationary phase planktonic cultures in all subsequent *G. mellonella* larvae infections. Using the above generated curve (Fig. 2.1) we were also able to determine the bacterial CFU ml^{-1} at any given OD_{600} nm, which was later used to determine bacterial inocula for *G. mellonella* infections.

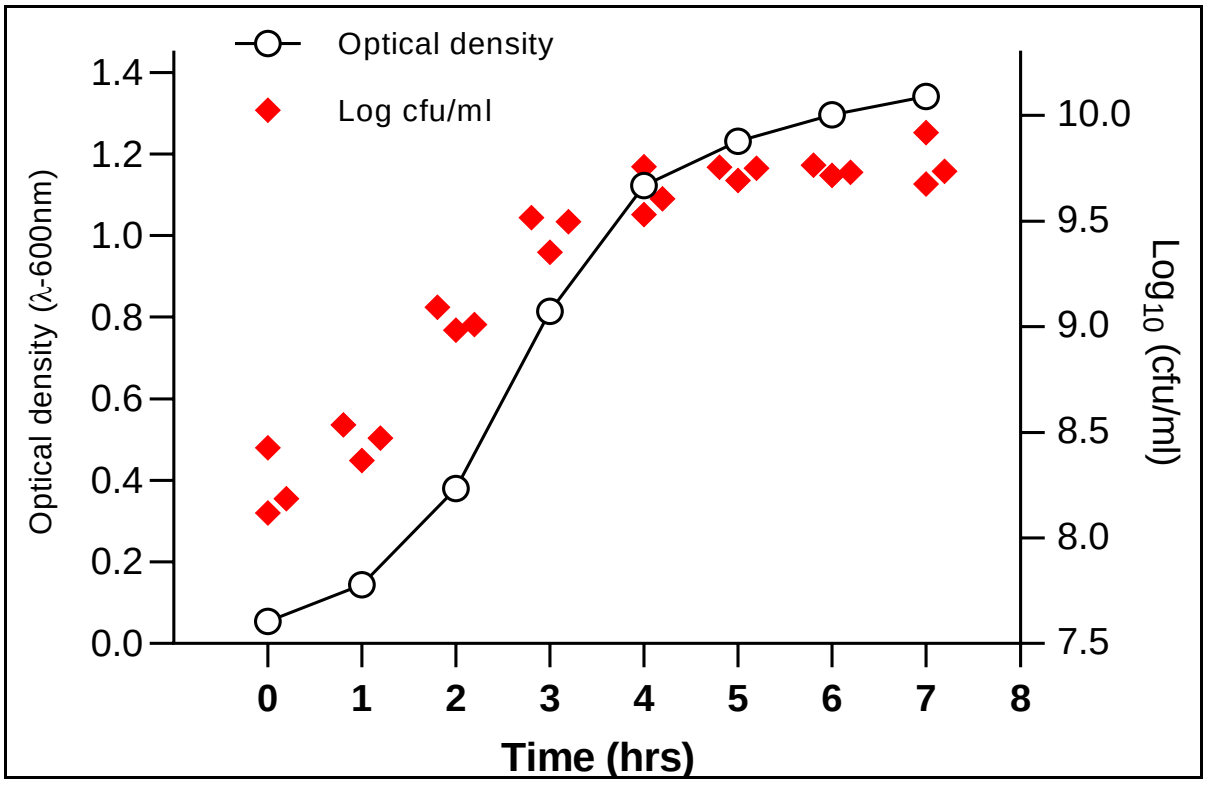


FIG. 2.1: *L. monocytogenes* strain EGD-e growth in BHI broth at 37^{0}C. Growth was monitored by absorbance measurements and plate counting at hourly intervals. Platting was carried out on Oxford agar supplemented with *Listeria* selective antibiotics (OXOID). Results represent three independent determinations.

2.3.2 *Galleria mellonella* larvae are sensitive to *L. monocytogenes* infection.

In order to determine if *G. mellonella* larvae could be used as a model to assess the virulence of *L. monocytogenes* strains, the well characterised *L. monocytogenes* laboratory strain EGD-e (reference strain, NCTC7973) was used to infect insect larvae. To evaluate the sensitivity of *G. mellonella* larvae to EGD-e infections we carried out dose-dependent survival assays. Larvae were delivered to the laboratory and incubated at 20ºC in the dark, with no food, for 7 d prior infection assays. Larvae (0.25 to 0.35g) were divided into three groups (*n* = 30 per group) and each group was inoculated with one of three EGD-e doses; 10^5 cfu/larva, 10^6 cfu/larva or 10^7 cfu/larva delivered in 20µl of PBS. A control group (*n* = 30) was inoculated with 20µl of PBS. Inoculations were carried out intrahaemocoelically via the last right proleg, and subsequently larvae were incubated at 37ºC. Mortality was assessed at 24, 48, and

72 h post-inoculation (p.i) by touch-induced lack of larval movement. No mortality was observed in the control PBS-inoculated larvae (Figure 2.2).

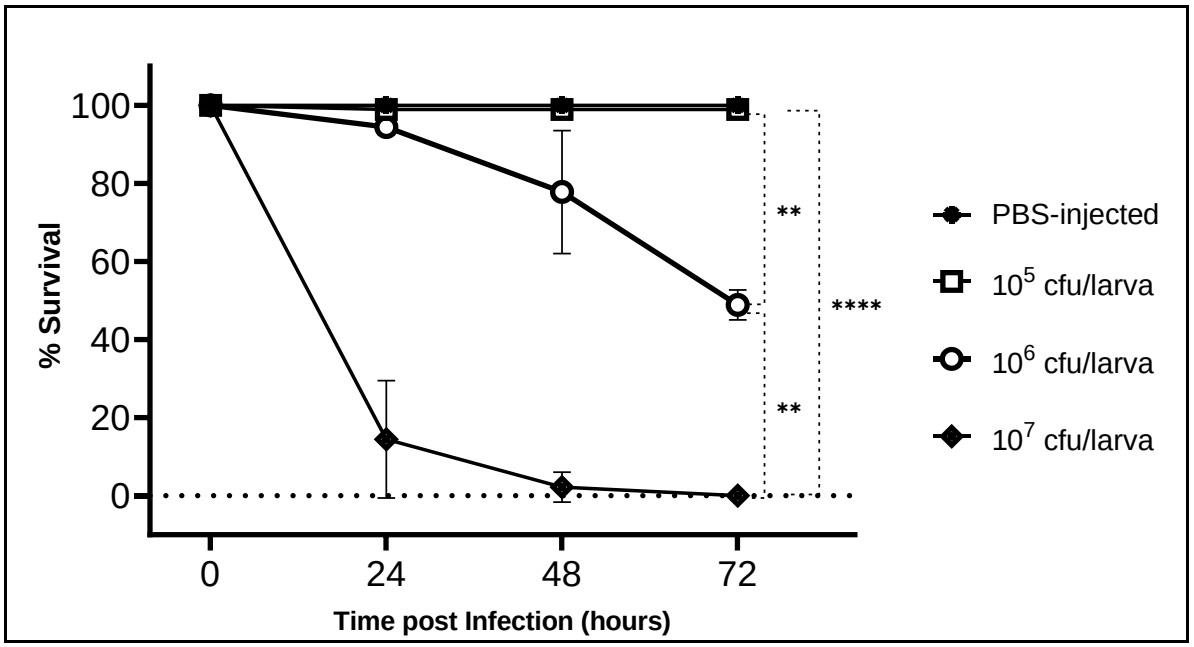


FIG 2.2: *Galleria mellonella* larvae susceptibility to EGD-e infection is dose-dependent. Bacterial cultures were grown in BHI to stationery phase, washed twice and re-suspended in PBS. Larvae were inoculated with a dose of 10^5 CFU, 10^6 CFU or 10^7 CFU, and controls with 20µl of PBS and subsequently incubated at 37°C. Results are Mean ± SD of three independent tests. Statistical comparisons are at 7 d p.i between EGD-e inocula. No significant differences was observed between PBS-inoculated larvae and 10^5 CFU (Two-Way ANOVA, **: $p < 0.01$, **** $P<0.0001$).

The results showed EGD-e has a time and dose-dependent virulence in *G. mellonella* as the effects of the three doses on larval survival varied at each time point and throughout the course of the investigation. For instance at 24 h p.i the 10^7 CFU only had a 14.4 ± 8.6% survival whilst 10^6 CFU had a 94.4 ± 1.1% survival. At the same time point the 10^5 CFU treatment was almost avirulent as a 98.9 ± 1.1% larval survival was observed. Survival rates continued to decline in the two highest doses over time. At about 72 h p.i a 50% mortality was observed for the dose of 10^6 CFU (48.9 ± 2.2%) whilst a mortality rate of 100% was seen with the 10^7 CFU treatment. The 10^5 CFU treatment approximates to a sub-lethal dose as only 1.1±1.1% mortality was observed at the end of the 72h period of the investigation.

These findings correlated with earlier reports by Mukherjee et al. (2010) who reported a 100% mortality at 72 h p.i for EGD-e using a dose of 10^7 CFU and no mortality caused by 10^5 CFU at the same time point. In contrast the 10^6 CFU had a higher survival rate (~80%) at 72 h p.i to that observed in this investigation. The time and dose-dependent survival of larvae to EGD-e infection, as observed here, was also as reported by Joyce and Gahan (2010). However, incubation temperature p.i was also reported in this study as inducing larval susceptibility to infection. All assays in this investigation were carried out three times and were reproducible. This demonstrated the sensitivity of *G. mellonella* larvae to bacterial infections and that it can be used as model for *L. monocytogenes* strains' virulence testing.

2.3.3 Modifying a model of *Listeria monocytogenes* infection in *G. mellonella.*

As indicated earlier, *G. mellonella* larvae used in the dose-dependent EGD-e assays were incubated at 20^0C for 7 d with no food prior bacterial challenge. However, as studies indicate that starvation, temperature, and prolonged incubation period before bacterial challenge all decrease *G. mellonella* larvae's ability to withstand microbial infections, we modified our experimental setup to minimise these effects in subsequent experiments.

It was found by Banville, Browne, and Kavanagh (2012) that larval susceptibility to infections increased significantly among larvae that had been food starved for 2, 4, or 7 d prior challenge with *Candida albicans* infections. At 48 h p.i larvae that had been starved for 2 and 4 d had 30% reduced survival as compared to non-starved larvae. Whilst larvae starved for 7 d were most susceptible to infection with a 16.7% survival rate at 48 h, non-starved larvae had a 60% survival rates at the same time-point. Similar findings were also later reported by Browne et al. (2015) who observed prolonged pre-incubation (as short as a week), significantly increased larvae susceptibility to *Candida albicans* and *Staphylococcus aureus* infections. A decrease in levels of proteins involved in metabolic pathways and decrease in density of haemocytes correlated with the prolonged incubation, resulting in decreased larvae survival. To minimise these influences and ensure consistency and reproducibility of results we resorted to using larvae for *L. monocytogenes* challenge a day after their delivery to the lab (2 days post-shipment from the producer) that are hereafter refer to as 'one day larvae'. This was observed to have significant effects on larval survival

when challenged with the same bacterial doses as carried out earlier. Except for the use of one day larvae all experimental conditions were maintained as earlier described in 2.3.1. Results (Figure 2.3) showed the LD_{50} for the dose of 10^7 CFU increased from under 15 h (Figure 2.2) to over 50 h, with the LD_{50} for 10^6 CFU increasing by over 24 h when one day larvae were used. Larval survival for 10^5 CFU (sub-lethal dose) was similar to that observed in PBS-inoculated control larvae during the 7d course of the experiment. A dose-dependent survival was observed using one day larvae as earlier seen in the use of 7 d old larva.

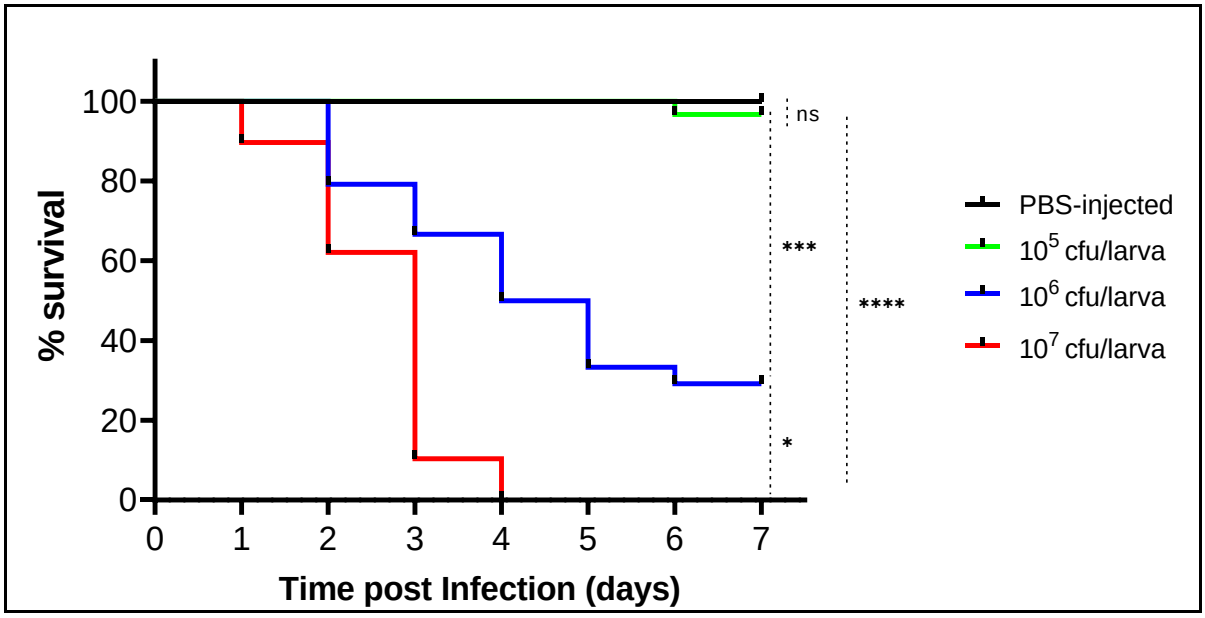


FIG 2.3: *L. monocytogenes* strain EGD-e infection of *G. mellonella* induces dose-dependent mortality. Kaplan-Meier survival curves of *G. mellonella* larvae post-inoculation with EGD-e. Bacterial cultures were grown in BHI to stationary phase, washed twice and re-suspended in PBS. Larvae were inoculated with one of three doses, controls with 20µl PBS and subsequently incubated at 37°C. All three doses caused time-dependent mortality of larvae with 10^7 CFU inducing the highest mortality. 10^5 CFU caused insignificant larval mortality and 10^6 CFU induced gradual larval death. Results are Mean of three independent tests. Statistical comparisons are survival rates at day 7 p.i (* $p < 0.05$; *** $P<0.001$; **** $P<0.0001$; ns, no significant differences).

