Investigation into the prevalence and persistence of *Listeria monocytogenes* in Irish seafood: Possible roles for $\sigma^B$ in environmental persistence

Kerrie NicAogáin

Thesis submitted for PhD by Research at the National University of Ireland, Galway.

Research was conducted in:

Microbiology,

School of Natural Sciences,

College of Science,

National University of Ireland, Galway.

Submission Date: 31st March 2017

Research Supervised by: Dr Conor P. O’Byrne
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Abstract

*L. monocytogenes* is a robust bacterial pathogen capable of surviving in a wide range of environments including food processing facilities. This pathogen is of concern for the food processing industry particularly producers of Ready to Eat (RTE) food products such as smoked salmon and soft cheeses. The ability of this organism to overcome and survive under harsh conditions is partially due to the alternative sigma factor, σB. In a stressful environment, σB interacts with RNA polymerase to initiate transcription of the General Stress Response (GSR) regulon helping the bacterium to overcome the encountered stress. Developing surveillance programmes to determine the prevalence of *L. monocytogenes* in RTE processing facilities can provide information to assist producers to combat contamination by this pathogen. Characterisation of isolates collected during these types of studies can provide useful information regarding persistence of *L. monocytogenes* in the processing environment.

This study aimed to conduct a surveillance programme to determine the occurrence of *L. monocytogenes* in Irish seafood facilities over the course of three years. We found that the overall occurrence was lower than that suggested for seafood facilities in other studies. We further aimed to characterise and investigate mechanisms leading to persistence of *L. monocytogenes* in processing environments using strains isolated during the surveillance study. Visible light has been shown to be a possible mechanism for controlling *L. monocytogenes* and here we demonstrate that blue light (460 – 470 nm) has an effect on the growth and survival of *L. monocytogenes* and this effect is dependent on the production of ROS. We also aim to characterise a possible P-type ATPase, Lmo0818 and its function in maintaining homeostasis of the cell. Results presented in this thesis highlight the prevalence of *L. monocytogenes* in Irish food processing facilities and the potential for novel treatment methods such as light to control this pathogen.
Acknowledgements

There are so many people who have helped me during the course of my PhD. I would like to take this opportunity to thank them all with the utmost gratitude for their support, help and guidance throughout. Without you all I would never have made it.

Firstly, my supervisor Dr Conor O’Byrne, thank you for giving me this opportunity to undertake this research project and for all your guidance and expertise throughout the years. Thank you for your day-to-day support, your enthusiasm and for keeping me motivated on the hardest days.

To all members of the Bacterial Stress Response group: Tara, Conor, Beth, Yinka and Amber. Thank you all for making the lab a happy working environment, just with the little comments of encouragement or trouble shooting discussions, it did not go unnoticed and without you all my PhD would have been much more difficult.

Both past and present members of Microbiology, staff, postgrads and students I worked with during my project, thank you for all you have done for me.

A special thanks to our collaborators on the FIRM project. Also to the members of the NSSLRL Niall and Joanne, for their help in setting up PFGE.

To the friends I have made in Galway, especially Justine, Laura, Hannah and Kate, thank you for the laughs and the positive comments to keep going. For the times I needed a pick me up, your kind words and the offer of an ear to listen to my many rants was greatly appreciated. And Justine, who would have thought we would be good housemates!

To my Micro buddies, Teresa and Marise, you have kept me sane throughout. I am so grateful to be friends with you guys. Just knowing you were at the other end of a text message made some days a lot easier.

Last but definitely not least, my family. To my extended family, thank you for your support throughout the past four years. Your visits to Galway were always welcomed with a break from everything else going on. But to my parents, my brother and sister, where to start. Mam and Dad, thank you so much for your love and support throughout and for giving us the confidence that nothing is unachievable. You made things that seemed out of reach, seem possible. I love you both and will be forever grateful for the chances you have given me.

Finally David and Shauna, I can’t put into words how lucky I am to have grown up with you two. Thank you for your support over the last few years and hopefully it is now my turn to return it.
List of Abbreviations

ADI       Arginine deaminase
5-ALA     5-aminolevulinic acid
Amp       Ampicillin
ATP       Adenosine triphosphate
ATR       Acid tolerance response
BHI       Brain heart infusion
Bp        Base pair
BSA       Bovine serum albumin
Cg-MLST   Core genome multi-locus sequence type
CFU       Colony forming unit
cm        Centimetre
Cml       Chloramphenicol
CT        Cg-MLST type
DM        Defined medium
DMTU      Dimethylthiourea
DNA       Deoxyribonucleic acid
EDTA      Ethylenediaminetetraacetic acid
eGFP      enhanced Green Fluorescent Protein
Erm       Erythromycin
FBO       Food business operator
FMN       Flavin mononucleotide
g         gram
GABA      γ-aminobutyric acid
GAD       Glutamate decarboxylase
GFP       Green fluorescent protein
GSR       General Stress response
h         Hour
HCl       Hydrochloric acid
HGF       Hepatocyte growth factor
<table>
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<tr>
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<td>HINS</td>
<td>High intensity narrow spectrum</td>
</tr>
<tr>
<td>Inl</td>
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<tr>
<td>kDA</td>
<td>Kilo-dalton</td>
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<tr>
<td>Kv</td>
<td>Kilovolt</td>
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<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriolysin O</td>
</tr>
<tr>
<td>LOV</td>
<td>Light, oxygen and voltage</td>
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<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
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<td>Pulsotype number</td>
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<td>Description</td>
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<tr>
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<td>-------------</td>
</tr>
<tr>
<td>QAC</td>
<td>Quaternary ammonium compound</td>
</tr>
<tr>
<td>RTE</td>
<td>Ready to Eat</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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Note. Sections 1.1 and 1.5-1.9 have recently been published in the review “The role of stress and stress adaptations in determining the fate of *Listeria monocytogenes* in the food chain” (NicAogáin and O’Byrne, 2016, Frontiers in Microbiology). Additional information has been included in sections 1.2-1.4 and 1.10-1.11.
1.1. Overview

*Listeria monocytogenes* is a robust bacterial pathogen that is widely found in the environment. Its ability to persist in a diverse range of niches is underpinned by a sophisticated ability to sense and respond to the physicochemical stresses it encounters (Gandhi and Chikindas, 2007; O'Byrne and Karatzas, 2008). The term “stress” in this context is intended to mean any environmental perturbation that reduces the growth rate (a mild stress) or negatively impacts cell survival (a more severe stress). In general stress imposes an energy cost on cells because they have to invest resources in protection (e.g. homeostasis, synthesis of new macromolecules, repair and replacement of damaged components) if they are to continue to survive and grow. The stress responses deployed when stress is encountered, confer on *L. monocytogenes* the ability to persist in soil environments, water, mammalian and avian faeces as well as in food and food processing environments. They also allow it to make a successful transition from food into the gastrointestinal tract of mammalian hosts, which is a prerequisite for establishing infections in immunocompromised individuals. The stress tolerance mechanisms at its disposal allow *L. monocytogenes* to withstand acidic conditions, environments with low water activity, desiccation, low temperatures and bile. Many of these stress tolerance mechanisms are under the control of an alternative sigma factor called sigma B (σB) whose role is to associate with RNA polymerase directing it to SigB promoters, which in turn leads to the reprogramming of the transcriptional profile of cells enabling the expression of protective functions (van Schaik and Abee, 2005; Chaturongakul *et al.*, 2008; O'Byrne and Karatzas, 2008). The genes under the control of σB, collectively known as the General Stress Response (GSR) regulon, are now well defined and many contribute to specific stress protective functions. Once within the host, an additional set of genes that allow cell invasion and systemic spread are expressed and these are regulated by a master transcriptional regulator called PrfA (Scortti *et al.*, 2007). The roles of most of the virulence genes under PrfA control have well defined roles in the intracellular life cycle of the pathogen and indeed their study has fuelled the development of new areas of cell biology (Cossart and Toledo-Arana, 2008).

Although food-borne infections caused by *L. monocytogenes* are comparatively rare they are associated with unusually high mortality rates; typically 20-30% of clinical cases result in mortality. Immunocompromised individuals are most at risk, especially those with reduced T-cell immunity including elderly or very young patients, pregnant women and individuals infected with HIV or on immunosuppressive treatment regimens (Lecuit, 2007). The organism is readily killed by normal cooking regimes including food processing treatments.
that use high temperatures (e.g. pasteurisation). Therefore, the main at-risk foods are the so-called ready-to-eat (RTE) foods, foods eaten without prior heating that have physio-chemical properties that can sustain the growth of *L. monocytogenes* (Chan and Wiedmann, 2008). Some of these foods include raw fruit and vegetables, dairy produce made with unpasteurised milk, minimally processed seafood, cold meats and pâtés (Farber and Peterkin, 1991; Lecuit, 2007). Although most countries enforce strict regulations on the tolerance for this pathogen in RTE foods, its prevalence in the environment means that it is very difficult, if not impossible, to eradicate it from the food chain. Within Europe, if a product is capable of supporting growth, the producer must be able to demonstrate that levels of *L. monocytogenes* will not increase higher than 100 CFU/g over the course of the shelf life by means of a challenge study. However if a RTE product is not capable of supporting growth, levels must not exceed 100 CFU/g during shelf life (EU, 2005). This differs from regulation in the US, where absence of *L. monocytogenes* is required in all RTE products (FSIS, 2014).