The use of one day larvae led to extension of the investigation period for all subsequent experiments, from 72 h to 168 h (7 days), providing more data collection points for larval survival, and bacterial recoveries and haemocyte density

quantification (discussed in chapter 4). This also enabled the use of the health index scoring system (HISS) which was significant in determining subtle differences in larval sensitivity to infection by different bacterial strains (Chapter 3-section 3.3.5) and as well as in the dose-dependent survival assays. As the 10^5 CFU dose was found to be sub-lethal and 10^7 CFU caused rapid larval death, the dose of 10^6 CFU, which showed a steady decline in larval survival, was to be used for all further virulence testing.

2.3.4 *G. mellonella* larvae health index assessment enables observation of subtle differences in EGD-e virulence.

In order to assess more subtle differences in larval sensitivity to EGD-e infection we further assessed larval health using a modified health index score system (HISS) earlier described by Loh et al. (2013). The index evaluates larval activity, cocoon formation, and cuticle melanisation in addition to survival (Fig 2.4). Larvae were examined individually and a score was assigned for each attribute and totalled to give a final health assessment of each larva. This was collated for each treatment group (n = 30 per treatment) to give a final health score per treatment group at each time point.

One day larvae weighing between 0.25 to 0.35g were divided into three treatment groups with each group treated with a different planktonic dose of EGD-e; 10^5 cfu/larva, 10^6 cfu/larva or 10^7 cfu/larva. A control groups was inoculated with 20µl of PBS and larvae were incubated at 37ºC. Larvae were examined individually on a daily interval. Larval melanisation over time was dose-dependent (not shown). The highest dose, 10^7 CFU, caused more melanisation than that observed in the 10^5 CFU and 10^6 CFU infections. Melanin is produced by larvae to aid microbial trapping and killing (Bergin et al., 2005), thus the increased dose-dependent melanisation observed here could indicate an increased larval immune response proportionate to the infection dose. In most cases larval melanisation correlated with reduced larval activity and was accompanied by death, as demonstrated for a dose of 10^6 CFU (Fig 2.5). The dose-dependent melanisation and larval death was also as observed for strain and dose-dependent virulence of *Acinetobacter baumannii* (Wand et al., 2012). It was observed that more virulent strains and higher bacterial doses caused increased melanisation resulting in higher mortality rates. *L. monocytogenes* induces the pro-phenoloxidase (PPO) system of *G. mellonella,* a process instituted by larvae for microbial phagocytosis and destruction (Bidla et al., 2009; Joyce & Gahan, 2010), which leads

to nodulation and then melanisation as observed here. This therefore served as an indicator of the larval immune system been overwhelmed by the EGD-e infections.

A

Category	Score	Description
Activity	0	No movement
	1	Minimal movement on stimulation
	2	Move when stimulated
Cocoon formation	0	No cocoon formed
	0.5	Partially formed cocoon
	1	Fully formed cocoon
Melanisation	0	Black larvae
	1	Brown larvae
	2	≥3 spots on beige larvae
	3	<3 spots on beige larvae
	4	No melaniSation
Survival	0	Dead alrvae
	2	Alive

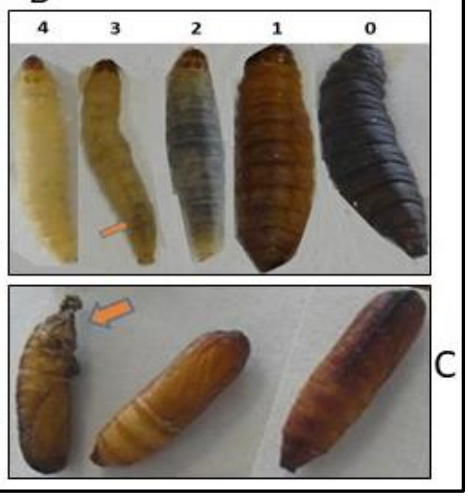


FIG. 2.4: *G. mellonella* health index scoring system. (A) Scores ascribed to different larvae attributes post-inoculation. (B) Larvae melanisation: uninfected larva showing no melanisation (1), infected larva with <3 melanisation spots (2), larva with >3 melanisation spots (3), brown larva (4), black and typically dead larva (5). (C) Pupae (fully formed cocoon) with arrow indicating silk formation. Table (A) is as adapted from Loh et al. (2013).

FIG 2.5: *L. monocytogenes* causes time dependent larval melanisation. Larvae were inoculated with stationary phase planktonic cultures of EGD-e at a concentration of 10^6 cfu/larva and incubated at 37ºC. Time dependent melanisation was observed with increased melanisation accompanied by larval death. Not all larval deaths were preceded by total darkening of larvae. Results are representative of general observations of larval challenge with EGD-e.

The overall health index of larvae (Fig 2.6) was found to be dose-dependent. As early as 24 h p.i 10^7 CFU had induced significant differences in larval health to that seen in the 10^5 CFU dose ($p < 0.01$), and larvae health declined rapidly post this time-point to a score of zero in about 4 days p.i On the other hand, stable health scores were observed for PBS-inoculated larvae (control) with insignificant variations for 10^5 CFU throughout the course of the experiment. No significant differences were observed between PBS-inoculated larvae and the 10^5 CFU dose ($p = 0.642$). Also, melanisation in PBS-inoculated larvae was insignificant, whilst all significant melanisations observed with 10^5 CFU resulted in larval death. The 10^6 CFU treatment showed a steady decline in larval health correlating with that observed for larval survival using the same dose. Though no studies on EGD-e dose-dependent health index assessment are available, the results observed in this study correlated with that observed in dose-pendent studies in group a streptococcus (Loh et al., 2013).

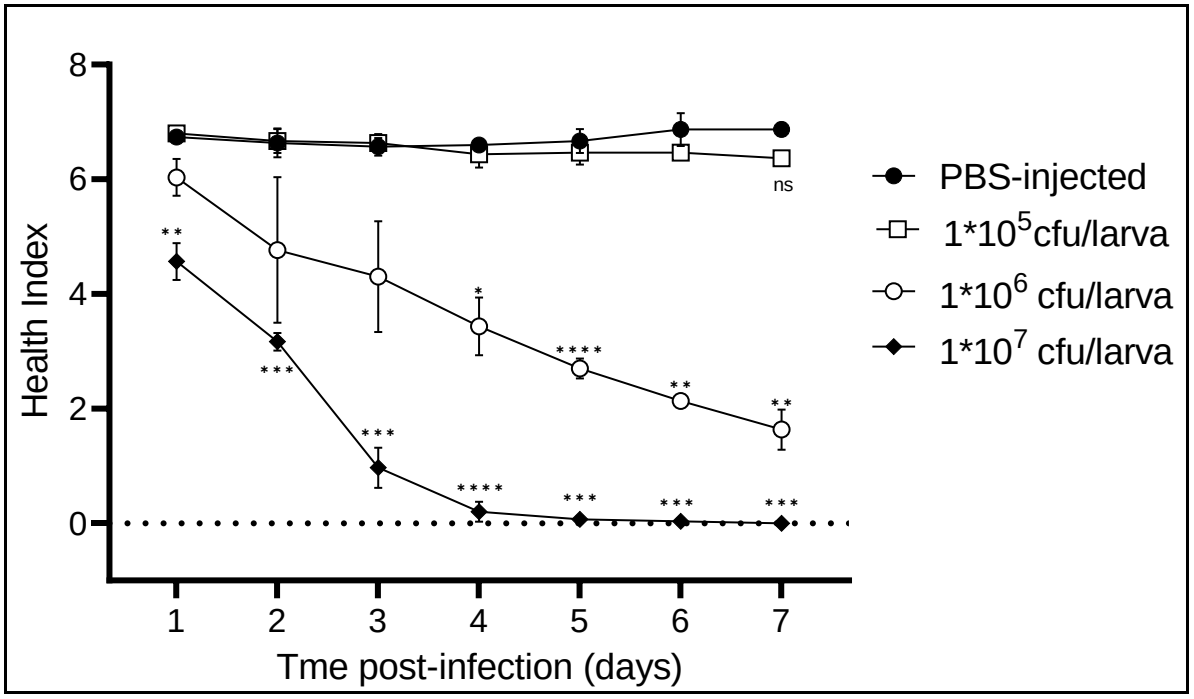


FIG 2.6: Dose-dependent effect *G. mellonella* larvae health post-infection with *L. monocytogenes* strain EGD-e. Larvae were inoculated with a dose of 10^5 CFU, 10^6 CFU or 10^7 CFU, and controls with 20µl of PBS and subsequently incubated at 37°C. Larvae health were scored base on activity, cocoon formation, melanisation, and survival. Results are Mean ± SD of three independent tests. Statistical differences are as compare to PBS-inoculated larvae (control). *p< 0.05; **p< 0.01; *** P<0.001; **** P<0.0001; ns, no significant differences (Two-Way ANOVA).

2.4 Conclusions

Whilst no significant larval death was observed in one day larvae infected with 10^5 CFU during the course of this investigation a dose of 10^7 CFU was found to be toxic and induced 100% morality in 4 d. By comparison 10^6 CFU only caused mortality in 50% of larvae at that time point and 70% by the end of the 7 d period. A dose of 10^7 CFU was found to be toxic whilst the dose of 10^5 CFU proved to be sub-lethal. Larvae infection with a dose of 10^6 CFU was found to be suitable for virulence testing due to the steady increase in mortality rates, which enabled the subsequent evaluation of other aspects of *L. monocytogenes* virulence using this model, such as larval immunological responses and bacterial growth *in vivo.*

Furthermore, the health index scoring system (HISS) was found to be useful in highlighting subtle differences in virulence in larvae that would otherwise be unnoticeable if using only binary assessments of alive or dead. At 1 d p.i significant differences in virulence were observed between larvae infected with 10^7 CFU and the other doses. Although no significant differences were observed between PBS-inoculated larvae and ones inoculated with 10^5 CFU differences were more apparent as compared to the survival assays. The HISS thus has potential to discriminate between *L. monocytogenes* strains that have similar virulence characteristics. Using different doses of EGD-e we therefore demonstrated that *G. mellonella* larvae are sensitive to *L. monocytogenes* infections and could potentially be used to determine differences in virulence of *L. monocytogenes* strains.

Chapter 3 - DETERMINING VIRULENCE OF *LISTERIA MONOCYTOGENES* STRAINS IN *GALLERIA MELLONELLA*

3.1 Introduction

Listeria monocytogenes consists of four lineages that comprises at least 15 serotypes of equal significance, at least for regulatory purposes, as causative agents of listeriosis (Okike et al., 2013; Vázquez-boland et al., 2001). However, *L. monocytogenes* serotypes of *L. monocytogenes* lineages I and II are the most frequent cause of listeriosis due to being responsible for more than 95% of sporadic and epidemic listeriosis outbreaks (Kathariou, 2002). This has led to a growing consensus that lineages I and II strains have higher virulence potential in animal hosts as compared to other *L. monocytogenes* lineages (Jeffers et al., 2001). However, it is also suggested that the increased prevalence of these lineages in listeriosis outbreaks is due to high exposures of humans to these isolates from food products (Roberts et al., 2006). While strains from both lineage I and II are prevalent in food products (Norton et al., 2001) lineage I strains are the most implicated in listeriosis outbreaks (EFSA & ECDC, 2018; Pérez-trallero et al., 2014), in contradiction to these widely held assumptions. Although a great deal of research efforts have been devoted to understanding the *in vivo* differences in virulence of *L. monocytogenes* strains and lineages, these studies have largely been carried out on clinical isolates. Hence, the virulence potential of *L. monocytogenes* strains from food and environmental sources remains to be evaluated. Therefore, this investigation aimed at determining the virulence potential of *L. monocytogenes* strains that were directly isolated from fresh produce (FP) foodstuff and from a food processing environment using the invertebrate model *Galleria mellonella.*

In Chapter Two we validated a model of *L. monocytogenes* infection, and in this chapter we used that model to test the pathogenic potential of nine *L. monocytogenes* strains of both lineages I and II. We compared the virulence potential of these strains to that of the well characterised *L. monocytogenes* strain EGD-e (NCTC7973, serotype 1/2a) whose virulence has widely been tested in this model (Martinez et al., 2017; Mukherjee et al., 2010).

3.2 Methodology

3.2.1. Bacterial isolates used in this study

The virulence of 10 *Listeria monocytogenes* strains and one *Listeria ivanovii* strain were used for this section of the investigation (Table 3.1). All strains, except EGD-e (reference strain), were obtained from a commercial food testing laboratory. These strains were isolated from various stages of the fresh produce supply chain (FPSC) around the UK between May 2016 and April 2017. Strains were isolated using ISO11290-2: 2017, species identification was carried out using biochemical tests (API *Listeria* , bioMerieux/Microbact *Listeria* , Thermo Scientific) and strain identification by whole genome sequencing, except *Listeria ivanovii*, in an earlier publication (Smith et al., 2019). The strains were processed for long term storage at Edinburgh Napier University (ENU) as described by Smith et al. (2019), suspended in BHI broth and 50% glycerol, and frozen at -80ºC until required for virulence testing.

Table 3.1: Virulence profiles of *L. monocytogenes* isolates used in this investigation and Stages in the FPSC they were isolated from. Strain lineage, sequence types and virulence profile of *L. monocytogenes* strains were determined by whole genome analysis and MLST in an earlier publication from this laboratory (Smith et al., 2019). RP = raw produce, FP = final produce, PC = post cooling.

Internal reference	Source of isolate	Lineage	Sequence type	Virulence profile
NLmo2	RP spinach (Unwashed)	I	ST-5	34
NLmo3	RP spinach (Unwashed)	I	ST-5	34
NLmo6	RP red leaf lettuce (Unwashed)	I	ST-4	41
NLmo7	PC spinach (Unwashed)	I	ST-1	41
NLmo14	FP Beetroot (Washed)	I	ST-1	41
NLmo20	FP Baby salad kale (Unwashed)	I	ST-6	41
NLmo4	Environmental swab (drain)	II	ST-325	31
NLmo5	RP Spinach (Unwashed)	II	ST-325	31
NLmo18	RP Baby salad kale (Unwashed)	II	ST-399	33
EGD-e	Laboratory (Reference strain)	II	ST-35	31

3.2.2. DNA extraction and 16S sequencing

The species identification of the *Listeria ivanovii* isolate was confirmed by 16S sequencing. From a long term stock, a streak plate on BHI agar was prepared before DNA extraction. DNA extraction was carried out using GeneElute Bacterial Genomic DNA kit (SIGMA-ALDERICH, UK) according to manufacturer's protocol. Amplification was performed using the 16S primers (27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, 5'-GGTTACCTTGTTACGACTT-3') with PCR conditions: 95^0C for 4 minutes (1 cycle); 95^0C for 1 min, 55^0C for 1 min, 72^0C for 2 min (30 cycles); and 72^0C for 2 min (final extension). The PCR product was cleaned using GeneJet Gel Extraction Kit (Thermo Scientific) and the sample sent to Edinburgh Genomics (Edinburgh, UK) for sequencing. BLASTN searches (NCBI: http://www.ncbi.nlm.nih.gov) was conducted to for species identification.