Here, we discuss the route that *L. monocytogenes* can use to enter the food supply chain and discuss the behaviour of the pathogen in foods. We outline the key stresses that *L. monocytogenes* must overcome to survive and grow in RTE foods and discuss the main protective systems that this pathogen uses to defend itself.

1.2. Classification of *L. monocytogenes*

*L. monocytogenes* belongs to the genus *Listeria* spp. Before 2009 it was well known that this genus consisted of six species, *Listeria monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria innocua*, and *Listeria grayi*. However, since then there has been a rapid increase in the identification of new species including *Listeria marthii*, *Listeria fleischmannii*, *Listeria floridensis*, *Listeria aquatica*, *Listeria newyorkensis*, *Listeria cornellensis*, *Listeria rocourtiae*, *Listeria weihenstephanensis*, *Listeria grandensis*, *Listeria riparia* and *Listeria booriae* (Orsi and Wiedmann, 2016). Of the 17 species, only two are considered to be animal pathogens, *L. monocytogenes* and *L. ivanovii*, although *L. monocytogenes* is considered to be the most important for human infection. Approximately 95% of all food or clinical isolates tend to belong to one of the following serotypes, 1/2a, 1/2b, or 4b (Seeliger and Höhne, 1979; Farber and Peterkin, 1991; Tappero *et al.*, 1995; Graves *et al.*, 2007).
Based on the idea that certain subtypes have a greater ability of causing human infection, four genetic lineages have been described to further characterise *L. monocytogenes* (Piffaretti *et al.*, 1989; Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997). Lineage I contains serotypes 1/2b, 3b, 4b, 4d and 4e; whilst Lineage II includes 1/2a, 1/2c, 3a, and 3c (Piffaretti *et al.*, 1989; Orsi *et al.*, 2011). Lineage III, which consists of 4a and 4c along with some 4b strains, was thought to contain three distinct groups, IIIA, IIIB and IIIC but recently IIIB has been renamed as the fourth lineage (Rasmussen *et al.*, 1995; Liu *et al.*, 2006; Roberts *et al.*, 2006; Ward *et al.*, 2008). Strains from Lineages I and II are most commonly associated with sporadic cases and outbreak cases of Listeriosis whilst Lineages III and IV are detected much less frequently (Wiedmann *et al.*, 1997; Orsi *et al.*, 2011).

### 1.3. Activation and Regulation of σ^B^  

Different bacteria have different mechanisms for overcoming stresses encountered in the environment. Many bacteria activate the GSR, which leads to the transcription of a subset of genes required for coping with the stress. For example, Gram negative bacteria such as *E. coli* utilise the sigma factor RpoS to activate the GSR by interacting with RNA polymerase to transcribe stress related genes (Lange and Hengge-Aronis, 1991; Battesti *et al.*, 2011), whilst Gram positive bacteria including *S. aureus, B. subtilis* and *L. monocytogenes* use the alternative sigma factor, σ^B^ (Hecker and Volker, 2001; Hecker *et al.*, 2007; O'Byrne and Karatzas, 2008). σ^A^ is the main housekeeping sigma factor of *L. monocytogenes* (Metzger *et al.*, 1994). When stress is encountered, σ^B^ becomes available and competes with other sigma factors for binding to RNA polymerase. When bound to SigB, the RNA polymerase core forms the holoenzyme and can recognise SigB promoter sequences to initiate transcription of the associated stress related genes (Borukhov and Nudler, 2003; 2008). σ^B^ activates the transcription of multiple genes allowing the bacteria to survive harsh conditions such as low pH, osmotic stress, oxidative stress, cold stress and many more as discussed in Section 1.7.

In *L. monocytogenes*, sigB is co-transcribed as part of an eight gene operon including genes rsbR, rsbS, rsbT, rsbU, rsbV, rsbW, sigB and rsbX (Ferreira *et al.*, 2004). These eight genes are cotranscribed from a σ^A^-dependent promotor located upstream of rsbR, whilst a σ^B^-dependent promotor located upstream of rsbV enhances transcription of rsbV, rsbW, sigB and rsbX (Glaser *et al.*, 2001; Ferreira *et al.*, 2004). The other genes in this operon encode proteins involved in regulating SigB similarly to *B. subtilis* (Ferreira *et al.*, 2004). As *B.
Subtilis has been studied more extensively than L. monocytogenes, it is often used as a model for understanding σ^B activation in L. monocytogenes. It is hypothesised that L. monocytogenes senses stress through a complex called the stressosome (Fig 1.1). This complex has been characterised in B. subtilis and has recently been confirmed in L. monocytogenes (Delumeau et al., 2006; Marles-Wright et al., 2008; Marles-Wright and Lewis, 2010; Impens et al., 2017). The stress sensing complex is comprised of RsbR and its paralogues, along with RsbS and RsbT (Impens et al., 2017). Five paralogues of RsbR have been identified in L. monocytogenes, RsbR, Lmo0161, Lmo0799, Lmo1642 and Lmo1842 (Kim et al., 2004b; Gaidenko et al., 2006; Hecker et al., 2007; Marles-Wright et al., 2008; Ondrusch and Kreft, 2011; Heavin and O’Byrne, 2012; Jurk et al., 2013). The stress is thought to be sensed by the protruding N-termini of these sensory proteins and the signal is transduced to the STAS domains found in the C-termini that are located in the core of the stressosome (Marles-Wright et al., 2008). This leads to a signalling cascade which ultimately results in the activation of σ^B. The stress signals detected by the sensory proteins have not yet been established with the exception of light, which is detected directly by Lmo0799 as discussed in Section 1.7.5 (Ondrusch and Kreft, 2011).

In an unstressed cell, RNA polymerase directs the transcription of housekeeping genes in conjunction with σ^A. In this case, σ^B is sequestered by the anti-sigma factor RsbW and is unable to interact with RNA polymerase (Benson and Haldenwang, 1993). However, when the cell senses a stress signal through the stressosome, RsbR and RsbS become phosphorylated by RsbT, which allows for RsbT to disassociate from this complex (Voelker et al., 1996; Akbar et al., 1997; Gaidenko et al., 1999; Chen et al., 2003; Kim et al., 2004b). RsbT then acts as a positive regulator activating RsbU, a phosphatase protein, through protein-protein interactions (Kang et al., 1996; Yang et al., 1996; Delumeau et al., 2004; Hardwick et al., 2007). RsbV is present in an unstressed cell in a phosphorylated form but when stress is encountered, RsbU dephosphorylates RsbV (Dufour and Haldenwang, 1994; Yang et al., 1996). RsbW has a higher affinity for the anti-antisigma factor RsbV in its unphosphorylated form than for σ^B. This leaves σ^B free to interact with RNA polymerase, thereby reprogramming the transcriptional response of the bacterium (Fig. 1.1; Dufour and Haldenwang, 1994).

The activation of σ^B has been shown to occur in a rapid and transient manner and occurs in both L. monocytogenes and B. subtilis (Boylan et al., 1993; Voelker et al., 1995; Utratna et al., 2011; Utratna et al., 2012). The level of activation has also been shown to increase in proportion to the stress sensed by the cell i.e. the more stress sensed by the cell, the higher the level of σ^B activation (Utratna et al., 2011; Utratna et al., 2012). When the stress
subsides, the GSR needs to reset itself as it is hypothesised that its activation consumes high amounts of energy (Chen et al., 2004; Xia et al., 2015). rsbX is the final gene in the sigB operon and encodes another phosphatase (Yang et al., 1996). In B. subtilis, RsbX dephosphorylates RsbS-P and RsbR-P in a negative feedback loop that restores the $\sigma^B$ system to its original state (Yang et al., 1996; Chen et al., 2004). It is also thought to act by similar means in L. monocytogenes, where it negatively regulates $\sigma^B$ in less stress environments. Xia et al. (2015) showed that this may be a way of conserving energy as they observed that there were lower levels of ATP intra- and extracellularly for $\Delta rsbX$ compared to the parent strain, 10403S. This suggests that deploying the stress response in L. monocytogenes is energetically costly to the cell which would favour a rapid return to the un-induced state when the stress conditions have been adequately dealt with.
Figure 1.1. Schematic representation of σ^B activation based on the *B. subtilis* model. In an unstressed cell, σ^B is bound to the anti-sigma factor RsbW. When stress is sensed by the stressosome complex, RsbV becomes dephosphorylated by the phosphatase RsbU. In its dephosphorylated form, RsbV sequesters RsbW leaving σ^B free to interact with RNA polymerase, leading to the transcription of the GSR regulon. When the stress subsides, RsbX dephosphorylates RsbS and RsbR to restore the stressosome to its original state and therefore allowing for RsbT to recomplex with the stressosome. In this state, RsbV rephosphorylates and σ^B is rebound to RsbW preventing transcription of the GSR. This model represents the regulation of σ^B in *B. subtilis*, *L. monocytogenes* does not possess RsbP or RsbQ and is thought to sense both environmental and energy stress through RsbU (Hecker *et al.*, 2007). Figure was adapted from Marles-Wright and Lewis (2010). Model of the stressosome is taken from Marles-Wright *et al.* (2008).
1.4. Virulence and Infection

Once inside the host, *L. monocytogenes* can invade host epithelial cells or macrophage cells. Internalisation into macrophages occurs by means of phagocytosis. However, invasion into non-phagocytic cells involves a complex mechanism requiring interactions between microbial factors and host cell receptors in a process known as induced phagocytosis. For the first step, *L. monocytogenes* cells must attach to the host cell before internalisation can occur. Proteins belonging to a leucine rich repeat (LRR) superfamily known as internalin (Inl) proteins including InlA (Gaillard *et al.*, 1991) and InlB (Dramsi *et al.*, 1995), initiate attachment of the bacteria to the host cell. These proteins recognise particular receptors on the surface of the host cell, E-cadherin and Met. E-cadherin is a Ca<sup>2+</sup>-dependent molecule involved in cell-cell adhesion, and is recognised by InlA (Mengaud *et al.*, 1996). InlB interacts with the hepatocyte growth factor (HGF) receptor Met (Shen *et al.*, 2000). There are at least 27 internalin proteins encoded in the *L. monocytogenes* genome including InlA, InlB, InlC, InlC2, InlD, InlE, InlF, InlG and InlH (Engelbrecht *et al.*, 1996; Dramsi *et al.*, 1997; Raffelsbauer *et al.*, 1998; Pizarro-Cerda *et al.*, 2012). Whilst it does not seem that any of these internalins are required for InlB mediated internalisation, it does appear that for InlA mediated phagocytosis, InlA requires support of InlB or InlC along with InlGHE (Bergmann *et al.*, 2002). It has been hypothesised that InlB can support endocytosis of cells after initial attachment by InlA. This occurs by accelerating the endocytosis of E-cadherin after the activation of Met by InlB (Pentecost *et al.*, 2010). When InlA interacts with E-cadherin, E-cadherin signals to induce a rearrangement of the actin cytoskeleton of the host cell. This process stimulates internalization of the bacterial cell (Lecuit *et al.*, 2000; Bonazzi *et al.*, 2009; Camejo *et al.*, 2011). A similar process occurs for InlB. The interaction between InlB and Met, triggers a signalling cascade that ultimately leads to rearrangement of the actin cytoskeleton, which is dependent on the Arp2/3 complex (Ireton *et al.*, 1996; Ireton *et al.*, 1999; Bierne *et al.*, 2001).

Following internalisation, the pathogen secretes the pore forming toxin listeriolysin O (LLO) and two phospholipases, PlcA and PlcB to escape from the phagosome into the cytoplasm. LLO is optimal at an acidic pH (<6) and consequently this indicates that it is active within the acidic environment of the phagosome (Vázquez-Boland *et al.*, 2001; Hamon *et al.*, 2006). Once free from the vacuole, the bacterial cell first multiplies and then utilises the surface protein ActA to polymerise actin from the host cell through recruitment of the Arp2/3 complex in order to form an actin tail (Hamon *et al.*, 2006). This helps to propel the bacterium around the cell and eventually the cell propels itself into the cell membrane and forms a protrusion into a neighbouring cell by pushing through the membrane. The
protrusion is taken into the neighbouring cell again through phagocytosis. This time, the bacteria containing phagosome is bound by a double membrane. *L. monocytogenes* escapes this vacuole using LLO and PlcB and begins to replicate further (Vázquez-Boland *et al.*, 2001; Hamon *et al.*, 2006; Disson and Lecuit, 2013). The ability of *L. monocytogenes* to spread from cell to cell without leaving the host epithelial cells means that the pathogen can avoid coming into contact with elements of the host’s immune system, a strategy that other bacterial pathogens also use – E.g. *Shigella flexneri* (Cossart and Sansonetti, 2004; Kuehl *et al.*, 2015).

![Image removed due to copyright restrictions](image.png)

**Figure 1.2. Schematic representation of cell-cell spread of *L. monocytogenes*.** The proteins indicated in red are implicated in the stage of the infectious cycle shown. InlA and InlB mediate uptake of the bacterial cell into the host epithelial cell. Once internalised, the bacteria need to escape from the phagosome using LLO along with PlcA and PlcB. Following this, the bacterial cells multiply and then polymerise host cell actin using the protein ActA to form an actin tail. This structure helps to propel the bacteria around the cell and eventually forms a protrusion into a neighbouring cell. *L. monocytogenes* must then escape from a double membrane bound vacuole using LLO and PlcA and PlcB again. From here the cycle repeats. Figure taken from Pizarro-Cerda *et al.* (2012).
1.5. Entry of *L. monocytogenes* into the food chain

1.5.1. Soil

During the 1970’s it was suggested that soil was a natural environment for *L. monocytogenes* (Welshimer and Donker-Voet, 1971; Weis and Seeliger, 1975). However, more recent studies have suggested that soil contamination by the organism may come from other sources such as sewage, animal manure and decaying plant vegetation (Fenlon *et al.*, 1996). Many studies have investigated the survival of *L. monocytogenes* in soil and have observed that the foodborne pathogen can survive over a period of time, although, soil type, water content, pH, and temperature can all influence the rate of survival (Ivanek *et al.*, 2009; McLaughlin *et al.*, 2011). For example, Locatelli *et al.* (2013) found that survival of *L. monocytogenes* was higher in fine soil with high clay content, which they suggest has a higher number of pores for protection against predation by protists and also has a cation content that is more compatible with long term survival.

Microflora within the soil can highly affect the survival of *L. monocytogenes* (Fig. 1.3). Interactions between *L. monocytogenes* and different types of protozoa have previously been demonstrated (Ly and Muller, 1990; Zhou *et al.*, 2007; Pushkareva and Ermolaeva, 2010). Sterilisation of soil can lead to an increase in growth of *L. monocytogenes* suggesting that the microflora of the soil such as bacteriophage or protozoa have an effect on persistence of the bacterium, although this effect has not yet been fully explained. McLaughlin *et al.* (2011) confirmed that the microbiota of the soil plays an important role on survival. In their study, they partially reconstituted sterile soil with culturable aerobic components of the soil microbiota and observed that this led to a decrease in survival at later time points in the experiment. They discuss the possibility that this decrease may be due to competition by different microflora for nutrients within the soil. Other factors which may affect the survival of the organism in soil include chemical properties as well as geographical and meteorological influences (Ivanek *et al.*, 2009; Strawn *et al.*, 2013). For example, Weller *et al.* (2015b) examined temporal factors (irrigation and rainfall) leading to contamination of pre-harvest spinach. There was a greater chance of isolating *L. monocytogenes* after irrigation than rainfall and this chance was highest within 24 hours of the event (Weller *et al.*, 2015b). Other studies have confirmed similarly that irrigation is a risk factor for contamination of pre-harvest foods (Généreux *et al.*, 2015; Weller *et al.*, 2015a). This is often due to the contamination of the water source used for irrigation of the fields (Strawn *et al.*, 2013; Généreux *et al.*, 2015). Along with irrigation, the use of manure as a fertiliser can
increase isolation of *L. monocytogenes* from produce production sites (Watkins and Sleath, 1981; Fenlon *et al.*, 1996; Garrec *et al.*, 2003). This is not surprising as animals are known reservoirs of the bacterium (Fenlon *et al.*, 1996; Esteban *et al.*, 2009; Mohammed *et al.*, 2010).

Spatial factors such as proximity to urban areas, farms or water sources can lead to higher detection of *L. monocytogenes* (Sauders *et al.*, 2012; Strawn *et al.*, 2013; Weller *et al.*, 2015b). One study conducted in New York State found that incidence of *L. monocytogenes* was much higher in samples taken from farms compared to a natural environment (defined as an undeveloped area with minimal human presence) suggesting that the presence of humans and animals is highly associated with isolation of *L. monocytogenes* (Chapin *et al.*, 2014).

### 1.5.2. Seawater

Many studies have shown that water sources such as rivers, ponds and creeks can act as reservoirs for *L. monocytogenes* (Schaffter and Parriaux, 2002; Lyautey *et al.*, 2007; Linke *et al.*, 2014). However one environment which has been considered to a much lesser extent is seawater. As isolation of this foodborne pathogen has been associated with seafood (Colburn *et al.*, 1990; Johansson *et al.*, 1999; González *et al.*, 2013; Leong *et al.*, 2015), it may be a source of contamination worth considering. As with rivers, it is possible that effluent and land run off may increase levels of contamination by this microorganism in coastal waters (Watkins and Sleath, 1981; Fenlon *et al.*, 1996). Some studies have shown isolation of *L. monocytogenes* from marine environments (Colburn *et al.*, 1990; Motes, 1991; Rørvik *et al.*, 2000; Rodas-Suárez *et al.*, 2006). Motes, (1991) found that *Listeria* spp. including *L. monocytogenes* could be isolated from fresh seafood and their harvest waters, suggesting that *L. monocytogenes* can survive in seawater for a period of time. Colburn *et al.*, (1990) also isolated *L. monocytogenes* from samples taken from an estuary and a bay in California. However, whilst some studies don’t dispute that *L. monocytogenes* can be isolated from water; they do disregard it as an important source of contamination in fish farms (Jemmi and Keusch, 1994; Rørvik *et al.*, 2000). More recent studies have also shown the survival of the pathogen in seawater, although many of these report that the survival of *L. monocytogenes* is strain and temperature dependent with lower temperatures correlating with higher survival (Bremer *et al.*, 1998; Hsu *et al.*, 2005; Hansen *et al.*, 2006). However, besides temperature, other factors must also be considered for the survival of *L. monocytogenes* in seawater. Those factors include osmotic stress, predation by protozoa,
nutrient availability, and solar irradiation (Smith et al., 1994; Tedetti and Sempéré, 2006). Data shown in Section 5.2.6 suggests that of these solar irradiation might be the major factor influencing survival in seawater as *L. monocytogenes* can survive for several weeks in seawater when it is shielded from sunlight.