3.2.3. Preparation of bacterial cultures for *Galleria mellonella* infections.

To prepare bacterial inoculum for *G. mellonella* larvae infection, 10ml of brain heart infusion (BHI) broth was inoculated with a single colony from a subculture and incubated in a shaken incubator (37ºC, 200rpm, aerobic conditions) for 16 h. The cultures were diluted in BHI broth to an OD_{600} of 0.42, which corresponds to 10^9 CFU/ml. Cells were harvested by centrifugation at 12,000g for 10 min at 22ºC, washed twice in phosphate buffered saline (PBS), and diluted to a concentration of 10^8 CFU/ml.

3.2.4. Infection of *Galleria mellonella* larvae and monitoring

G. mellonella larvae were purchased from UK Waxworms Ltd (Sheffield, UK). Larvae were stored at 20ºC and used for bacterial challenge within a day after their delivery to the lab. Only healthy looking larvae weighing between 0.25-0.35g with no signs of melanisation were used, and a new batch of larvae was used in each of the three experimental replicates. For each strain *G. mellonella* larvae (n = 30) were injected with 10^6 CFU/larva delivered in 20ul through the last right proleg using an insulin syringe, as described previously (Joyce & Gahan, 2010). Each time 100ul of the bacterial inoculum was serially diluted and plated to confirm the CFU counts injected into larvae. As a control, larvae (n = 30) were injected with 20ul of PBS, larvae were incubated at 37ºC in 9cm petri dishes lined with Whatman paper p.i.

Galleria mellonella larvae were examined individually on a daily basis p.i. Larval mortality was assessed by turning larvae on their backs and checking for leg movement; health larvae upright themselves with dead ones showing no movement. In order to evaluate more subtle differences in larvae health status the health index scoring system (HISS), earlier described by Loh et al. (2013), was applied by examining the following attributes: larvae activity, cocoon formation, cuticle melanisation, and survival, as discussed in section 2.2.4 and demonstrated in Fig 2.4.

3.3 Results and Discussion.

3.3.1. Sequencing and *Listeria ivanovii* identification

To test virulence of *L. monocytogenes* strains in the *G. mellonella* model a non *L. monocytogenes* species was to be included that can be used as a second reference point to ensure potential difference in virulence observed are not species dependent. As *L. monocytogenes* strains to be tested were obtained from predominantly food sources a *L. ivanovii* isolate obtained from a final product spinach sample was selected. To identify this isolate a 16S ribosomal sequencing was carried out and BLASTn search was conducted for sequence alignment **(Fig 3.1**). Using the Query blast on the NCBI website an Expected value (E-value)-probability of such an alignment been found by chance in the database-of 0.0 was obtained with a Query coverage of 99% and percentage identity of 99.98% for the isolate. The isolate was thus identified as *Listeria ivanovii* strain indoniensis (NCTC12701).

Score	Expect	Identities	Gaps	Strand
909 bits(492)	0.0	496/498(99%)	0/498(0%)	Plus/Minus

```
Query  15    GCTGGCTCCTAAAAGGTTACCCTACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACG  74
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1411  GCTGGCTCCTAAAAGGTTACCCTACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACG  1352

Query  75    GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAG  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1351  GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAG  1292

Query  135   CGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTATG  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1291  CGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTATG  1232

Query  195   GGATTGGCTCCACCTCGCGGCTTCGCTACCCTTTGTACCATCCATTGTAGCACGTGTGTA  254
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1231  GGATTGGCTCCACCTCGCGGCTTCGCGACCCTTTGTACCATCCATTGTAGCACGTGTGTA  1172

Query  255   GCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGCTTGCACC  314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1171  GCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGCTTGCACC  1112

Query  315   GGCAGTCACTTTAGAGTGCCCAACTAAATGCTGGCAACTAAAATCAAGGGTTGCGCTCGT  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1111  GGCAGTCACTTTAGAGTGCCCAACTAAATGCTGGCAACTAAAATCAAGGGTTGCGCTCGT  1052

Query  375   TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCA  434
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1051  TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCA  992

Query  435   CTTTGTCCCCGAAGGGAAAGCTCTGTCTCCAGAGTGGTCAAAGGATGTCAAGACCTGGTA  494
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  991   CTTTGTCCCCGAAGGGAAAGCTCTGTCTCCAGAGTGGTCAAAGGATGTCAAGACCTGGTA  932

Query  495   AGGTTCTTCGCGTAGCTT  512
             ||||||||||||| ||||
Sbjct  931   AGGTTCTTCGCGTTGCTT  914
```

FIG. 3.1: BLASTn alignment for *L. ivanovii* (Accession number: NCTC12701) using the NCBI online software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.2. *L. monocytogenes* virulence in *G. mellonella* is strain and time dependent.

Prior work on genomic analysis of the *L. monocytogenes* strains used in this study suggested there exists differences in their virulence potentials (Smith et al., 2019). However, the virulence potential of these strains had not been determined in this model, as is also the case for numerous other preceding *in silico* studies of *L. monocytogenes* strains of foodstuffs and environmental sources (Liu, 2006; Poimenidou et al., 2018). We therefore intended to test for differences in their virulence potential using the *G. mellonella* model. Nine *L. monocytogenes* strains were tested in addition to a *L. monocytogenes* reference strain (EGD-e) and also a non *L. monocytogenes* strain (*Listeria ivanovii*) that was included as a second reference strain. As previously described (Chapter 2, section 2.3.1) bacterial CFU ml^{-1} values corresponding to given OD_{600} nm were determined in order to guarantee that the same amount of CFU is injected into larvae for all the bacterial strains in this investigation (Table 3.1). The inoculating dose (10^6 CFU/larvae-as determined in section 2.3.2) was also confirmed each time by serial dilution and plating of 100µl of bacterial inoculum on Oxford agar before larval infection. Briefly, to determine the differences in virulence of *L. monocytogenes* strains in *G. mellonella,* 30 larvae were inoculated with 10^6 CFU/larvae of each tested strain and their survival and health status monitored for 7 d. For a control group 30 larvae were inoculated with 20µl of PBS.

PBS-inoculated larvae remained alive during the 7 d course of this investigation with similar observations also made for *L. ivanovii* inoculated larvae 96 ± 4.4% survival at 7 d. *L. monocytogenes* inoculated larvae showed increasing mortality rates over time indicating cumulative bacterial pathogenesis, which was analogous to earlier reports using clinical and mutant isolates of *L. monocytogenes* in *G. mellonella* (Martinez et al., 2017). Inter-strain strains comparisons revealed strain specific variations in virulence based on the mortality rates and overall larvae survival at the end of the 7 d time course. Inoculation with different *Listeria* strains caused significant differences in *G. mellonella* larval mortality (F = 23.69, df = 11, 66, $p < 0.0001$) (Fig 3.3). Notably, NLmo4, NLmo5 & NLmo7 induced >90% mortality at 7 d p.i while a 4.4 ± 4.4% mortality was observed in *L. ivanovii* inoculated larvae at the same time point.

All *L. monocytogenes* strains induced significantly higher larvae mortality than *L. ivanovii* ($p < 0.05$), and all, except NLmo20, were also found to be more virulent in *G. mellonella* than the reference strain (EGD-e). Amongst the investigated *L.*

monocytogenes strains NLmo4 and NLmo5 induced the highest mortality rates (98.8 ± 1.1% and 96.7 ± 1.9%, respectively) and NLmo20 caused the least larval deaths (65±2.9%), an over 30 percentage points variation indicating significant differences in strains virulence. 'Epidemic clones' of *L. monocytogenes* of the same sequence type (ST6) as NLmo20 have been reported to be hyper virulent, causing central nervous system (CNS) infections (Koopmans et al., 2013; Maury et al., 2016), contrary to the low mortality rates observed in this model. Interestingly, NLmo4 and NLmo5 are of the same sequence type, and both strains along with NLmo20 belong to the same *L. monocytogenes* lineage (II). Additionally, varied mortality rates were observed among *L. monocytogenes* lineage I strains, albeit were statistically insignificant ($p \geq 0.05$). And when compared to lineage II isolates statistical significance was only observed between NLmo6 (lineage I) and NLmo4 and NLmo5 (both lineage II).

L. monocytogenes* strains + *L. ivanovii	**Days post-inoculation**						
	1	2	3	4	5	6	7
PBS-inoculated (control)	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
L. ivanovii	100±0.0	97±2.2	97±2.2	97±2.2	97±2.2	97±2.2	96±4.4
NLmo20	100±0.0	96±1.1	82±6.1	66±4.0	47±1.9	36±2.2	34±2.9
EGD-e (Reference strain)	98±1.1	97±0.0	84±2.2	70±5.8	53±6.7	41±6.2	28±4.0
NLmo18	100±0.0	98±1.1	92±2.9	67±0.0	47±3.3	34±7.3	21±6.8
NLmo2	98±1.1	97±1.9	76±5.9	60±11	30±1.9	23±6.9	20±5.1
NLmo3	100±0.0	96±1.1	90±5.1	63±8.8	39±11	30±12	19±7.8
NLmo6	100±0.0	98±1.1	86±1.1	51±4.8	31±2.2	20±1.9	18±1.1
NLmo14	100±0.0	94±1.1	71±12	49±12	31±8.7	21±9.9	16±6.8
NLmo7	100±0.0	94±1.1	77±3.8	48±9.1	19±8.7	12±7.8	9±4.8
NLmo5	100±0.0	97±1.9	72±8.0	39±8.0	14±6.2	7±1.9	3±1.9
NLmo4	100±0.0	97±0.0	69±2.9	39±6.2	17±5.1	2±1.1	1±1.1

Larval survival (%)

100	90	80	70	60	50	40	30	20	10	0

FIG. 3.3: *Listeria* strains induced significant and varied differences in virulence in *Galleria mellonella* larvae. Bacterial cultures were grown in BHI to stationary phase, washed twice and resuspended in PBS. Larvae were inoculated with 10^6 CFU/larva, and control with 20µl/ of PBS. All *Listeria strains* caused time-dependent mortality of larvae with NLmo5 & 5 inducing higher mortality rates. *L. ivanovii* caused insignificant larval mortality and was similar to PBS-injected larvae. Results are Mean ± SEM of three independent tests.

In agreement with previous reports (Martinez et al., 2017; Mukherjee et al., 2010) *L. ivanovii* was found to be less virulent in *G. mellonella* than *L. monocytogenes* strains.

A survival rate of over 90% was reported for *L. ivanovii* at 7 d p.i (Mukherjee et al., 2010), near identical to that observed in this study. Similar outcomes to those observed in this study for the reference strain (EGD-e) was also reported at a dose of 10^6 CFU, all indicating consistency of *G. mellonella* as a virulence testing model. These results thus allow us to conclude that the larvae mortality kinetics observed here were strain specific and that these *L. monocytogenes* strains have significantly different virulence potentials. Moreover, as correlations have already been established between virulence variations observed in *G. mellonella* larvae and that observed in mammalian models for several human pathogens (Brennan et al., 2002; Cotter et al., 2000; George et al., 2000; Mukherjee et al., 2013; Slater et al., 2011) our data suggest the existence of potential differences in pathogenicity of *L. monocytogenes* strains from food sources to induce infections in humans.

3.3.3. *L. monocytogenes* lineage types do not correlate with virulence.

Differences in virulence of *L. monocytogenes* lineages I and II strains (as a collective) were assessed by comparing mean survival rates and larvae health status using the HISS. Briefly, for each strain one day old larvae (n = 30) were intrahaemocoelically inoculated through the last right proleg with a stationary phase infectious dose of 10^6 CFU/larvae delivered in 20µl, and control larvae (n = 30) were inoculated with 20µl of PBS. Larvae were incubated at 37ºC and monitored daily for survival by touch-induced lack of larval movement, and overall health status by examining larval activity, cocoon formation, melanisation and mortality for 7 d (as demonstrated in Chapter 2-section 2.3.4). The survivals and health indices were determined for each strain and averaged for all strains belonging *L. monocytogenes* lineage I (six strains) and lineage II (three strains). No mortality was observed in PBS-inoculated larvae (control) during the course of the 7d investigation (Fig 3.4A). In contrast, a gradual increase in mortality rates were observed in bacterial inoculated larvae of lineages I and II reaching 80.74 ± 0.68% and 91.48 ± 6.33%, respectively, at 7 d. Whilst significant differences in mortality rates were observed between PBS-injected larvae and *L. monocytogenes* lineages ($p < 0.0001$), no significant differences were observed between mean mortality rates of lineages I and II ($p = 0.567$, Log-rank test). However, although lineage II strains induced higher mean mortality rates than lineage I strains, high variability in mortality rates of up to 10.4% was observed in lineage II strains; and these

were consistent with observations on the overall larvae health status (Fig 3.4B), albeit differences in lineages virulence were more apparent.

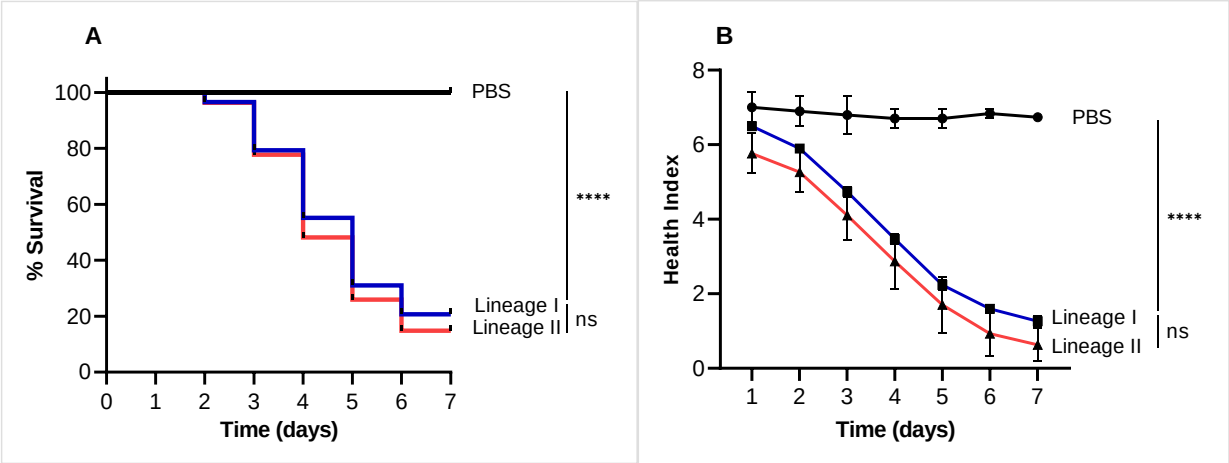


FIG 3.4: Virulence of *L. monocytogenes* lineages in *Galleria mellonella.* Virulence of six *L. monocytogenes* lineage I and four lineage II strains was assessed. Larvae were inoculated with a dose of 10^6 CFU/larvae of stationary phase cultures and monitored daily for 10 d. (A) Kaplan-Meier survival curves. ****$p < 0.0001$, ns, not significant (Log-rank test). (B) Mean ± SEM health index scores of larvae. **** $p< 0.001$, ns not significant (Two-Way ANOVA). Results represent three independent experiments.

3.3.4. *L. monocytogenes* virulence in *G. mellonella* using HISS is strain and time dependent, correlating with survival assays.

In order to evaluate more subtle differences that *L. monocytogenes* strains may exhibit in *G. mellonella* we assessed larval health using a modified HISS that examines larval health by assigning scores (Loh et al., 2013). Briefly, for each larva four major observations are made: mobility/activity, cocoon formation, melanisation as observed on the cuticle, and survival. Larvae (n = 30) were treated with standardised doses of 10^6 CFU for each strain and incubated at 37ºC, and health status was monitored daily for 10 days. Results presented are up to 7 d p.i to ease comparisons with other experiments and also due to a 4.0 ± 1.1% mortality been observed in the control group at 9 d p.i (see appendix for the complete 10 d trials). Cocoon formation and higher larval activity indicate high health status and such larvae most commonly score 8-9, and highly melanised larvae typically score 0-1 indicating poor health status. The index

thus correlate with different stages of bacterial pathogenicity in the larvae serving as a marker of overall larval health.

PBS-injected larvae were observed to have stable health statuses during the 7d course of this investigation and were closely comparable to that observed in *L. ivanovii* inoculated larvae with statistically no significant differences ($p \geq 0.05$) (Fig 3.5 A-D). In contrast, wide-ranging variations were observed in *L. monocytogenes* inoculated larvae, mainly emerging after 5 d of incubation. In the first 3 d p.i most inter-strains comparisons and comparisons with control subjects showed less significant variations (See Appendix 1) and differences became more apparent with increasing incubation time. The time-dependent variation with increasing statistical significance could indicate progressive bacterial pathogenicity in larvae as this correlated with larval death. This phenomenon was also as reported by Sousa et al. (2018) when determining virulence of *Legionella pneumophila* isolates. Thus, increasing virulence correlated well with decreasing larvae health index scores.

A notable characteristic was also the gradual increase in cuticle melanisation in bacterial inoculated larvae, chiefly amongst *L. monocytogenes* strains. This was observed to start with distinctive black spots around the tail region (inoculation site) and increased in size and numbers culminating to entire larval melanisation (dark brown and then completely black, typically). Melanisation is reportedly employed by larvae to enable killing of encapsulated microbes through the synthesis and deposition of melanin (Barnoy et al., 2017). This process is preceded by the oxidisation of phenolic substances to quinones and melanin by phenoloxidase (PO) (Pereira et al., 2018). Therefore, it is intuitive that as the infection progresses more microbial encapsulations will occur leading to further nodulations (black spots) characteristics of what is observed in this study. However, although increasing melanisation correlated with larval death the reverse was not always true. For instance, at 4 d p.i the proportion of larvae mortality in NLmo4 inoculated larvae was 0.61/1 as compared to the proportions of completely melanised larvae of 0.12/1, and for NLmo14 inoculated larvae it was 0.51/1 to 0.21/1, respectively, with also similar observations made for NLmo18 (death: 0.33/1, completely melanised 0.04/1) at the same time point.

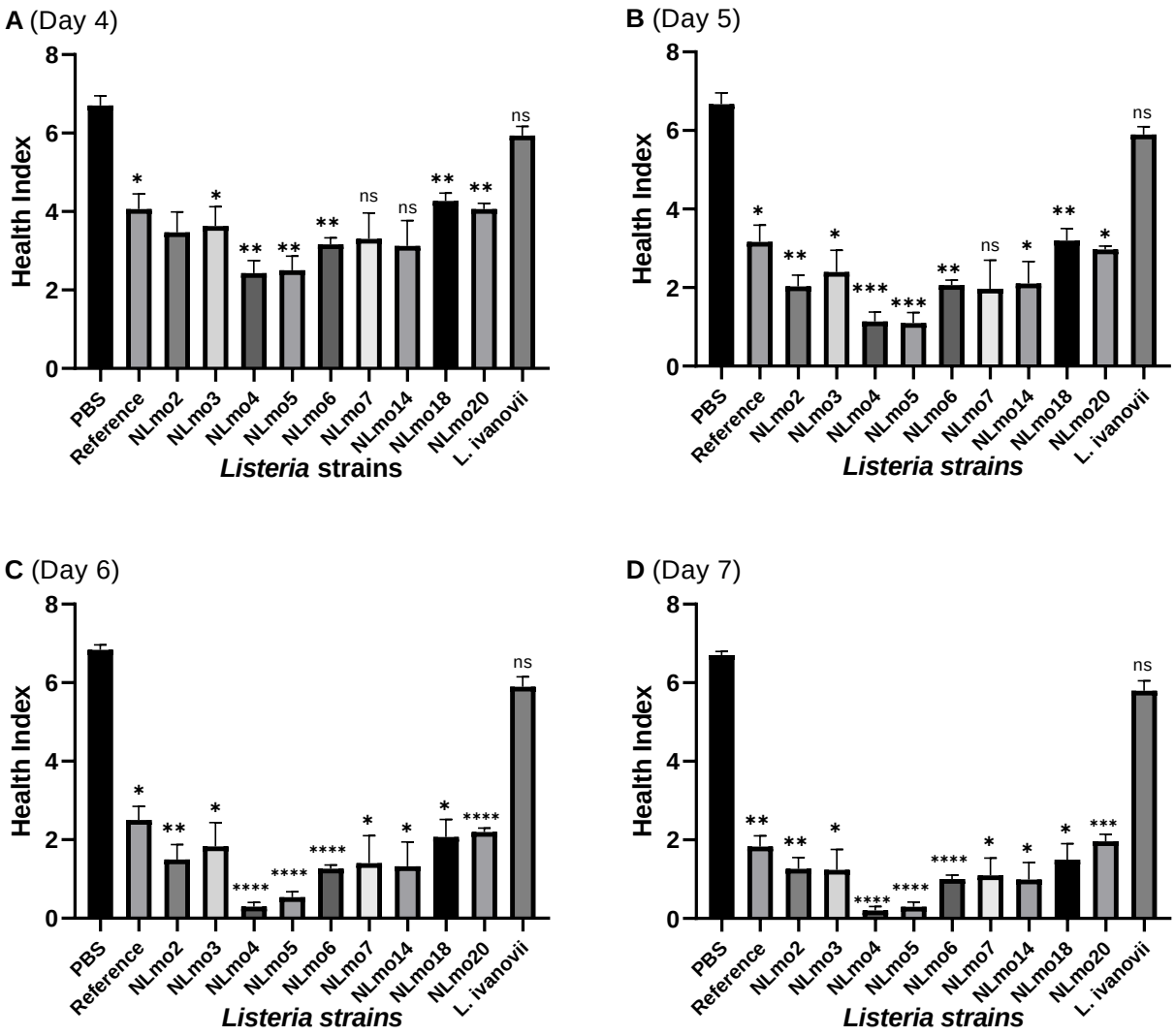


FIG. 3.5: Strain and time-dependent effect of infection on *G. mellonella* larvae health status. Larvae were inoculated with a stationary phase dose of 10^6 CFU for each of the tested strains, and controls with 20µl of PBS and subsequently incubated at 37°C. Larvae health were scored base on activity, cocoon formation, melanisation, and survival. Results are Mean ± SD of three independent tests. (A-D) overall larvae health status at 4, 5, 6, and 7 days post infection, respectively. Statistical differences are as compare to PBS-inoculated larvae (control). *p< 0.05; **p< 0.01; *** P<0.001; **** P<0.0001; ns, no significant differences (Two-Way ANOVA).

3.3.5. *Listeria* infection causes opposing developmental effect in *G. mellonella*

Inoculating *G. mellonella* larvae with *Listeria* isolates was observed to cause opposing developmental effects to the larval life cycle. Last-instar larvae are predominantly used in disease modelling, antimicrobial and virulence testing. The last-instar stage is also

the final stage before *G. mellonella* larvae start producing silk and forming cocoons which then pupate to moths (Kwadha et al., 2017). In this study, we observed that inoculating larvae with *Listeria* strains cause developmental arrest in larvae. After 10 d incubation 26% of PBS-inoculated larvae had either formed partial or full cocoon. In contrast, only 2% of larvae inoculated with NLmo20, or *L. ivanovii* had at least a partially formed cocoon. More so, no cocoon formation was observed in larvae inoculated with the remaining 9 *L. monocytogenes* strains (Fig 3.6). Interestingly, NLmo20 and *L. ivanovii* were the least virulent of the bacterial strains in this investigation, in both larval survival and overall health status. This suggests more virulent strains are capable of inducing arrest to larval metamorphosis. Our observations are consistent with a study (Mukherjee et al., 2013) that showed inoculating larvae with *L. monocytogenes* EGD-e can postpone development of *G. mellonella* larvae. It was also demonstrated in that study that inoculating larvae with heat-killed EGD-e accelerates larval development. Yet, strain comparisons were not conducted in the study as only EGD-e was used. However, at the moment we do not know what applicability our data may have though it suggests upon infection these strains could impact the endocrine system interfering with developmental processes (Mukherjee et al., 2013; Ruang-Rit & Park, 2018).

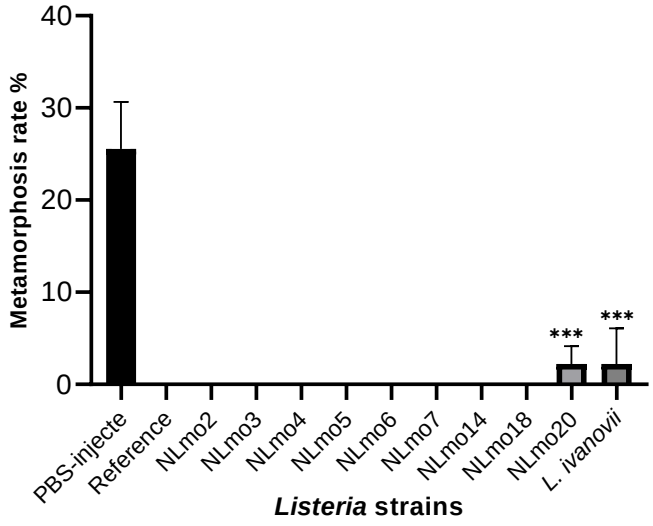


FIG. 3.6: Metamorphosis of *G. mellonella* larvae following infection with *Listeria* isolates. Last-instar larvae were inoculated with a stationary phase dose of 10^6 CFU for each of the tested strains, and controls with 20µl of PBS and subsequently incubated at 37°C. Larvae health were monitored for cocoon formation as part of a health status assessment. Results represent Mean ± SD of three independent trials at 10 d p.i Statistical differences are as compare to PBS-inoculated larvae (control) (*** p = 0.0006, One-Way ANOVA).

3.4 Results summary and conclusions

Prior work provided evidence that *L. monocytogenes* strains have different pathogenic potentials when tested in *Galleria mellonella* larvae (Mukherjee et al., 2010), but to the best of our knowledge, virulence potential of various *L. monocytogenes* strains from environmental and food sources has not been determined previously. We therefore evaluated the virulence potential of nine *L. monocytogenes* strains (eight from food products and one from a drain swab) belonging to *L. monocytogenes* lineages I and II. To test pathogenicity and also determine differences in virulence of these strains we carried out infection assays in the *Galleria mellonella* model. We compared virulence of these strains with the *L. monocytogenes* strain EGD-e (reference strain), whose pathogenic potential in the model is well characterised (Joyce and Gahan, 2010; Mukherjee et al., 2010), and also a non *L. monocytogenes* strain (*Listeria ivonovii*) known to rarely cause clinical cases and less virulent in *G. mellonella* larvae (Mukherjee et al., 2010).

It was observed that *L. monocytogenes* lineages do not correlate with virulence in this model with no significant differences in mean mortality rates been found between *L. monocytogenes* lineage I and II. However, significant differences in virulence were observed in inter-strain comparisons. Notably, NLmo4, NLmo5, and NLmo7 were most virulent inducing highest mortality rates (98.8 ± 1.1%, 96.7 ± 1.9% and 91 ± 4.4%, respectively) and NLmo20 was least virulent with 66 ± 2.9% mortality at the end of the 7d course of the investigation. The observed mortality rates strongly correlated with overall larvae health status for all the strains. Also, data obtained for the reference strains EGD-e and *L. ivanovii*, were consistent with prior reports using these strains, and when compared to the *L. monocytogenes* strains used in this investigation significant differences in virulence were also noted. We therefore report that there exists significant differences in virulence potential of *L. monocytogenes* strains from food sources and that these could cause varied pathogenicity in human infections.

Additionally, *L. monocytogenes* strains of the same sequence type, example NLmo2 and NLmo3, NLmo4 and NLmo5, and NLmo7 and NLmo14, were found to have closely comparable virulence characteristics base on both larval survival and overall health status with no significant differences among any pair throughout the course of the 7 d investigation. It is also noteworthy that each of these pairs have the same virulence profile (equal number of examined virulence factors) and were isolated from

different environments, except NLmo2&3. Therefore, we propose that *L. monocytogenes* sequence types could be dependable genomic predictors of *L. monocytogenes* strains virulence, and suggest for further investigation using a larger sample size of isolates from diverse food sources be carried out to ascertain this hypothesis.

Chapter 4 : IMMUNOLOGICAL RESPONSES OF *G. MELLONELLA* LARVAE TO *L. MONOCYTOGENES* INFECTIONS.