1.5.3. Food processing environments

Within food production facilities, it is known that *L. monocytogenes* can survive over long periods of time; however, the source of contamination is often unknown (Fig. 1.3). Persistence is often defined as a particular subtype re-isolated from the same environment over an extended period of time (Carpentier and Cerf, 2011; Ferreira et al., 2014; Larsen et al., 2014). It is often difficult to determine whether a particular strain is persisting within an environment such as a food processing environment or if the strain is being reintroduced into the facility at different times. It is also disputed if a genotype associated with persistence exists or whether *L. monocytogenes* can colonise specific favourable niches in a processing environment and therefore “persist” over a longer period of time (Carpentier and Cerf, 2011; Ferreira et al., 2014). Studies have compared phenotypic characteristics that cause strains to persist compared to non-persistent strains (Lundén et al., 2008; Ringus et al., 2012; Magalhães et al., 2016). One inherent limitation of this sampling process is that only a subset of the population is sampled and persistent clones may be missed on multiple sampling occasions. Therefore, categorising strains as non-persistent can be difficult as it may happen that a persistent strain was only isolated sporadically during a study (Ferreira et al., 2014; Larsen et al., 2014). Another challenge is that apparent persistence could be caused by the repeated introduction of the same strain to a food production facility, which could happen if contaminated personnel, equipment or product serve as a vector to continually introduce the same strain from some reservoir outside the plant.

Different studies have been conducted to investigate the main sources of contamination within food processing facilities (Johansson et al., 1999; Hansen et al., 2006; Leite et al., 2006; Ho et al., 2007; Chen et al., 2010a; Rivoal et al., 2010). Hansen et al., (2006) found that there was evidence of strains isolated from the outside environment also being identified within fish slaughterhouses. Other studies have shown that operators within a facility or different pieces of equipment may also be considered sources of contamination (Leite et al., 2006; Lomonaco et al., 2009; Chen et al., 2010a). Lomonaco et al., (2009) isolated *L. monocytogenes* from locker rooms, hallways and toilets in a gorgonzola producing facility
suggesting the possibility that personnel within the factory contributed to the problem of contamination. Chen et al., (2010b) found that water used to chill fish products along with a weighing table, were important sources of contamination within their facility.

It is still disputed as to whether seasonal variation has a contributing role in the isolation of L. monocytogenes from food processing environments. Many studies show no correlation between seasonal variation and occurrence of L. monocytogenes (Garrec et al., 2003; Ho et al., 2007; Esteban et al., 2009; Mohammed et al., 2010; Leong et al., 2014; Leong et al., 2017) but others have disputed these findings by showing a link between the two (Rivoal et al., 2010).

Figure 1.3. Factors influencing the survival and transmission of Listeria monocytogenes in the environment and food chain. The survival of L. monocytogenes in the soil is influenced by factors such as the composition of the soil and the competing microbiota present. Its presence in this environment is also influenced by weather events (sunshine and rainfall), irrigation from contaminated sources, as well as human and animal faecal contamination. Therefore, agricultural produce can be contaminated with this pathogen at the point of harvest. This can introduce the pathogen into the food processing environment or the produce can become contaminated there if adequate cleaning and decontamination practices are not in place. Ready-to-eat food produce that can support the growth of L. monocytogenes is a particular risk to the consumer, especially those that are immunocompromised (NicAogáin et al., 2016).
1.6.  **L. monocytogenes in seafood**

RTE seafood products are of particular concern regarding contamination by *L. monocytogenes*. According to a recent EU report, detection of *L. monocytogenes* occurs in 10.6% of RTE fish products in Europe (EFSA, 2015). This figure was significantly higher than for other food categories with 2.7% and 1% detection reported for RTE meat products and soft and semi-soft cheeses respectively (EFSA, 2015). Surveillance studies have produced varied rates of occurrence for this pathogen in seafood products (Johansson *et al.*, 1999; Lambertz *et al.*, 2012; González *et al.*, 2013; Leong *et al.*, 2014). González *et al.* (2013) showed that the prevalence of *L. monocytogenes* in smoked salmon in Spain was 4.8%, whilst Lambertz *et al.* (2012) showed that Swedish seafood had the highest rate of occurrence (12%) compared to other types of RTE foods including cheese (0.2%) and meat (1.2%) products. It is possible that the prevalence of *L. monocytogenes* in RTE seafood may be different depending on the type of seafood. González *et al.* (2013) sampled smoked salmon products and surimi (crab sticks and eel) and they reported that none of the 125 surimi samples tested were positive for *L. monocytogenes*. To determine if a product is a high risk to a consumer, challenge studies can be conducted to determine whether a food can support the growth of *L. monocytogenes* (Uyttendaele *et al.*, 2009; Rosshaug *et al.*, 2012; Leong *et al.*, 2014). These tests can help to determine if numbers of *L. monocytogenes* could exceed the limit of 100 CFU/g during the shelf life of the product. Other studies have investigated differences between the types of smoking process used. Products that are cold-smoked appear to have a higher rate of occurrence than hot-smoked products (Johansson *et al.*, 1999; Lambertz *et al.*, 2012). This may be because the cold-smoking process lacks a heat step or any other type of antimicrobial step to remove *L. monocytogenes* from the product increasing the risk to the consumer (Rotariu *et al.*, 2014).

1.7.  **Stresses encountered in food**

1.7.1.  **Osmotic shock**

As salt is widely used in the preservation of food, osmotic stress is an important stress that *L. monocytogenes* must overcome to survive within many foods. This foodborne pathogen can survive salt concentrations as high as 3 M NaCl (Cole *et al.*, 1990). It has been suggested that *L. monocytogenes* has a so-called primary and secondary response to osmotic shock. The primary response involves the influx of K⁺ and glutamate into the cell, whilst the
secondary response involves the uptake of small molecules known as compatible solutes (Kallipolitis and Ingmer, 2001; Brøndsted et al., 2003). These methods of combating osmotic shock play a role in helping the bacterium to restore turgor pressure, cell volume and also help to stabilise cell protein structure and function (O’Byrne and Fraser, 2000; Sleator et al., 2003).

_L. monocytogenes_ accumulates the compatible solutes, glycine betaine and carnitine, in hyperosmotic environments (Fraser et al., 2000; Sleator and Hill, 2001; Wood et al., 2001; Sleator et al., 2003). These solutes can often be found in different foods, with glycine betaine commonly found in foods of plant origin and carnitine from foods of animal origin (Sleator et al., 2003). The presence of these osmolytes in foods can help to enhance the growth of _L. monocytogenes_ in the presence of hyperosmotic conditions. Besides these main osmolytes, other compatible solutes including proline, proline betaine, acetylcarnitine, gamma-butyrobetaine and 3-dimethylsulfonylporpionate have also been found to help the growth of _L. monocytogenes_ in osmotic stress conditions (Bayles and Wilkinson, 2000). Uptake of compatible solutes occurs via three main transporters, Gbu, BetL and OpuC (Sleator et al., 1999; Fraser et al., 2000; Gerhardt et al., 2000; Angelidis et al., 2002; Angelidis and Smith, 2003b). BetL or Betaine Porter I is one of two systems involved in the transport of glycine betaine into the cell (Gerhardt et al., 1996; Sleator et al., 1999) and is dependent on the presence of Na⁺ (Gerhardt et al., 1996). Gbu, the second system involved in betaine uptake, is an ATP-dependent transporter that can be activated independently of Na⁺ in response to osmotic shock by excess sucrose or KCl (Ko and Smith, 1999; Gerhardt et al., 2000). Finally, OpuCA has been characterised as a carnitine transporter (Fraser et al., 2000; 2003). Deletion of genes encoding these transporters leads to an increase in generation time of the bacteria in the presence of hyperosmotic stress when incubated with glycine betaine and carnitine (Angelidis and Smith, 2003a). Interestingly, SigB promoter sites have been identified upstream of each of these genes and deletion of σ^B leads to reduced survival in response to high salt concentrations (Sleator et al., 1999; Fraser et al., 2003; Cetin et al., 2004). Further studies have shown that _opuC_ and _gbuA_ are under the control of σ^B, but despite the presence of the putative SigB dependent promoter site upstream of _betL_ this gene does not appear to be under SigB control. Utratna et al. (2011) showed that transcription of _opuC_ in response to osmotic shock occurred in a transient manner and the level of σ^B activity observed also appeared to be proportional to the level of osmotic stress encountered.

Along with overcoming osmotic upshock, some bacteria have mechanisms to deal with hypoosmotic conditions. Mechanosensitive channels can allow the controlled release of osmolytes and water from the cell to aid the survival of a rapid increase in turgor pressure.
that occurs during osmotic downshock (Wood et al., 2001). Not much information is known about the existence of these channels in L. monocytogenes but two genes, lmo1013 and lmo2064, have been identified as having homology to genes encoding mechanosensitive channels in E. coli and S. pneumoniae (Sleator et al., 2003). Rapid efflux of osmolytes, glycine betaine and carnitine, has also been observed in L. monocytogenes cultures exposed to hypoosmotic conditions, providing evidence for the presence of systems involved in downshock survival (Verheul et al., 1997).

L. monocytogenes also exhibits an adaptive response to NaCl known as osmoadaptation, where treatment of cells with a sub-lethal level of NaCl can offer increased survival following further exposure to lethal salt concentrations (Faleiro et al., 2003). A cross protection between osmotolerance and other stresses has also been confirmed. Schmid et al. (2009) found that csp genes are upregulated in the presence of either cold shock or osmotic shock. Deletion of some of these genes can lead to stunted growth when treated with low temperatures or high salt concentrations, leading this group to hypothesise that the use of the CSP proteins may help to offer cross protection between osmotolerance and cold shock or vice versa depending on the condition encountered first by the bacterium (Schmid et al., 2009).

1.7.2. Cold Shock

L. monocytogenes is capable of growth at temperatures as low as -0.4°C (Walker et al., 1990). Various studies have demonstrated growth of this foodborne pathogen in different foods at refrigeration temperatures. However, at these temperatures the doubling time of the bacterium can be up to 50 hours or more (Angelidis and Smith, 2003a). During an encounter with cold temperatures, bacterial membranes become more rigid, the rate of enzymatic reactions reduces and the level of uptake and transport of molecules is also decreased (Graumann and Maraheil, 1996). The bacterium must modulate its gene expression to mitigate the effect of these physical changes. Changes in expression usually occur for genes involved in cell membrane function, lipid, carbohydrate and amino acid synthesis, ribosomal structure and biogenesis and motility (Chan et al., 2007b; Cordero et al., 2016).

During exposure to cold temperature, one of the methods used by L. monocytogenes to combat cold shock is the accumulation of low molecular weight solutes such as glycine betaine and carnitine. High amounts of these solutes are found in various foods (Zeisel et al., 2003; Demarquoy et al., 2004), which may help to promote the survival and growth of
this pathogen in foods at refrigeration temperatures. The generation time of *L. monocytogenes* reduces by more than 20 hours at 4°C when cells are incubated in the presence of compatible solutes (Angelidis and Smith, 2003a). The BetL glycine betaine transporter (Section 1.7.1) does not seem to be involved in cryotolerance (Sleator *et al.*, 2003). Chan *et al.*, (2007b) identified increased expression of both Gbu and OpuC but not BetL in response to cold shock, while a metabolomics study also showed increased quantities of glycine betaine and carnitine present within *L. monocytogenes* when grown at 8°C compared to 37°C (Chan *et al.*, 2007b; Singh *et al.*, 2011). The increase in solute levels within the cell may help to decrease loss of intracellular water from the cell when temperatures drop.

A number of studies have investigated the role of σ^B^ in adaptation to cold stress, but the data show conflicting results. It seems likely that survival, during exposure to cold temperatures, is controlled in a manner that is at least partly σ^B^-dependent. For example, Chan *et al.*, (2007a) demonstrated that while some cold-induced genes were under σ^B^ control (*opuCA*) or were preceded by a σ^B^-dependent promoter site, they could be activated in a σ^B^-independent manner at 4°C, indicating that genes responding to cold shock may be partially under σ^B^ control. They also showed that a mutant lacking *sigB* did not have reduced growth at 4°C compared to the wild-type (Chan *et al.*, 2007a). Utratna *et al.*, (2014) showed σ^B^ does not play a large role in survival at low temperatures. They also showed that σ^B^ could be activated at 4°C in a manner that was independent of RsbV without levels of RsbW being affected (Utratna *et al.*, 2014). Other systems that have been suggested to play a role in adaptation to cold stress include the two component regulatory systems, YycGF and LisRK (Pöntinen *et al.*, 2015). Transcript levels of the *yycF* gene were shown to be increased at 4°C (Chan *et al.*, 2007b) and Pöntinen *et al.*, (2015) suggested that YycF was more involved in survival of initial cold stress than acclimation over time, whilst LisRK seems to be more involved in acclimation.

### 1.7.3. Low pH

*L. monocytogenes* can often encounter acidic conditions either in food matrices or in the gut of the host. These acidic conditions can arise from either weak organic acid such as lactate, benzoate, acetate or sorbate, or by strong acids like hydrochloric acid. Once *L. monocytogenes* enters the host following the ingestion of contaminated food, it encounters acidic conditions firstly within the stomach but also within the vacuole of the macrophage phagosome after intracellular uptake. The bacterium possesses a variety of different
mechanisms including the adaptive acid tolerance response (ATR), the Glutamate Decarboxylase (GAD) system and the Arginine Deaminase (ADI) system, to help it overcome these acidic environments (Davis et al., 1996; Cotter et al., 2001a; Ryan et al., 2009).

1.7.3.1. ATR

Davis et al. (1996) first confirmed the presence of the Adaptive Acid Tolerance Response in L. monocytogenes. This study showed that when exponential cells were pre-exposed to a sub-lethal pH (pH 5.0) for one hour prior to exposure to a lethal pH (pH 3.0), cells exhibited a much higher survival rate compared to unexposed cells (Davis et al., 1996). The ATR results from pre-exposure of cells to a sub-lethal pH, typically between pH 5-6, before exposure to more lethal acids (Davis et al., 1996; Skandamis et al., 2012). Some studies have shown how this response can help L. monocytogenes survival on low pH foods (Gahan et al., 1996; Skandamis et al., 2012), whilst other studies also showed that this protective effect could be extended to other stresses such as heat and osmotic shock (O’Driscoll et al., 1996; Skandamis et al., 2009). Ferreira et al. (2003) have investigated whether the general stress response has a role in the ATR. They suggest that whilst an isogenic ΔsigB mutant survives less than the parental strain after being pre-exposed to sub-lethal pH, survival increases after pre-exposure suggesting that there are other σB-independent mechanisms working on survival against acid (Ferreira et al., 2003).

1.7.3.2. ADI System

The ADI system is involved in enhanced survival at low pH in a variety of Gram positive microorganisms including L. monocytogenes (Cunin et al., 1986). The system works by converting molecules of arginine into ornithine using three enzymes encoded for by the arcABC operon. A membrane antiporter ArcD transports a molecule of arginine into the cell which is then converted to ornithine, CO₂, ammonia and ATP. Ornithine is then transported back out of the cell in exchange for another molecule of arginine. During this process, the by-product ammonia can associate with intracellular protons to form NH₄⁺ and this leads to an increase of the cytoplasmic pH (Cunin et al., 1986). Many studies have investigated the role of the ADI system in acid survival in L. monocytogenes (Ryan et al., 2009; Chen et al., 2011a; Cheng et al., 2013). Ryan et al. (2009) first showed the presence of a functional ADI system within L. monocytogenes and demonstrated that it is implicated in survival at low pH
and virulence in vivo. They also identified ArgR as a regulator of the ADI system. Another study showing the role of Lmo0036 (ArcB) in acid tolerance also confirms the role of the ADI system (Chen et al., 2011a). Interestingly, the transcription of arcA and argR, have been shown to be SigB and PrfA-dependent (Ryan et al., 2009). Hain et al. (2008) and Bowman et al. (2010) also identified arcA as being under SigB control, whilst Miloanic et al. (2003) identified it as being controlled by PrfA suggesting a role for the ADI system in both stress response and virulence.

1.7.3.3. GAD System

Another system identified in L. monocytogenes to help to maintain pH homeostasis within the cell, is the GAD system, known to be important for survival within synthetic gastric fluid, infection in mouse models and also in acidic foods (Cotter et al., 2001a; Cotter et al., 2001b; Feehily et al., 2014). It works to increase the internal pH of the organism in the presence of extracellular acidic conditions. However, it has been suggested that the GAD system is only responsible for survival of strong acidic conditions (below pH 4.5) and does not have a role to play in tolerance to weak acids (Heavin et al., 2009; Karatzas et al., 2010). The process works by the utilisation of glutamate, which is present in all foods and living organisms. Under acidic conditions an extracellular molecule of glutamate is taken up by an antiporter (GadT) and then converted into γ-aminobutyric acid (GABA) by a decarboxylase enzyme, GadD. GABA is then exported back out of the cell in exchange for another molecule of glutamate. The decarboxylation process consumes one proton, thereby leading to an increase in intracellular pH (Lund et al., 2014). In L. monocytogenes, there are five genes involved in the Gad system, three genes encoding glutamate decarboxylases (GadD1, GadD2, GadD3) and two encoding antiporters (GadT1 and GadT2). These genes are arranged into three operons, gadD1T1, gadT2D2 and gadD3 (Cotter et al., 2005). Whilst gadT2D2 and gadD3 are possessed by all strains, serotype 4 strains lack gadD1T1. This trait is not limited to this serotype, as the presence of these genes vary within strains of other serotypes too (Cotter et al., 2005). GadD1T1 seems to be required for growth at mild pH conditions while GadD2T2 is important in more severe acidic conditions. GadD2T2 and GadD3 have been shown to be at least partially under σB regulation (Kazmierczak et al., 2003; Wemekamp-Kamphuis et al., 2004), but little more is known about the regulation of the GAD system in L. monocytogenes.
1.7.4. Nisin