4.1 Introduction

Mammalian models have been the gold standard for pathogenicity testing and understanding of the mechanistic host response to infections. However, with mounting costs and the social and ethical issues associated with animal testing insect models, such as *Galleria mellonella*, have been introduced as alternatives. Although insects diverged from vertebrates about 500 million years, their innate immune response to microbial infections still share a lot of commonalities with mammalian systems (Kavanagh & Reeves, 2007; Muller et al., 2008). In recent years *G. mellonella* larvae have widely been used as a model to study microbial disease pathology of various human pathogens (Barnoy et al., 2017; Harding et al., 2012; Li et al., 2018; Mukherjee et al., 2013; Senior et al., 2011). Though *G. mellonella* lack an adaptive immunity and specialised phagocytic cells, as opposed to mammalian systems, it mounts linked cell-mediated and humoral immune responses effective in clearing microbial infections. A major component in the *G. mellonella* immunological response to infection are the haemocyte cells, which are analogous mammalian phagocytes (Pereira et al., 2018). Total and viable haemocyte counts in addition to immunocytochemistry characterisation of these cells have therefore been used to unravel many aspects of their function and deduce the host responses to pathogens (Browne et al., 2013; Browne & Kavanagh, 2013; Chain & Anderson, 1983; Fallon et al., 2011). Haemocytes secreted antimicrobial peptides (AMPs), such as galiomycin, gallerimycin, and insect metalloproteinase inhibitor (IMPI), amongst others, are also recurrently used to assess *G. mellonella* responses to specific microbial species.

Additionally, haemocyte are commonly used in conjunction with microbial load post larvae infection to correlate microbial generation time, disease progression, and host mortality, with corresponding immunological response (Barnoy et al., 2017; Cools et al., 2019; Polenogova et al., 2019). Therefore, based on the virulence profile of *L. monocytogenes* strains determined earlier (sections 3.2.2 & 3.2.3), representative *L. monocytogenes* strains were selected for investigating *G. mellonella* larvae immune responses to strains of significantly different virulent potential. The growth rates of these strains *in vivo* and *in vitro* were determined, and haemocyte density post larvae

infection quantified and compared to controls and reference strains. Additionally, induction of immune response associated genes were also evaluated.

4.2 Methodology

4.2.1 *In vitro* growth rate of *L. monocytogenes* strains

Base on the virulence profile of *L. monocytogenes* strains, as determined in Chapter 3 (sections 3.2.2 & 3.2.3), three *L. monocytogenes* strains (NLmo4, NLmo14, and NLmo20) were selected as representative strains for further investigation. NLmo4 induced the highest mortality rates and NLmo14 induced intermediate mortality rates relative to other strains whilst NLmo20 infection resulted in the least larval deaths at 7 d p.i. Growth rate of these strains *in vitro* were hence assessed and compared to the *L. monocytogenes* reference strains EGD-e and *L. ivanovii.*

Microbiological media preparation and bacterial growth conditions were as described previously (Chapter 2, section 2.2.2) with modifications. Overnight planktonic cultures were diluted to a starting absorbance (OD_{600} nm) of 0.01, and OD_{600} was determined at hourly intervals for 12 h whilst CFU enumerations were carried out at bi-hourly intervals. Calibration curves of Absorbance and Log CFU were generated to determine bacterial growth over time.

4.2.2 Preparation of bacterial cultures for *G. mellonella* inoculation.

Planktonic bacterial cultures were prepared by seeding 10ml BHI broth with one CFU and incubating at 37ºC in a shaking incubator (200 rpm, aerobic conditions). The planktonic cultures, in stationary phase, were diluted in fresh BHI to an OD_{600} of 0.42 which corresponds to 10^9 CFU ml^{-1}, as determined from growth curves (section 4.2.1). Cells were harvested by centrifugation (12000 x g, 10 min, 22ºC). Prior larval inoculation the growth media was removed by washing cells twice and re-suspending in PBS at 10^8 CFU ml^{-1}.

4.2.3 *G. mellonella* larvae infection

Last instar *Galleria mellonella* (*G. mellonella*) larvae were purchased from UK Waxworms Ltd (Sheffield, UK) and stored at 20ºC. Larvae weighing between 0.25-0.35 g were selected and used within a day of receipt. For *Listeria* l load (3.2.4) and haemocyte density determination (3.2.5) 75 healthy larvae per treatment were

inoculated to ensure sufficient survival for experimentation at 7 d, and *n* = 30 were used per treatment for RNA assays. Larvae were inoculated with 10^6 CFU dissolved in 20µl PBS through the last right proleg using an insulin syringe as described previously (Joyce & Gahan, 2010). Control groups were inoculated with 20ul of PBS, and larvae were incubated in the dark at 37ºC in 9cm petri dishes lined with Whatman paper.

4.2.4 Determination of *Listerial* load in *G. mellonella* larvae

Listeria monocytogenes bacterial burden was evaluated in larvae inoculated with 10^6 CFU at fixed time points daily for 7 d. Three larvae were homogenised using a Stomacher (Stomacher® 80 Biomaster, Seward, UK) in 3 ml of sterile PBS. This was serially diluted with PBS and aliquots of 100 µl plated on Oxford *Listeria* plates containing amphotericin B (10ug/ml), colistin sulphate (20ul/ml), acriflavin (5ul/ml), ceotetan (2ul/ml), and fosfomycin (10ul/ml) to inhibit growth of native larval flora and allow *Listeria* selection. Plates were incubated at 37^0C for 48 h and *Listeria* CFU per larvae was enumerated base on the number of CFU counts growing at specific dilutions (Sheehan and Kavanagh, 2018). Experiments were performed independently three times using a different batch of larvae each time and the means ± SD determined.

4.2.5 Determination of larval haemocyte density post *Listeria* inoculation

Larvae were inoculated with 10^6 CFU and the haemocyte density was assessed daily for 7 d. At each time point p.i three alive larvae were pierced at the side of the head with a sterile needle and the haemolymph pooled together into a pre-chilled Eppendorf containing phenylthiourea granules to prevent melanisation, as carried out previously (Mowlds et al., 2010). Haemolymph was diluted in PBS containing 0.37% (v/v) 2-Mercaptoethanol and cell density assessed using a haemocytometer. Three independent experiments were performed and the means ± SEM were expressed as haemocytes per ml of haemolymph.

4.2.6 Haemocyte cells staining and morphology

Haemolymph was collected and processed as discussed in section 4.2.3 above. Before haemocyte density was determined cells were washed in PBS, using a 1:10

haemolymph to PBS dilution, and cells harvested by centrifugation (1000 x g, 20ºC, and 5 min). Haemocytes were resuspended in PBS and density determined as 4.2.3, thereafter concentrations were normalised to 5 x 10^5 haemocytes/ml. Using 150µl of cell suspension cells were concentrated to a glass slide using a Shandon cytospin 3 cytocentrifuge (110 x g for 5 min). Cells were air-dried, fixed using methanol (30 sec), and stained with eosin (repeatedly dipping and withdrawing slide for 30 sec) and haematoxylin (repeatedly dipping and withdrawing slide for 30 sec). Slides were rinsed in buffer, air-dried, and cells were viewed under a light microscope.

4.2.7 Semiquantitative RT-PCR of insect immunity-related genes.

Three genes previously detected as part of larval immune response were investigated. These were genes coding for the antimicrobial peptides galiomycin, gallerimycin, and the insect metalloproteinase inhibitor (IMPI). RNA was extracted from larvae inoculated with either *Listeria sp.* or PBS at 6 h and 24 h post- inoculation. Total RNA was isolated from whole larvae using RNeasy® Mini Kit (Qiagen, UK) according to manufacturer's protocol with modification. Three larvae were homogenised in 1ml PBS using a Stomacher (Stomacher® 80 Biomaster, Seward, UK) at each time point. 150 mg of homogenate was weighed out and spun at 12000 g (60 sec) and 60 mg of supernatant collected into a new 1.5ml microcentrifuge tube. 600µl of RLT lysis buffer (supplied with kit) was added and sample was left at room temperature for 20 min with manufacturer's protocol been followed afterwards. The first strand cDNA synthesis kit (Fisher Scientific) was used for reverse transcription using normalised samples of 800 ng RNA. RT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) and Rox SYBR Green master mix (Primer Design, UK) following the manufacturer's instructions. This was done relative to the house keeping gene β-actin using gene specific primers as listed in Table 4.1.

Table 4.1: Primers used for real-time RT-PCR analysis.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Galiomicin	TCC AGT CCG TTT TGT TGT TG	CAG AGG TGT AAT TCG TCG CA
Gallerimycin	GAA GAT CGC TTT CAT AGT CGC	TAC TCC TGC ACT TAG CAA TGC
IMPI	AGA TGG CTA TGC AAG GGA TG	AGG ACC TGT GCA GCA TTT CT
β-actin	ATC CTC ACC CTT AAA TAC CC	CGA CAC GGA GCT CAT TGT A

4.3 Results and Discussion

4.3.1 *Listeria* isolates have similar growth kinetics *in vitro*

Given the earlier determined inter strain differences in virulence (Chapter 3) isolates representative of the least to the potentially most virulent strains were selected for further assessment. It has been reported that bacterial growth rate *in vitro* can be used as a predictor of strain virulence in hosts. *Flavobacterium columnare* strains that exhibited significantly varied virulence in rainbow trout (*O. mykiss*) and zebra fish (*D. rerio*) hosts were reported to have different replication rates in media (Kinnula et al. 2017). This phenomenon was also true for the bacterial insect pathogen *Xenorhabdus nematophila* when grown in liquid Luria Bertani medium (LB) and later tested in *in vivo* assays using *G. mellonella* larvae (Chapuis et al., 2011). To therefore establish whether *L. monocytogenes* strains used in this study have different growth capabilities in laboratory media, mean growth rates in BHI broth was determined over a 12 h time course as described (section 4.2.1).

Starter cultures, at OD_{600} of 0.01, contained mean CFU of 10^7 per ml (Fig. **4.1**). Rapid growth was observed amongst all *L. monocytogenes* strains within the first 6 h (exponential growth), consistent with reported growth rates for *L. monocytogenes* EGD-e in BHI media at 37ºC (Rea et al., 2004). Over the same time points *L. ivanovii* exhibited slower but gradual growth dynamics. Inter strain comparisons showed no statistical significance in replication rates amongst the *L. monocytogenes* strains within the first 6 h ($p \geq 0.05$), except between EGD-e and NLmo14 at 6 h ($p < 0.05$). In contrast, significant differences in viable CFU counts were observed when *L. monocytogenes* strains were compared to *L. ivanovii* at both 4h and 6h with differences been more significant at 6 h especially between EGD-e and NLmo14 as compared to *L. ivanovii* ($p < 0.0001$, Two-Way ANOVA, Fig 4.1). At ~7h all isolates, except *L. ivanovii,* had reached station growth as observed in both CFU ml^{-1} and OD_{600} nm. Though *L. ivanovii* had a slow growth rate after 9h incubation it was observed to have CFU counts parallel to that observed in the other isolates ($p \geq 0.05$, Two-Way ANOVA). During the 12h time course bacterial growth increased by ~2 Logs for all isolates plateauing at 10^9 CFU ml^{-1}. Though NLmo14 and NLmo20 attained the most mean CFU ml^{-1} no significant differences were observed amongst all the strains investigated at the stationary phase as all isolates reached the same CFU ml^{-1} and

final OD_{600} nm by 12 h. Therefore, the bacterial replication *in vitro* did not correlate with the prior observed differences in virulence by number of larval mortalities caused.

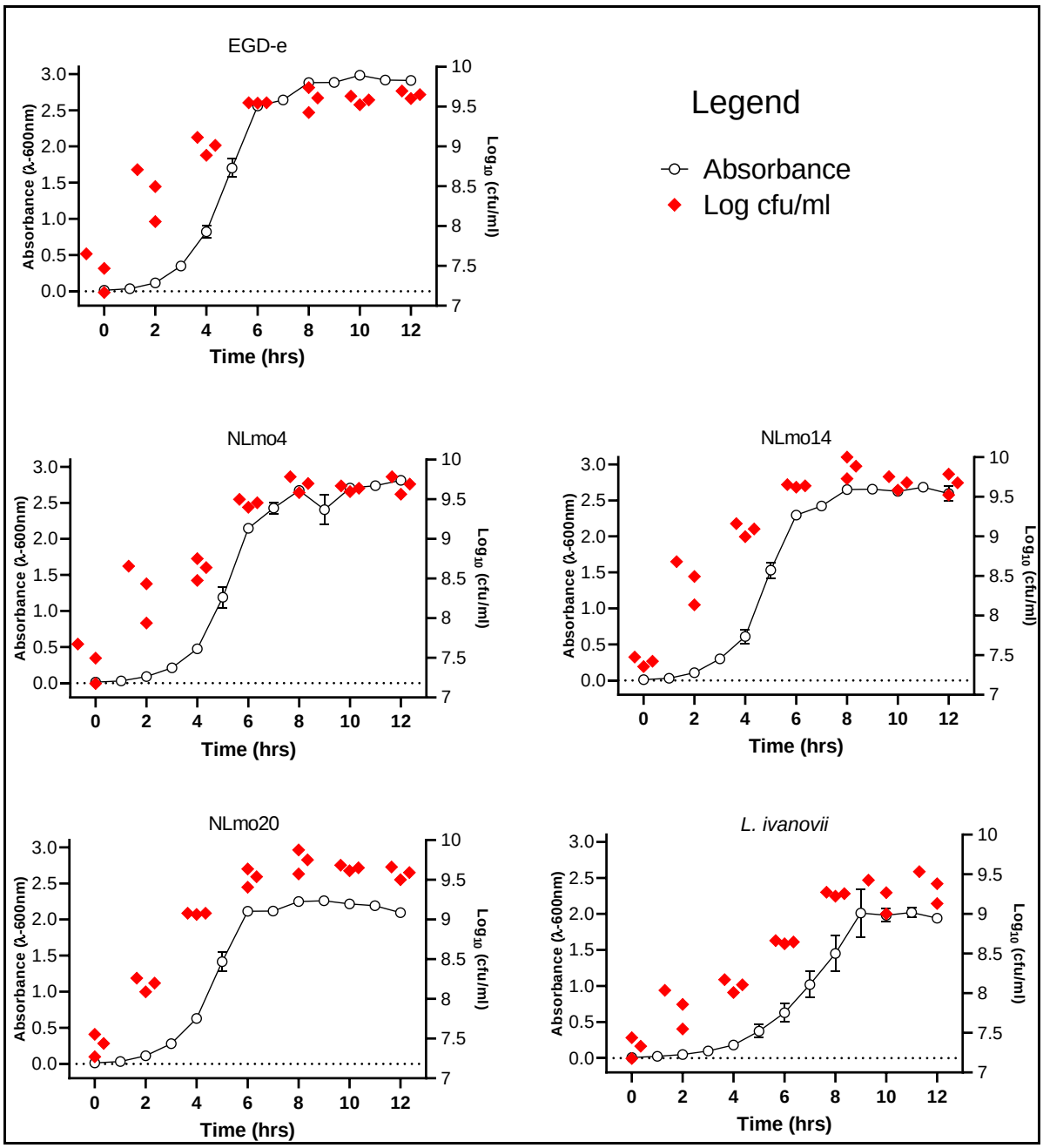


FIG. 4.1: Growth of *L. monocytogenes* strains EGD-e, NLmo4, NLmo14, and NLmo20, and *L. ivanovii* (absorbance and CFU counts) as a function of time. BHI broth was seeded with bacterial cultures in stationary phase to a starting absorbance (600 nm) of 0.01 and incubated at 37ºC (200 rpm, aerobic conditions). Absorbance was measured hourly and CFU ml^{-1} at bi-hourly intervals. Results represent individual replicates (CFU ml^{-1}) and mean ± SEM values of three independent determinations.