Different antimicrobial compounds including bacteriocins have been studied extensively over the past decades as a method of controlling bacterial contamination within food products. Some examples of antimicrobials which have been proven to be active against \textit{L. monocytogenes} include lauric arginate, chitosan, pediocin and nisin (Kaur \textit{et al.}, 2013; Kang \textit{et al.}, 2015). Nisin is one of the most common antimicrobials used in the food industry especially within dairy products and acidic foods (Delves-Broughton \textit{et al.}, 1996). It is a bacteriocin that is produced by the lactic acid bacterium, \textit{Lactococcus lactis}. Compared to other bacteriocins, nisin has been shown to be most effective at reducing numbers of \textit{L. monocytogenes} (Kaur \textit{et al.}, 2013). However, when used in combination with other antimicrobials, levels of inhibition increase further (Tokarskyy and Marshall, 2008; Kaur \textit{et al.}, 2013). It has also been observed that \textit{L. monocytogenes} isolates can develop resistance to nisin, which is potentially a worrying prospect for the food industry (Gravesen \textit{et al.}, 2002). Cross resistance can also develop between bacteriocins meaning that combinations of different bacteriocins may not always be feasible (Kaur \textit{et al.}, 2013).

The antimicrobial effect of nisin involves interference with cell wall biosynthesis, disruption of the cell membrane by the formation of pores and consequent disruption of cell membrane associated processes (Bruno \textit{et al.}, 1992; Abee \textit{et al.}, 1994). It has been suggested that resistance of \textit{L. monocytogenes} to nisin may arise due to changes within the cell wall composition which stops the bacteriocin from gaining access to the cell and therefore increasing survival (Kaur \textit{et al.}, 2012). Different systems including two component regulatory systems and the general stress response have been implicated in \textit{L. monocytogenes} resistance to nisin. Kang \textit{et al.} (2015) showed that a mutant deficient in the response regulator, VirR, had a greater loss of membrane integrity compared to the wild-type strain, while Begley \textit{et al.} (2006) found that a sigB mutant had decreased growth and survival in response to nisin. However, Palmer \textit{et al.} (2009) reported data that conflicted these results. They suggested that σB contributes to nisin resistance in \textit{L. monocytogenes} but only when it is deleted in a background lacking another alternative sigma factor, SigL (σL). When ΔsigB is solely deleted, growth and survival actually increases in response to nisin. These data suggest that both σB and σL have a role to play in nisin resistance in \textit{L. monocytogenes} (Palmer \textit{et al.}, 2009). Thus the actual role of σB in the response of \textit{L. monocytogenes} to nisin has yet to be determined.
1.7.5. Light

Light has recently been described as being a possible method of combating bacterial contamination with both visible light and UV light being used in different studies (Ozer and Demirci, 2006; Maclean et al., 2010; Hosein et al., 2016; Xu and Wu, 2016; Vollmerhausen et al., 2017). The UV spectrum includes wavelengths between 100 – 400 nm and is split into three different categories, UV-A, UV-B and UV-C. UV-A, which ranges from 320 – 400 nm, has mainly been used in studies to combat bacterial contamination (Ozer and Demirci, 2006; Vollmerhausen et al., 2017). More recently, visible light (400 -700 nm) has been investigated as an alternative to UV light with many studies using blue violet light in the range of 405 nm (Ozer and Demirci, 2006; Maclean et al., 2010; Maclean et al., 2014; O’Donoghue et al., 2016; Vollmerhausen et al., 2017).

Within environments where L. monocytogenes can persist, the bacterium can encounter varying amounts of light. Light has previously been used as a method of bacterial decontamination both within clinical environments and on food products and therefore, may be useful as a means of controlling L. monocytogenes contamination within the food industry. Recently, it has been shown that blue light triggers the activation of the general stress response within L. monocytogenes and therefore should be considered as a stress for the bacterium (Ondrusch and Kreft, 2011; Tiensuu et al., 2013). It is known that many bacteria have light sensing mechanisms which help them to overcome this stress. In Bacillus subtilis, a light sensing protein, YtvA, has been discovered (Losi et al., 2002; Ávila-Pérez et al., 2006; Ávila-Pérez et al., 2009). This protein is present in a stress sensing complex known as the stressosome which is composed of the proteins RsbR and its paralogues as well as RsbS and RsbT (Gaidenko et al., 1999; Kim et al., 2004b; Hecker et al., 2007; Marles-Wright et al., 2008; Jurk et al., 2013). The stress signals are thought to be sensed by the protruding N-termini of these sensory proteins and are transduced into the core of the stressosome (Marles-Wright et al., 2008). This leads to a signalling cascade downstream of the stressosome which ultimately leads to the activation of σB in response to the stress (Ávila-Pérez et al., 2009). This complex has recently been confirmed in L. monocytogenes (Impens et al., 2017). In L. monocytogenes, the paralogues of RsbR include, Lmo0799, Lmo0161, Lmo1642 and Lmo1842 (Ondrusch and Kreft, 2011; Heavin and O'Byrne, 2012). Although it is not clear what stress signals most of these proteins sense, Lmo0799, a homologue of YtvA in B. subtilis, has been confirmed as a blue light photoreceptor (Ondrusch and Kreft, 2011). Mutants lacking Lmo0799 have been shown to have similar phenotypes to a ΔsigB mutant with higher levels of motility in the presence of blue light and have lost the ability to form rings in response to cycles of light and dark (Ondrusch and
The protein consists of an LOV (Light, Oxygen and Voltage) domain at its N-terminus and a STAS domain at its C-terminal region. LOV domain proteins belong to the Per-Arnt-Sim (PAS) superfamily and can bind a flavin cofactor such as Flavin mononucleotide (FMN), to facilitate light sensing (Christie et al., 1999; Herrou and Crosson, 2011). During light exposure it is thought that a covalent bond forms between a thiol residue of a conserved cysteine residue at position 56 of the Lmo0799 protein and the FMN molecule located in the binding pocket of the protein (Chan et al., 2013). Recently, O’Donoghue et al. (2016) constructed a mutant with a missense mutation, changing Cys56 to an alanine. When tested in response to light this mutant showed similar phenotypes to both ∆sigB and ∆lmo0799 suggesting that this residue is indeed required for light sensing by this protein (O’Donoghue et al., 2016). Interestingly, while it has been shown that σB is activated in response to light, it has also been demonstrated that virulence genes have also been activated. Ondrusch and Kreft, (2011) investigated the transcription levels of the internalin genes (inlA and inlB) that are involved in invasion of L. monocytogenes into epithelial cells (Section 1.4). Transcription of both inlA and inlB was increased in response to blue light in combination with 0.3M NaCl and invasion into Caco-2 enterocyte-like human cells was also increased under these conditions. These data suggest that along with activating the stress response, blue light may also play a role in activation of virulence genes within L. monocytogenes.
Figure 1.4. Sensing and consequences of blue light in the cell. *L. monocytogenes* senses blue light via the photoreceptor Lmo0799, which is thought to be part of the stressosome complex. Other stress signals can be “sensed” by RsbR and its paralogues in the stressosome. The light signal causes a conformation change in the stressosome complex that triggers the release of RsbT, which in turn activates a phosphatase called RsbU. RsbU acts on to dephosphorylate RsbV, an anti-anti sigma factor that antagonises the anti-sigma factor RsbW. The interaction between RsbV and RsbW liberates SigB to interact with RNA polymerase and consequently leads to the transcription of the general stress response regulon. Blue light can also interact with photosensitisers in the cell which, in the presence of oxygen, can lead to the production of Reactive Oxygen Species (ROS). The ROS produced can cause damage of cellular macromolecules, which in extreme cases results in cell death (NicAogáin et al., 2016).

### 1.8. Implications for food safety

As RTE foods are of major concern for contamination by *L. monocytogenes*, it is beneficial to investigate how the bacterium behaves in such foods. This section looks at the expression of stress-related and virulence genes within different food matrices and how these could prime the bacterium for survival within the host. We also discuss the relationship between σ^B^ and PrfA within the host and how this may aid survival and pathogenesis.