4.3.2 *L. monocytogenes* infection of *G. mellonella* larvae was accompanied by bacterial growth.

L. monocytogenes virulence factors are reportedly expressed at low levels during growth in laboratory media but are upregulated during infections (Camejo et al., 2009; Ermolaeva et al., 2004; Riedel et al., 2009). We therefore tested whether the differences in *L. monocytogenes* induced *G. mellonella* mortality observed previously depended on bacterial replication and persistence within *G. mellonella* larvae. *G. mellonella* larvae were inoculated with 10^6 CFU/larva as described (section 4.2.3) using 75 larvae per treatment. Three alive larvae were crushed at each time point p.i and bacterial cells harvested at 1hr, 6hr, and thereafter at fixed time points daily for 7 d. To ensure the bacteria recovered from these larvae were *Listeria* samples were plated on *Listeria* selective media that inhibit growth of the natural larval flora.

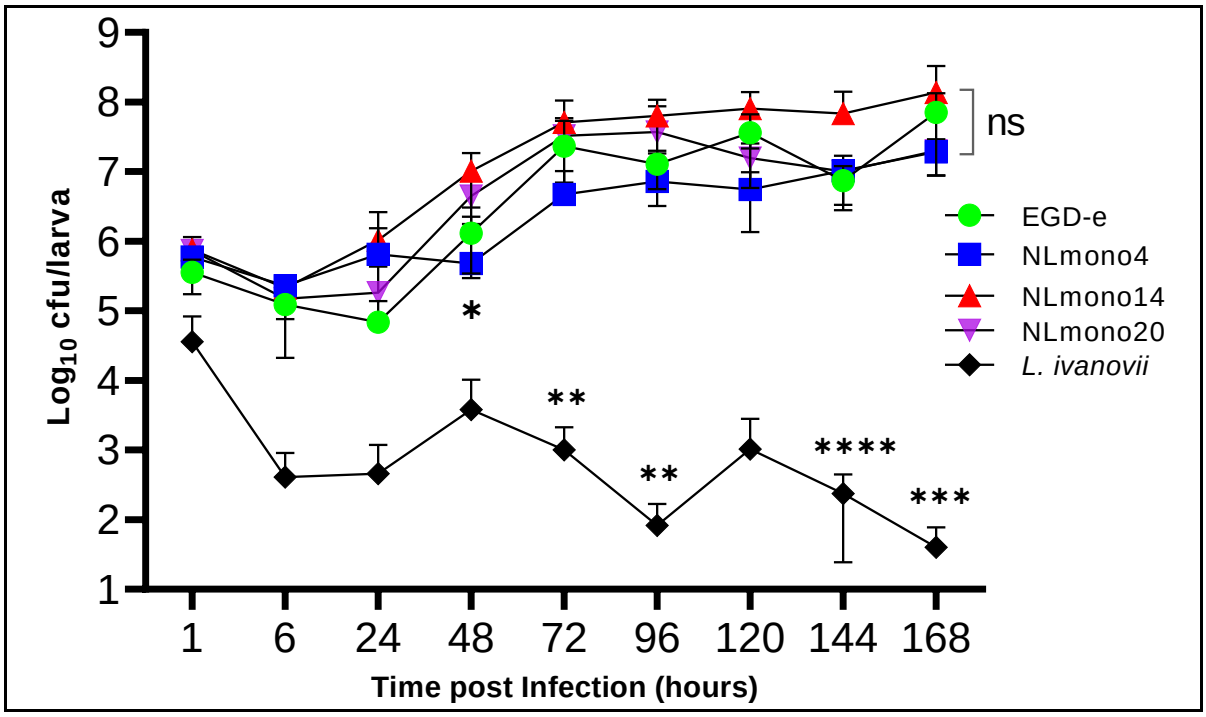


FIG. 4.2: Bacterial replication after *Listeria* infection of *G. mellonella*. Larvae were inoculated with 10^6 CFU and incubated at 37˚C. At each time point larval bacterial load was assayed by crushing 3 live larvae in 1ml PBS and plating on *Listeria* selective Oxford agar. The experiments were repeated 3 times and values represent Mean ± SEM. A Mixed-effects 2-Way analysis was used to assess statistical significance and data shown is of *L. ivanovii* as compared to the *Listeria* strain with most significant difference at that time point (*p<0.05; ** p< 0.01; ***p< 0.001; ****p<0.0001; ns, no significance).

Viable CFU counts decreased for the first 6h p.i for all isolates with replication rates varying thereafter (Figure 4.2). However, recovered bacterial counts from larvae in the first 6h p.i did not significantly differ from the dose used to initiate infection for all strains, except *L. ivanovii* ($p = 0.001$ and $p < 0.0001$, at 1hr and 6 hr p.i, respectively). After 24h bacterial burden was observed to increase rapidly in all *L. monocytogenes* infected larvae and plateaued after 72h. This corresponded to ~2 Logs increase in bacterial burden from the infecting bacterial dose. Though NLmo14 consistently exhibited higher replication rates *in vitro* final bacterial burden in *G. mellonella* did not significantly differ amongst *L. monocytogenes* strains at the end of the 7d time course ($p = 0.488$, one-Way ANOVA). In contrast, bacterial burden significantly differed between *L. monocytogenes* and *L. ivanovii* inoculated larvae. Viable *L. ivanovii* CFU counts gradually declined during the 7d time course decreasing by a total of 5 Logs at 7 d p.i. This strain was unable to establish an infection indicating it is relatively avirulent at a dose of 10^6 CFU in *G. mellonella* larvae, and these results corresponded with its relatively low mortality rates. *G. mellonella* was therefore effective at clearing the *L. ivanovii* infection, which was demonstrated by a lack of recoverable CFU even in neat samples at certain time points in the investigation.

The *in vivo* bacterial generation time observed in this investigation was proportional to that seen in *in vitro* growth rates for all the investigated strains, except *L. ivanovii.* It is a classic virulence theory that pathogens with higher growth rates within hosts have higher virulence potential than slower growing pathogens. This trade-off theory assumes that parasites with higher internal growth rates have higher transmission rates but also cause increased host mortality (Anderson & May, 1982; Frank, 1996). However, in this study the contrary was observed when assessing *L. monocytogenes* strains, though such can be presumed when compared to the *L. ivanovii* strain. Amongst *L. monocytogenes* strains no positive correlation between bacterial generation time and mortality rates in *G. mellonella* larvae was found. Instead, amongst the *L. monocytogenes* strains the strains with the highest observed mortality rates (NLmo4) had the least recoverable bacterial load, albeit differences were not significant when compared to other isolates. This was also as reported recently by Leggett et al. (2017) who analysed data of bacterial growth rates in relation to virulence for 61 human pathogens that included *L. monocytogenes*. A significantly negative correlation between microbial growth and higher host mortality rates was reported. It has also been reported that *Flavobacterium columnare* strains that induced significant

high and low mortality rates in zebra fish had no significant differences in growth rates *in vivo* (Kinnula et al., 2017). Such was also observed for *Xenorhabdus nematophila* isolates exhibiting significant differences in virulence in *G. mellonella* larvae that had similar growth characteristics in LB media (Chapuis et al., 2011). The latter study, however, did not evaluate *in vivo* generation time of *X. nematophila* isolates. Nevertheless, increased mortality rates corresponding to higher bacterial burden have been demonstrated for yeast strains, *Burholderia cepacia* species, and *Actinobacillus pleuropneumoniae* strains in *G. mellonella* larvae (Bergin et al., 2003; Seed & Dennis, 2008; Terra et al., 2015). In summation, though fast bacterial growth could be one way to be virulent, these results suggest this is, at least in *L. monocytogenes,* a multifactorial trait dependent on other strain specific factors.

4.3.3 Inoculation of *G. mellonella* larvae with *Listeria* leads to alterations in haemocyte density

Haemocytes are the main cellular component in *G. mellonella* immunity and they mediate the larvae immune response to infections (Fallon et al., 2011). Fluctuations in *G. mellonella* haemocyte densities following exposure to a range of microorganisms have been demonstrated (Matha & Áček, 1984; Morton et al., 1987; Mowlds et al., 2010). In fact these fluctuations, in addition to microbial load, have been suggested as indicators of microbial pathogenicity in *G. mellonella* larvae (Bergin et al., 2003). Hence, having determined mortality rates and *Listerial* burden we assayed how these could correlate with haemocyte density. The objective was to understand larval immunological response to *L. monocytogenes* infections and also determine relative pathogenicity of the isolates. *G. mellonella* infection and haemocyte quantification is as described (sections 4.2.3 & 5) using 75 larvae per bacterial treatment, 30 for controls, and extracting haemolymph from 3 alive larvae at each time point for a duration of 7 d. Experiments were carried out three times using different batches of larvae.

Before larval inoculation haemocyte density was quantified, and this represented 0 hr for each tested isolate. The results (Figure 4.3) indicate at 0 hr larvae haemocyte density was $3.15 \pm 0.96 \times 10^7$ per ml of haemolymph. In *Listeria* inoculated larvae a gradual decline in haemocyte density was observed within the first 24 h p.i and thereafter increased to pre-inoculation levels, except in EGD-e and *L. ivanovii.* However, in EGD-e and *L. ivanovii* inoculated larvae the rapid decline continued till 48

hr p.i. before stabilising. In contrast, a decline in haemocyte density in PBS-inoculated larvae was only observed in the first 1 h p.i which gradually increased to pre-inoculation levels within 24 hr. Though haemocyte density levels increased in *L. monocytogenes* inoculated larvae after 48 hr p.i this was mostly followed by a gradual decrease and was more apparent in NLmo14 and NLmo20 towards the conclusion of the time course. Nonetheless, mean haemocyte density comparisons amongst treatment groups showed PBS inoculated larvae had higher haemocyte densities than *Listeria* inoculated larvae throughout the course of the investigation, albeit differences were insignificant at most time points. Notwithstanding, though differences in haemocyte density was observed between all strains these were largely between PBS and *L. ivanovii* inoculated at most time points larvae and were more apparent at 24-72 hr p.i. After 7 d incubation however, haemocyte densities amongst treatment groups did not significantly differ from pre-inoculation levels nor were there any significant inter-strain differences at this time point ($p < \geq 0.05$, Two-way ANOVA). Interestingly, while *L. ivanovii* had the least recoverable CFU and exhibited slower growth rates *in vitro* relative to *L. monocytogenes* strains it induced the most sustained decrease in haemocyte density.

A correlation between strain virulence and haemocyte density was not observed in *G. mellonella* larvae for the investigated *L. monocytogenes* isolates in this study. It was also recently reported that *Streptococcal* strains of different virulence potentials that included a heat-killed bacterial dose exhibited no significant differences in haemocyte densities (Cools et al., 2019). Also, a study using a wild type (WT) *Legionella pneumophila* serogroup I strain (130b) and a mutant (ΔDotA) lacking a type IV secretion system (T4SS) that induced 70% differences in *G. mellonella* mortality rates at 18 h p.i reported these strains had no significant differences in haemocyte density at the same time point (Harding et al., 2012). In contrast, other studies (Bergin et al., 2003; Joyce & Gahan, 2010; Terra et al., 2015) have reported a linear relationship exist between strain virulence and haemocyte density. These suggest mortality rates do not correlate with decreased haemocyte density in all microbial spp. The observed differences in this study in relation to the findings by latter research groups could also be as a result of the methodology used. Whilst we determined total haemocyte counts in this study, live (viable) haemocyte counts were assessed in one of the latter studies (Joyce & Gahan, 2010).