#### 1.8.1. Behaviour of *L. monocytogenes* within food matrices
Studies have been conducted to investigate whether the transcriptional response of stress related and virulence genes of *L. monocytogenes* differ within various food matrices (Olesen *et al.*, 2010; Bae *et al.*, 2011; Alessandria *et al.*, 2013). Within RTE foods, the bacterium encounters many of the stresses discussed in this thesis. Therefore, transcriptional studies can provide information on genes involved in allowing the survival of *L. monocytogenes* in the presence of these stresses *in situ*. Importantly, virulence of *L. monocytogenes* has been shown to be heterogeneous between strains and between food matrices (Duodu *et al.*, 2010; Olesen *et al.*, 2010; Rantsiou *et al.*, 2012a; Rantsiou *et al.*, 2012b; Hadjilouka *et al.*, 2016). Virulence genes have been reported to be more highly induced under laboratory conditions than in food matrices, (Olesen *et al.*, 2010; Rieu *et al.*, 2010) and this was confirmed by a study which tested the effects on mice fed with broth cultures compared to contaminated food (Mahoney and Henriksson, 2003). The mice that were fed with fermented salami batter spiked with *L. monocytogenes* had a lower rate of infection than mice intragastrically challenged with a broth culture (Mahoney and Henriksson, 2003). However, mixed results have been found for the levels of *sigB* transcription when comparing broth cultures to food matrices. When grown on RTE deli turkey meat, transcriptional levels of *sigB* and related genes remained unchanged when compared to cultures grown in BHI broth (Bae *et al.*, 2011). Rantsiou *et al.* (2012b) observed that *sigB* transcript levels are generally upregulated in food matrices incubated at low temperature compared to BHI broth at 37°C, whilst in contrast Olesen *et al.* (2010) showed that the level of *sigB* transcription is increased in BHI broth compared to standard liver pâté. Somewhat surprisingly when NaCl concentration was reduced in the pâté compared to the standard pâté, which contained 3.66% (w/v) NaCl in the water phase, *sigB* transcript levels were significantly increased in some strains (Olesen *et al.*, 2010). Rantsiou *et al.* (2012b), suggest that temperature is the main variable contributing to the differences in *sigB* transcription that they observe. Differences in upregulation of expression of stress related genes and virulence genes have also been observed in other studies but often no particular pattern can be established (Duodu *et al.*, 2010; Hadjilouka *et al.*, 2016). Overall these studies show that different stresses encountered within foods can influence the induction of stress related genes and therefore have the potential to influence the gastrointestinal stages of a food-borne infection by *L. monocytogenes*.

### 1.8.2. Overlap between Stress response and virulence

Tolerance to environmental stress and virulence can be considered to be overlapping facets of the biology of *L. monocytogenes* (O'Byrne and Karatzas, 2008). Firstly, without a robust
stress response this pathogen would not be able to survive and persist in the food chain sufficiently well to allow it to gain access to a mammalian host. Secondly, the stresses encountered within the host, especially the in the upper gastrointestinal tract, represent a significant barrier that must be overcome in order for *L. monocytogenes* to establish an infection. Particular challenges are presented by the acidic pH of the stomach, the osmolality and presence of bile in the ileum. As discussed earlier, (See Sections 1.7.1 and 1.7.3) *L. monocytogenes* has specific mechanisms for coping to acid and osmotic stress, some of which are under the control of σ^B_. This pathogen is also remarkably tolerant to bile. It can colonise the murine gall bladder (Hardy *et al*., 2004), aided by its bile salt hydrolase (BSH; Begley *et al*., 2005; Sue *et al*., 2004), a bile exclusion system called BilE (Sleator *et al*., 2005) and two efflux pumps (MdrM and MdrT; Quillin *et al*., 2011). The *bsh* gene and the *bilE* operon are both under σ^B_ control (Fraser *et al*., 2003; Sue *et al*., 2004; Begley *et al*., 2005), while the efflux pumps are under the control of BrtA, a TetR-type transcriptional regulator (Quillin *et al*., 2011).

Having survived the stresses imposed by the GI tract the next step in establishing an infection is the invasion of epithelial cells in the intestinal villi (Cossart and Toledo-Arana, 2008). Invasion of epithelial cells is dependent on a surface protein called internalin (encoded by the *inlA* gene) whose expression is dependent on σ^B_ (Kim *et al*., 2004a; 2005). It is interesting that the regulator of the general stress response has been co-opted to participate in regulating the expression of a dedicated virulence gene and perhaps suggests that escape from the harsh conditions in the lumen of the gastrointestinal tract can be partly viewed as a response to stress (O’Byrne and Karatzas, 2008). The transcriptional regulator PrfA, a member of the Crp/Fnr family of regulators, is the master regulator controlling expression of virulence genes required for the intracellular stages of the infection caused by *L. monocytogenes* (reviewed in Scortti *et al*., 2007). PrfA expression is activated at 37°C by a thermal sensing switch in the 5’UTR region of the *prfA* transcript (Johansson *et al*., 2002) and is also influenced by the CodY transcriptional regulator under conditions where branched chain amino acid levels are low (Lobel *et al*., 2015). The activity of PrfA is also modulated post-translationally by an association with a ligand whose identity has been elusive for many years. Recently however, glutathione was identified as an allosteric modulator of PrfA activity (Reniere *et al*., 2015).

A number of lines of evidence indicate that there is regulatory cross talk between PrfA and SigB but the precise nature of this link has been difficult to define (O’Byrne and Karatzas, 2008). A number of transcriptomic studies have identified sets of genes whose regulation is influenced both by PrfA and by σ^B_ (Kazmierczak *et al*., 2003; Milohanic *et al*., 2003;
Ollinger et al., 2009; Toledo-Arana et al., 2009; Chaturongakul et al., 2011). $\sigma^B$ contributes directly to the regulation of a number of virulence genes including the inlAB operon (Kim et al., 2004a; 2005) and prfA itself (Nadon et al., 2002; Rauch et al., 2005; Schwab et al., 2005). Although prfA is preceded by a $\sigma^B$-dependent promoter (designated prfAP2) the significance of this promoter in vivo remains unclear since it overlaps with a $\sigma^A$ promoter and it can be deleted without an obvious effect on haemolysis (Nadon et al., 2002). Overall it appears that the dominant role for $\sigma^B$ is during the gastrointestinal stage of the infection (Garner et al., 2006) whereas PrfA dominates after the intestinal barrier has been breached (Toledo-Arana et al., 2009). But the multiple and complex regulatory inputs that exist to control PrfA expression and activity probably allow $\sigma^B$-mediated fine tuning of the PrfA regulon under certain conditions.

1.9. Methods of controlling L. monocytogenes

Measures to control L. monocytogenes in the food chain mainly focus on the food processing environment, including personnel and the formulation and processing of the product itself. Here we review some of the sanitizers that are in common use to control L. monocytogenes in food processing environments and consider some novel control strategies that are beginning to show promise and that might find application at different points in the food chain in the future.

1.9.1. Sanitizers

Different sanitizers such as quaternary ammonium compounds (QACs), hydrogen peroxide, peracetic acid and sodium hypochlorite are often used to disinfect areas within food processing environments. It is known that these sanitizers are effective at killing planktonic L. monocytogenes cells (Kastbjerg and Gram, 2012; Rückerl et al., 2014) and their effectiveness does not seem to differ between persistent and non-persistent strains of L. monocytogenes isolated from food environments (Magalhães et al., 2016). Development of resistance against different sanitizers has also been investigated but the overall conclusion is that resistance does not seem to occur (Kastbjerg and Gram, 2012). Therefore no correlation between persistence and resistance to sanitizers has been discovered (Rückerl et al., 2014). Different sanitizers have different mechanisms of inhibition. For example, QACs such as benzalkonium chloride attack the cell membrane of cells leading to cell leakage, whilst
peracetic acid and sodium hypochlorite tend to act as oxidising agents, creating ROS which lead to damage of cellular components (McDonnell and Russell, 1999).

To date, very little is known about the role of $\sigma^B$ in the mechanism of resistance of L. monocytogenes to sanitizers. However, it has been observed that $\sigma^B$ does play a role in the resistance of both planktonic and biofilm cells to benzalkonium chloride and peracetic acid over short periods of time (Ryan et al., 2009; van der Veen and Abee, 2010). Deletion of sigB reduces the levels of resistance against these sanitizers but does not affect growth in sub-lethal concentrations, whilst complementation of the mutation restores or even increases the resistance compared to the wild-type (van der Veen and Abee, 2010). Whilst no studies have shown a correlation between $\sigma^B$ and resistance to sodium hypochlorite, it is considered that it may have a role to play as genes involved in oxidative stress are under $\sigma^B$ control (Ferreira et al., 2001; Boura et al., 2016). It is important to note that other systems controlled independently of $\sigma^B$ (e.g. the efflux pumps QacH, MdrL and Lde) have also been observed to impact survival in the presence of sanitizers such as benzalkonium chloride (Romanova et al., 2006; Muller et al., 2013; Muller et al., 2014).

### 1.9.2. Photodynamic inactivation

Alongside the discovery that several bacterial strains respond to light as a stress agent, interest has developed in the possible use of light as a bacterial containment method. Specifically, photodynamic inactivation (PDI) has been shown to be effective in the treatment of different bacteria, including antimicrobial resistant strains of bacteria (Maclean et al., 2010; Luksiene and Paskeviciute, 2011; Endarko et al., 2012; Murdoch et al., 2012; Bumah et al., 2015; Hosein et al., 2016). In the case of L. monocytogenes, light can decrease cell numbers in liquid culture, on surfaces and decrease its biofilm production meaning that PDI could be a very useful way of treating Listeria monocytogenes contamination in the food production environment (Murdoch et al., 2012; McKenzie et al., 2013; O'Donoghue et al., 2016). This treatment involves the use of a photosensitizer in combination with light and oxygen. The photosensitizer can be added to the medium or can be found naturally within cells in the form of endogenous molecules such as porphyrins (Hamblin et al., 2005; Buchovec et al., 2010; Luksiene et al., 2010). The mechanism of PDI involves a photosensitizer becoming activated by the absorption of photons and this leads to the creation of a singlet state of the photosensitizer which can decay and omit fluorescence as it
returns to the ground state, or it can form an excited triplet state. From this triplet state, photooxidation can occur via two different pathways leading to the formation of reactive oxygen species (ROS) or singlet oxygen (Sibata et al., 2001; Luksiene, 2003; Luksiene and Zukauskas, 2009; Robertson et al., 2009). The generation of ROS in response to light can lead to interactions with lipids and proteins within the cell membrane and also lead to DNA damage, which can result in cell death (Fig. 1.4). Addition of reactive oxygen scavengers to quench the effects of ROS has been shown to increase growth and survival of L. monocytogenes in the presence of blue light suggesting that ROS contribute to inhibition by visible light (Endarko et al., 2012; O’Donoghue et al., 2016). Interestingly, Tiensuu et al., (2013) found that many genes activated by Lmo0799 and σB in response to blue light, are involved in combatting oxidative stress.