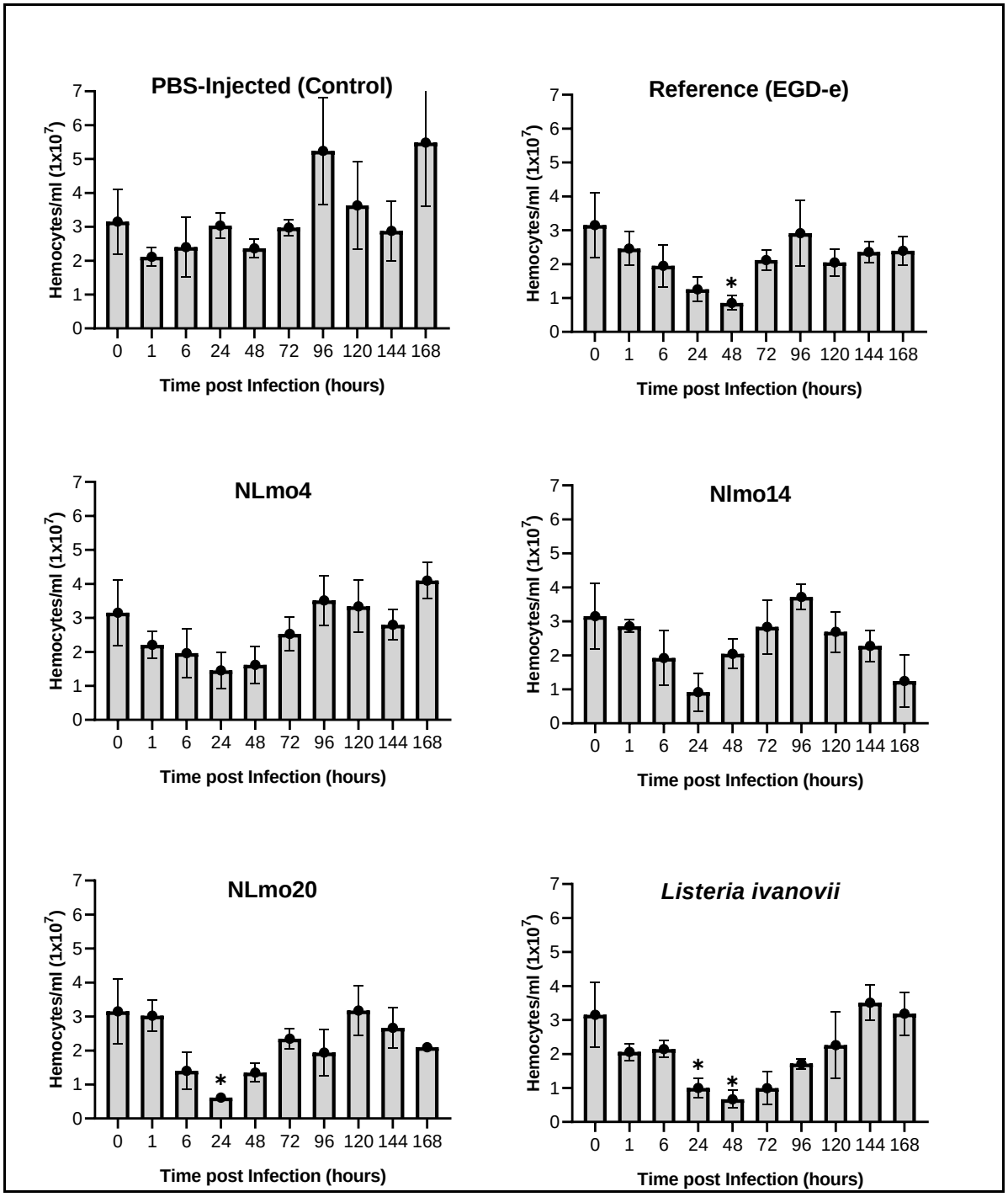
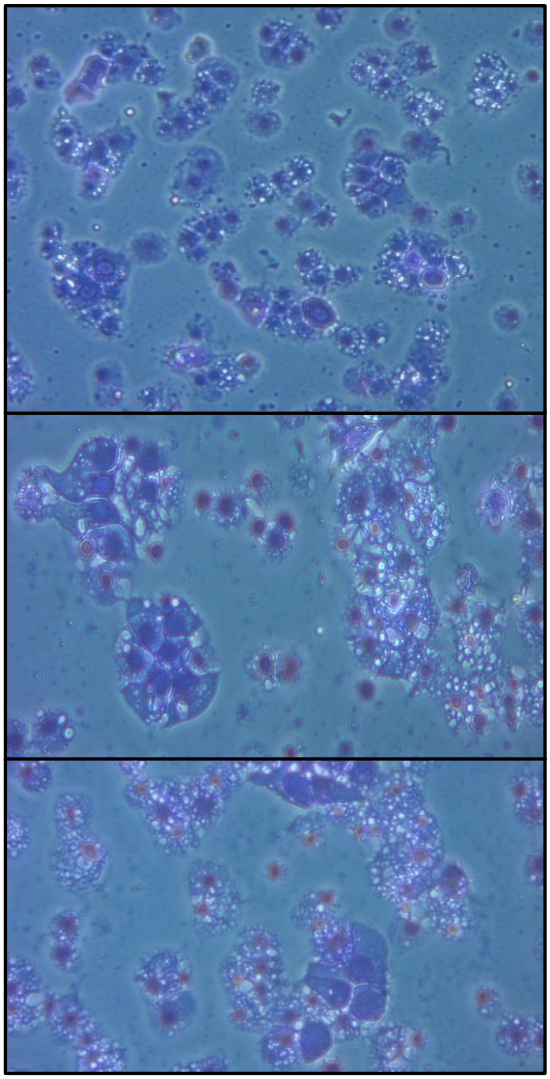


FIG. 4.3: Fluctuations in haemocyte density in larvae inoculated with *Listeria* strains and PBS. Bacterial cultures were grown to stationary phase, washed, and resuspended in PBS. Larvae were inoculated with a dose 10^6 CFU/larvae, and Controls with 20µl of PBS, thereafter incubated at 37°C. At each time-point haemolymph was collected from 3 alive larvae, and haemocytes quantified on a haemocytometer chamber. Statistical significance was tested by comparing haemocyte density of *Listeria* inoculated larvae to PBS at each time point (* $p < 0.05$, Two-Way ANOVA). Results represent mean ±SEM of three independent determinations.

4.3.4 *L. monocytogenes* infection of *G. mellonella* larvae induces morphological and population changes of haemocytes.

An attempt was made to characterise haemocytes post-larval inoculation through cell staining. The *L. monocytogenes* reference strain (EGD-e) and the *L. monocytogenes* strain with highest observed mortality rates (NLmo4) were tested. *G. mellonella* larvae were inoculated with 10^6 CFU and controls with 20µl PBS, and at 24 h p.i haemolymph was collected and cell staining using haematoxylin and eosin carried out as described (section 4.2.6). Different haemocyte morphologies were observed in the different treatments (**Figure 4.4**)



(a)

(b)

(c)

FIG. 4.4: Light microscopy of circulating *G. mellonella* haemocytes at 24 hr post-inoculation. Images (400X magnification) are of haemocytes stained with haematoxylin-eosin. (a) PBS inoculated larvae (control), (b) EGD-e inoculated larvae, (c) NLmo4 inoculated larvae. Results are representative of haemocyte subpopulations and not total haemocyte count (THC).

Haemocytes stained with haematoxylin and eosin showed many cytoplasmic granules of variable sizes and also cells without granules. In haemocytes of larvae inoculated with *L. monocytogenes* strains more granulation was observed (Figures 4.4(b) and 4.4(c)). NLmo4 inoculated larval haemocytes had less visible nuclear membranes as

compared to controls, higher numbers of granules were also present (Figure 4(c)). This was consistent with reviewed literature on mechanistic haemocyte response to pathogen invasion. Reportedly, following microbial infections granular *G. mellonella* haemocytes recognise and encapsulate pathogens. Upon contact these cells lyse or degranulate to release enzymes, such as phenoloxidase (Battistella et al., 1996), that promote plasmatocytes to attach and aid microbial killing (Browne et al., 2013; Joyce & Gahan, 2010). Compared to PBS controls, larger nucleated cells of varying shapes were also observed in *L. monocytogenes* inoculated larvae with smaller and more spherical cells seen in the PBS controls. Haemocyte subpopulations were also more diverse in PBS-inoculated larvae than seen in ones inoculated with *L. monocytogenes* strains, suggesting *L. monocytogenes* infections only induce increased production of certain haemocyte subtypes. However, given time limitation of this project no attempts were made to identify the different haemocyte subtypes. Nonetheless, the results suggest *L. monocytogenes* strains induce varied larval immunological responses. The use of Flow Cytometry and differential staining (Estrada et al., 2016) is proposed to quantify haemocyte subpopulations and identify haemocyte cell types.

4.3.5 *L. monocytogenes* infections induce expression of AMPs

The innate cellular response of insects is reportedly followed by synthesis of a broad range of antimicrobial peptides (AMPs) in response to microbial infections (Sheehan & Kavanagh, 2018). AMPs, secreted by haemocytes in *G. mellonella*, are a major component of immune defence to injury and microbial invasion and an increase in their expression levels can mediate larvae survival (Bergin et al., 2006; Kelly & Kavanagh, 2011). Since *Listeria* infection was accompanied by a decreased in haemocyte density within the first 24 hr p.i we investigated whether this has opposing effect to expression levels of AMPs. We used the *L. monocytogenes* reference strain EGD-e and the strain found to induce the highest mortality rates, NLmo4, and also compared these to *L. ivanovii* to evaluate whether the response could also be strain or species dependent. Larvae were infected with 10^6 CFU and RNA was extracted at 6 and 24 hr p.i with gene expression evaluated by semiquantitative RT-PCR. The fold change in transcriptional activation of the AMPs galiomycin and gallerimycin (also sometimes referred to in the literature as defensin 1 & 2, respectively), and the metalloproteinase inhibitor IMPI relative to PBS inoculated larvae were assessed and normalised to the house keeping *β-actin* gene (Figure 4.5).

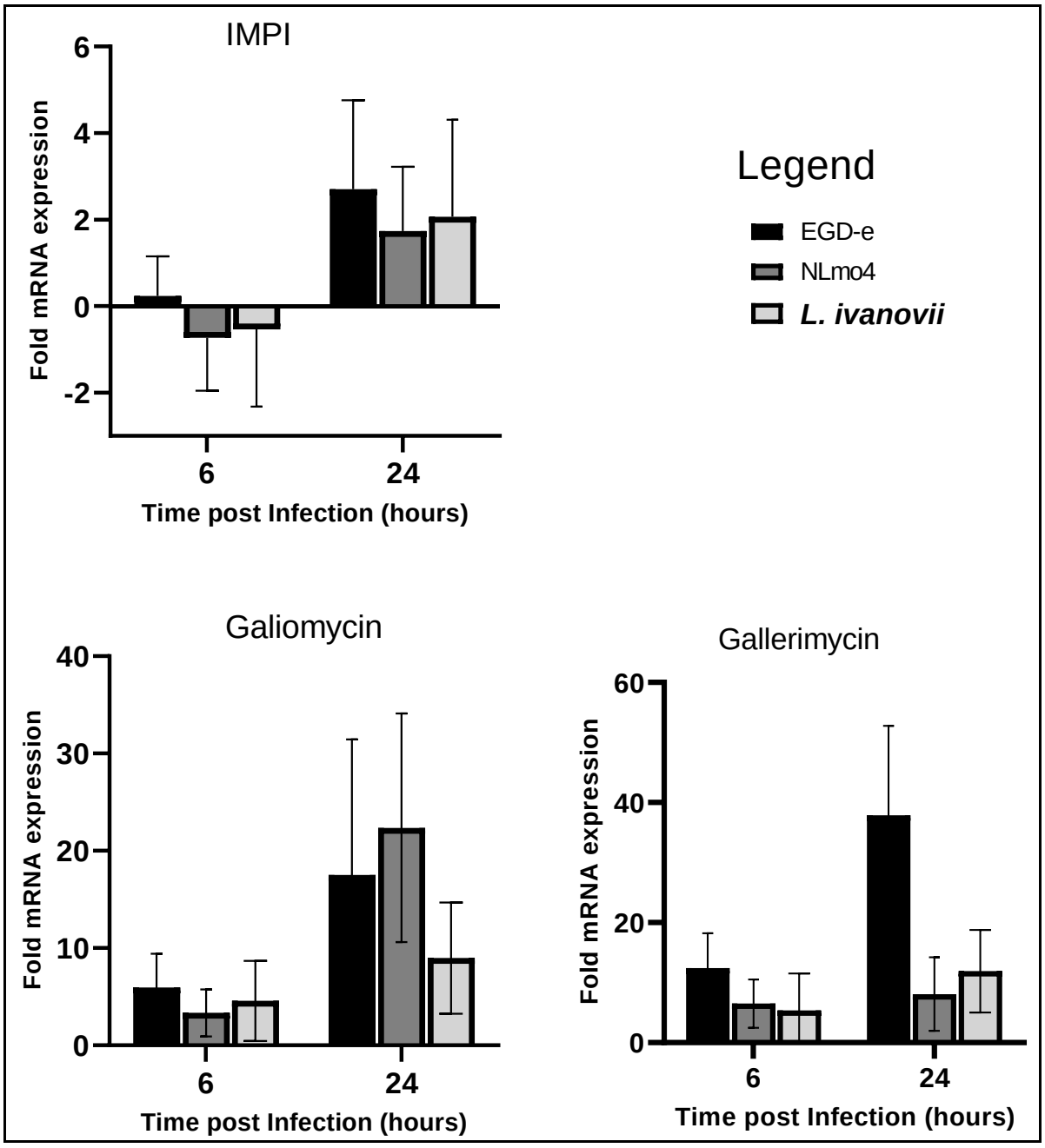


FIG. 4.5: Semiquantitative determination of induction of *G. mellonella* immune response genes after challenge with *Listeria* isolates. Larvae were inoculated with *L. monocytogenes* reference strain EGD-e, NLmo4 or *Listeria ivanovii.* Whole larvae RNA extraction of three live larvae at the indicated time points was carried out. The transcriptional levels of *Galiomycin, Gallerimycin,* and IMPI were determined by real time RT-PCR analysis and the results are relative to PBS inoculated larvae. These were normalised to expression levels of actin mRNA and the results are mean of three independent determinations ± SEM.

Expression levels of galiomycin and gallerimycin were upregulated for all bacterial isolates at all given time point p.i. In contrast, a down regulation of IMPI expression was observed amongst all isolates at 6 hr p.i. Expression levels of all AMPs tested for increased over the course of *Listerial* infection and significant mean differences were observed between expression levels at 6 hr and 24 hr (p = 0.031, Two-way ANOVA). At any time point EGD-e was found to elicit the most AMPs expression, except at 24 hr for galiomycin, with a highest fold change of 37.9 been observed at 24 hr p.i in gallerimycin. Interestingly, an earlier report using EGD-e found galiomycin and gallerimycin to be most induced at 6 hr p.i though a gradual increase in gene expression, as found in this investigation, was also reported (Mukherjee et al., 2010). Though lower AMPs induction fold changes were reported using the same EGD-e inoculum, except for gallerimycin, the 18S mRNA used for normalising expressions in that study could be a reason for the observed differences. In a later study that used heat-killed *Listeria monocytogenes* (Mukherjee et al., 2011), mRNA fold-changes in the AMPs investigated were significant less than that observed in this study at 24 hr p.i, suggesting immune induction is dependent on strain virulence. However, as the observed differences in AMPs inductions in this study were not proportional to earlier determined inter-strain virulence differences it indicates expression levels cannot be used to correctly predict differences in virulence in otherwise all virulent strains.

4.4 Results summary and Conclusions

Our results show that *Listeria* isolates have different growth kinetics in laboratory media but can grow to comparable CFU counts within 12 h. Though these *in vitro* growth dynamics were proportional to that observed in *in vivo* assays for *L. monocytogenes* isolates, bacterial generation time was longer *in vivo* with ~2 Logs CFU less than *in vitro* growth at the point when bacterial growth plateaued. Virulence of *L. monocytogenes* strains did not correlate with bacterial growth during the time course of this investigation. In contrast, though *L. ivanovii* had similar growth characteristics to *L. monocytogenes* strains *in vitro* a rapid decline in growth was observed *in vivo. G. mellonella* larvae were effective in clearing the *L. ivanovii* infection keeping CFU counts at sub lethal numbers which correlated with earlier observed mortality rates. Interestingly, the potentially most virulent *L. monocytogenes* strain

(NLmo4) was found to have the least viable CFU counts amongst the *L. monocytogenes* isolates *in vivo.*

Additionally, infection of *G. mellonella* larvae with *Listeria* isolates resulted in fluctuations in haemocyte density but fluctuations were mostly insignificant amongst *L. monocytogenes* infected larvae. Nonetheless, significant differences were observed between *L. ivanovii* and PBS controls with lower haemocyte densities in the *L. ivanovii* inoculated larvae. These indicate larvae response to infection is species dependent, and that though fluctuations haemocyte densities could be virulence dependent total haemocyte counts do no correlate with the severity of the pathogenic potential of strains. Similar deductions can also be made for the ability of *L. monocytogenes* strains to induce expression of immune response associated AMPs. However, these observations require further investigation using more diverse *L. monocytogenes* isolates with larger sample size.

Chapter 5 – GENERAL DISCUSSION.

Differences in pathogenic potential of *Listeria spp.* is an area of ongoing research and still of limited understanding. More so is our understanding of virulence differences of *L. monocytogenes* strains, which, as a consequences, means all *L. monocytogenes* strains are still treated as the same for regulatory purposes. Of the characterised *L. monocytogenes* strains, whose determined virulence are used to predict the pathogenic potential of other *L. monocytogenes* isolates and disease pathology, nearly all are from clinical sources. Therefore, studied *L. monocytogenes* strains are mainly of already disease causing capabilities whilst isolates from environmental and food sources that could be avirulent or of different virulent profiles to clinical isolates are yet hardly characterised. This study thus investigated the pathogenic potential of *L. monocytogenes* strains isolated from fresh leafy produce at different stages of the fresh produce supply chain (FPSC) and another isolate from a drainage at a food processing environment. These included strains belonging to *L. monocytogenes* lineages I or II. The aim of the project was to determine the differences in pathogenic potential of these strains in the *G. mellonella* infection model.

Numerous methods have previously been used to determine the virulence potential of *L. monocytogenes* strains, including the chicken embryo test (Gripenland et al., 2014; Lattmann et al., 1989; Quereda et al., 2018), Anton's test (Abdeltawab et al., 2015; Bhat et al., 2011), cell lines based assays (Rupp et al., 2017; Van Langendonck et al., 1998), laboratory animals (Bécavin et al., 2014; Brosch et al., 1993; Joyce & Gahan, 2010; Maury et al., 2016), and in recent times invertebrate models such as *G. mellonella* (Joyce & Gahan, 2010; Mukherjee et al., 2013). *G. mellonella* as an infection model is now routinely used to assess virulence due to the commonalities it shares with mammalian models, ethical acceptance of its use, ease of handling, cheap to acquire, and low rearing costs (Bergin et al., 2003; Browne et al., 2013; Mukherjee et al., 2015; Scully & Bidochka, 2006). Studies using *G. mellonella* as a model host commonly induce infections by inoculating larvae through the haemocoel with viable *L. monocytogenes* strains. Microbial virulence in the model has mostly been evaluated by either comparing the total mortality rates or 50% lethal dose, determining the microbial burden in larvae, larval health index score (HIS), or by quantifying haemocyte density and other immunological responses such as expression of AMPs following microbial infections. As each of these variables can be used to determine the

pathogenic potential of microorganisms, we recognised that a combination of these assays may enable consistent determination and better discrimination between strains of different virulence profiles. This is also as has been carried out in earlier studies that determined differences in virulence of other bacterial and fungal pathogens, as well as in correlation studies that demonstrated that virulence observed in the model parallels those seen in mammalian infection models (Brennan et al., 2002; Seed & Dennis, 2008; Slater et al., 2011; Wand et al., 2011).

Key to our investigation was to test whether *L. monocytogenes* strains exhibit different virulence potentials in *G. mellonella.* As depicted in Figure 3.3, significant differences in virulence were observed between *L. monocytogenes* strains ($p<0.05$) based on the rates of larvae mortality induced. *L. monocytogenes* strains induced mortality rates in the range of 65 ± 2.9% to 98.8 ± 2 1.1% by the end of the 7 d time course. All *L. monocytogenes* strains, except NLmo20, were found to cause higher mortality rates in *G. mellonella* larvae than the reference strain used in this in study, EGD-e. Also, mortality rates observed for EGD-e corresponded to that reported in other studies at the same time point (Martinez et al., 2017; Mukherjee et al., 2010). A correlation with the *L. ivanovii* strain used in this study as a second reference strain was also found in these studies. These demonstrated reproducibility and consistency of the *G. mellonella* model but also confirming that observed differences in virulence between *L. monocytogenes* strains are strain-dependent.

L. ivanovii was found to be the least virulent isolate relative to the investigated *L. monocytogenes* strains in this study, causing mean mortality rates of 4.4 ± 4.4% at 7 d. *L. ivanovii* is known to be of low pathogenic potential relative to *L. monocytogenes* in animal models (Mukherjee et al., 2010). This *spp*. of *Listeria* rarely causes clinical cases as of the nine reported clinical cases involving the bacterium since 1970 only one fatality was detailed (Beye et al., 2016). The low pathogenicity of *L. ivanovii* is mainly predicated on the low number of virulence factors found in strains of this bacterium relative to *L. monocytogenes* (Beye et al., 2016). *L. ivanovii* strains contain only two of the identified four *Listeria* pathogenic islands (LIPI) with far less virulence factors present in its core genomes as compared to *L. monocytogenes.* Thus, this study in addition to prior *in vivo* studies (Martinez et al., 2017; Mukherjee et al., 2010) supported the postulate that virulence of *Listeria* strains is dependent on number of virulence factors found in their genomes. However, the *in silico* predicted virulence of

the *L. monocytogenes* strains did not correlate with *in vivo* virulence in our investigation.

The number of identified virulence factors from whole genome sequencing (WGS) and other genetic based analysis tools such as PFGE have routinely been used to predict pathogenic potential of *L. monocytogenes* isolates (Klaeboe et al., 2006; Smith et al., 2019; Wiedmann et al., 1997). *L. monocytogenes* isolates used in this study (Table 3.1) were of different *in silico* predicted virulence stratifications (Smith et al., 2019). Of the 42 virulence factors (genes) tested for NLmo6, NLmo7, NLmo14 and NLmo20 carried the highest number of copies of those (41) whilst NLmo4 and NLmo5 possessed the least (31). Contrary to what we expected, NLmo4 and NLmo5 were the most virulent in *G. mellonella* and the strains of highest predicted virulence mostly exhibited medium (NLmo6 and NLmo14) and low virulence (NLmo20) relative to NLmo4 and NLmo5. In a literature search no prior studies testing for correlation between *in silico* predictions and *in vivo* virulence for such a wide range of determined virulence factors of *L. monocytogenes* were found. However, clinical strains of different virulence profile for eight *L. monocytogenes* virulence associated genes (*inlA, inlB,* and the six genes of LIPI-1) have been evaluated (Franciosa et al., 2005). All 27 isolates in that study were equally pathogenic in mice, more so, no correlation was found between *in silico* predictions and strains' virulence in a mouse model. Similar findings have also been reported for *Coxiella burnetii* strains in a mouse model (Melenotte et al., 2019). A *C. burnetti* strain (Guiana Cb175) that had 77 times less virulent genes compared to two other strains, *C. burnetti* German (Z3055) and *C. burnetti* Nine Mile (RSA 493), was found to be the most virulent causing 100% mortality at 4 week p.i whilst at the same time point a 0% and 75% mortality was reported for the two other strains. These correlated with the findings of this study, suggesting that total number of virulence factors is an inaccurate predictor of *in vivo* virulence, at least in *L. monocytogenes*.

Listeria strains were investigated for their growth rates *in vitro* and *in vivo* as a means to explaining the differences in mortality rates they induced. However, similar growth rates *in vitro* were observed for all strains and viable CFU recovered from larvae were only significantly different when *L. ivanovii* was compared to the *L. monocytogenes* strains (Figure4.2). Though bacterial burden was not significantly different in larvae inoculated with the most and least virulent *L. monocytogenes* strains, relative to the mortality rates they induced, the most virulent strain, NLmo4, had the least recoverable

bacterial CFU at 7 d p.i when compared to the least virulent NLmo20 strain. This indicated that increased numbers of viable bacterial CFU do not correlate with increased mortality rates in the larvae. In addition, *G. mellonella* response to infection and bacterial burden in larvae can also be quantitatively inferred from the rate of larval melanisation (Terra et al., 2015). After infection haemocytes are recruited which bind to and limit bacterial growth and dissemination (Bidla et al., 2009; Joyce & Gahan, 2010). This opsonisation process that initiate bacterial killing causes nodulations, an increase in nodule formation is visualised by the increase in larvae melanisation (Bergin et al., 2005; Pereira et al., 2018). High bacterial load thus correlate with increased melanisation, which was as observed on the dose dependent assays carried out in this study (section 2.3.4) as well as in reports by other research groups (Terra et al., 2015; Wand et al., 2012). In the HISS analysis conducted in this study, however, significant differences in larval melanisation between NLmo4 and NLmo20 inoculated larvae was not observed (melanisation data not shown). This further indicate that though mortality rates are dependent on bacterial burden reaching a lethal threshold, mortality rates beyond such a limit are independent of the bacterial replication rates; thus suggesting an intrinsic expression of virulence factors by bacterial strains to drive the differences in mortality rates seen between strains of similar growth characteristics. Given the determined negative correlation between high presence of virulence factors and increased mortality rates, strains were also evaluated for the presence of non-chromosomal factors from WGS data. The two most virulent strains in this study (NLmo4 and NLmo5) were also the only strain reported to carry plasmid derived QAC resistant genes (Smith et al., 2019). However, these plasmid were not characterised in the study. Nonetheless, plasmids are known reservoirs of bacterial virulence factors and their role in enhancing strain virulence in *in vivo* models, including *G. mellonella*, have been reported in other human pathogens including *Salmonella* (Barnoy et al., 2017)*, Staphylococcus aureus* (McCarthy & Lindsay, 2012)*, Escherichia coli* (Wijetunge et al., 2014)*,* and *Pseudomonas aeruginosa* (Rodríguez-Andrade et al., 2016). It was thus theorised that plasmid derived virulence in *L. monocytogenes* strains NLmo4 and NLmo5 could have caused the virulence phenomena observed in this study.

Notwithstanding, a reason for *in silico* predictions not correlating with *in vivo* virulence of *L. monocytogenes* strains from food and environmental sources could also be as a result of differences in ability of strains to modulate certain virulence determiners. *L.*

monocytogenes lives as a non-pathogenic saprophyte in the environment but transitions into a disease causing intracellular life cycle upon infecting a host by modulating the transcriptional regulatory *prfA* gene (Freitag et al., 2009; Gray et al., 2006). The *L. monocytogenes* PrfA regulon encompasses 10 genes, including genes of the *Listeria* pathogenic island 1 (LIPI-1), and is directly regulated by PrfA. PrfA is found in all *L. monocytogenes* strains and it also regulates 145 other putative genes in both the core and accessory *L. monocytogenes* genomes (Scortti et al., 2007). However, demonstrations of transcriptional activation of PrfA amongst *L. monocytogenes* strains in *in vivo* models have already been carried out and significant differences in its modulation have been documented (Fang et al., 2015; Sokolovic et al., 1996). Also, differences in *prfA* expression was reported by Sokolovic et al. (1996) to result in differences in virulence of *L. monocytogenes* strains in a mouse model and a Caco-2 line. The mechanisms behind modulations of this factor that underpins *L. monocytogenes* transition from a saprophyte to a disease causing bacterium remains to be elucidated, but differences in the ability of *L. monocytogenes* isolates from food sources to modulate its expressions could be key to their *in vivo* virulence, which would help explain the differences in virulence observed in this study.

Nevertheless, a limitation of this study based on the route chosen to establish *L. monocytogenes* infection could also be an underlying factor to the negative correlation of *in silico* prediction to *in vivo* virulence observed in this study. *L. monocytogenes* is a foodborne pathogen thus infections by the bacterium is primarily via the oral route. In listeriosis when the bacterium is ingested through contaminated food it traverses the intestines into the blood stream, subsequently infecting the liver, cerebrospinal fluid, meninges, and spleen. In one such pathological process the bacteria has been found to colonise the Peyer's patches in the small intestine upon its ingestion (Marco et al., 1992). It is postulated that *L. monocytogenes* uses the membranous or microfold (M) cells epithelium as an entry portal to cross the intestinal barrier, an entry route that has however been observed to be inefficient for successful infection (Pron et al., 1998). In a model using rabbit ileal loop it was found to transverse the intestinal barrier by binding to epithelial (E)-cadherin receptors through the bacterial surface protein Internalin A [InlA] (Pentecost, Otto, Theriot, & Amieva, 2006). In either routes, failure to clear the infection by immune cells (macrophages, neutrophils, etc.) in the liver following this process could lead to severe listeriosis as the bacterium gets released into circulation. In pregnant women *L. monocytogenes* spread from the maternal

bloodstream to placental villi and eventually into the foetal bloodstream, aided by the bacterial surface internalin proteins InlA and InlB to traverse the fetoplacental barrier (Lecuit, 2005) where it induces abortion, stillbirth, and neonatal septicemias (Gray et al.,1955). However, in this study infections were established by injecting bacterial inocula directly into the haemocoel which bypasses the required intestinal barrier breach required for the bacterium to successfully establish an infection. The differences in virulence of *L. monocytogenes* strains could thus be significantly dependent on their ability to traverse the intestinal walls. As such, inoculating bacteria directly into the haemocoel would eliminate a key first line virulence determiner and minimise the potential of identifying significant differences in virulence that are dependent on the variable virulence factors found in *L. monocytogenes* strains.

5.2 Concluding remarks

L. monocytogenes remains a priority pathogen and poses significant health risks globally. As global human population increases leading to an increasing need for food production, there is a significant need to: (a) determine differences in virulence of *L. monocytogenes* isolates found in food and food processing environments, and (b): test the pathogenicity of these isolates in animal model hosts to extrapolate the imminent danger they pose human health. This will enable better risk assessments and the delivery of safe food products to consumers, and enhance source tracking of *L. monocytogenes* outbreaks to curtail and prevent epidemics.

The first part of this thesis validated a model of *L. monocytogenes* infection in in *G. mellonella.* This was in turn used to determine that *L. monocytogenes* food products and environmental sources have significantly different virulence potential. This highlighted the need for research to heed the significant of *L. monocytogenes* and lays the groundwork for how future work could be used in this model to assess virulence of this bacterium. Potential reasons for differences in virulence of *L. monocytogenes* strains from food sources were discussed and suggestions of future work that could enhance our understanding of the pathogenicity of *L. monocytogenes* were made in this process.

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