Gram positive bacteria have been shown to be more susceptible to PDI than Gram negative possibly due to differences in cell wall composition or due to different amounts of endogenous porphyrins being produced within the cell (Nitzan et al., 2004; Maclean et al., 2009). Many different bacteria including foodborne pathogens such as L. monocytogenes, Bacillus cereus and Salmonella enterica have been inhibited in various studies using visible light (Luksiene and Paskeviciute, 2011; Endarko et al., 2012; Murdoch et al., 2012; O’Donoghue et al., 2016). Endogenous porphyrins are produced through the heme biosynthetic pathway in bacteria and act as natural photosensitzers within the cell. Some studies have proposed boosting porphyrin production within cells by adding increased amounts of 5-aminolevulinic acid (5-ALA), a precursor of the heme biosynthetic pathway (Nitzan et al., 2004; Buchovec et al., 2010). Other studies have sought to test whether the addition of exogenous photosensitzers to the medium could increase the sensitivity of Listeria to PDI (Romanova et al., 2003; Luksiene et al., 2010; Lin et al., 2012). Luksiene and Paskevicituc (2011) successfully used light in combination with Na-chlorophyllin to reduce levels of contamination by L. monocytogenes on strawberries, proving that PDI could also be used in combination with approved food additives to control the growth and survival of L. monocytogenes on food products.

1.9.3. **Innovative strategies for reducing the risk of L. monocytogenes**

Strategies aimed at reducing the risk of L. monocytogenes contamination in foods, usually focus on the elimination of the organism at the stage of food processing as well as designing food preservation regimes that don’t support the growth of L. monocytogenes. Food preservation systems generally employ generic stress “hurdles” that act synergistically to
inhibit microbial growth (Leistner, 2000), (e.g., reduced water activity combined with acidic pH), but the next generation of food preservatives might usefully target specific protective mechanisms and thereby prevent food pathogens from protecting themselves. As discussed in section 1.7.4, nisin is increasingly being used to prevent the growth of *L. monocytogenes* in food (reviewed in Cleveland *et al*., 2001). Its inhibitory mode of action is twofold; it interferes with cell wall biosynthesis and also disrupts the cytoplasmic membrane (McAuliffe *et al*., 2001). This dual action renders the cell vulnerable particularly when additional preservation-related stresses are also present in the food matrix. Lytic bacteriophages that target *L. monocytogenes* have also been considered for biocontrol of this pathogen. For example, broad host-range phages such as A511 and P100 have been shown to be effective at reducing viable *L. monocytogenes* cells to undetectable levels in some RTE foods (Carlton *et al*., 2005; Guenther *et al*., 2009; Bigot *et al*., 2011). In the future it might also be possible to target the regulators that control stress tolerance (σ^B^) and virulence (PrfA). A small molecule that blocks σ^B^ activity and reduces host cell invasion has recently been described (Palmer *et al*., 2011). The compound, 2 fluoro-phenyl-styrene-sulfonamide (FPSS), apparently blocks the release of σ^B^ from its anti-sigma factor RsbW, thereby preventing it from participating in transcription (Ringus *et al*., 2013), but the precise mode of action has not yet been established. Blocking the expression of virulence functions might also be a viable means of reducing the risk to food consumers. Recently a class of ring-fused 2-pyridone molecules have been identified that bind to PrfA and decrease its affinity for its consensus binding site on DNA (Good *et al*., 2016). A structural analysis of the interaction of PrfA with one of these molecules revealed that it interacts at two different sites on the protein that could prevent both allostERIC activation of PrfA and also the correct alignment of the DNA binding helix-turn-helix domain, thereby interfering with its ability to stimulate virulence gene expression. Additional work will be needed to develop these molecules further as potential therapeutic agents or even as designer food-preservatives.

### 1.10. Identification of novel genes that potentially contribute to σ^B^ activation

Recently studies have been conducted to investigate the role of genes involved in the activation of σ^B^ (Ondrusch and Kreft, 2011; Tiensuu *et al*., 2013). It has been shown that σ^B^ can be activated by the blue light sensor Lmo0799 (Section 1.7.5; Ondrusch and Kreft,
2011). Tiensuu et al. (2013) recently found that *L. monocytogenes* forms a ring phenotype on low agar plates in response to oscillating cycles of light and dark, in an Lmo0799/ σB dependent manner. To try to determine the mechanism involved in the formation of this phenotype, they constructed a mariner transposon mutagenesis library. They found 48 of these mutants did not form rings in response to cycles of light and dark. Genes affected by the insertion of the transposon were characterised into three groups, genes encoding proteins involved in (i) σB activation or repression, (ii) transport or cell wall maintenance proteins and (iii) regulatory proteins. Following on from this, they wanted to investigate the level of σB activation in the transposon mutants from categories (ii) or (iii) as category (i) was assumed to affect the function of σB. For the majority of the transposon mutants tested, they found that H2O2 – induced σB activation was affected. This suggested that the proteins encoded for by the transposon mutants act on the level of σB activity. Further work to characterise the function of some of these proteins including Lmo0818 (a possible P-type ATPase) and their involvement in the activation of σB is being conducted.

1.10.1. P-type ATPases

P-type ATPases are membrane spanning proteins that are critical to the survival of all living organisms. They provide a homeostatic mechanism to maintain the internal environment of a cell by transporting ions across cellular membranes (Kühlbrandt, 2004; Bublitz et al., 2011). Transportation of ions by P-type ATPases is dependent on ATP hydrolysis (Kühlbrandt, 2004; Bublitz et al., 2010). The structure of a typical P-type ATPase consists of a transmembrane domain and a cytoplasmic domain. The transmembrane domain consists normally of 10 α-helices but it is possible to have more or less helices in some P-type ATPases (Kühlbrandt, 2004; Bublitz et al., 2011). Three domains are associated with the cytoplasmic domain; the nucleotide binding domain (N), the actuator domain (A) and the phosphorylation domain (P) (Kühlbrandt, 2004; Bublitz et al., 2010; Bublitz et al., 2011). The sequences of each of these domains are highly conserved between different types of P-type ATPases. Five different groups of these membrane proteins have been characterised based on their substrate specificity. Type I P-type ATPases pump heavy metals, Type II are calcium, sodium and potassium pumps, Type III are Magnesium pumps, Type IV transport phospholipids and no specific substrate has been determined for Type V as of yet (Axelsen and Palmgren, 1998; Palmgren and Axelsen, 1998). To transport ions, P-type ATPases alternate between two different states, E1 and E2. The state E1, involves the transfer of a phosphate from ATP to the P domain in the cytoplasm to form a high energy phosphate
intermediate. In this state, the ATPase has a high affinity for the cation substrate. E2 is the dephosphorylation of the P domain returning it to the ground state and reducing the affinity for the ion. This return to a low energy state is thought to lead to the opening of the channel and therefore expel the ion across the membrane (Kühlbrandt, 2004; Bublitz et al., 2010; Bublitz et al., 2011).

Ca\(^{2+}\)-dependent ATPases will be the focus of Chapter 6 in this study, as the function of the studied protein Lmo0818 has been suggested previously to be this type of P-type ATPase (Hein et al., 2012). Within bacteria, it has been shown that calcium is required for multiple processes including spore formation, chemotaxis, motility, virulence and can also influence gene regulation (Raeymaekers et al., 2002; Dominguez, 2004; Sarkisova et al., 2005; Patrauchan et al., 2007; Guragain et al., 2013; Dominguez et al., 2015). Calcium concentrations can be present in the millimolar range within a host’s blood and mucosa, which suggests that pathogens must employ systems to reduce intracellular Ca\(^{2+}\) levels (Goldstein, 1990; Vanthanouvong and Roomans, 2004). As suggested by the name, Ca\(^{2+}\) ATPases either transfer calcium ions across the cell membrane, or require calcium for Ca\(^{2+}\)-dependent phosphorylation. There have been few Ca\(^{2+}\) ATPases characterised in prokaryotes and their function is not well understood. PA2435 and PA3920 were characterised in Pseudomonas aeruginosa and when mutated led to increased levels of intracellular calcium and also affected the swarming motility of the strain (Guragain et al., 2013). CaxP was shown to be involved in survival of Streptococcus pneumoniae in the host environment with a caxP mutant demonstrating attenuated virulence in a mouse model (Rosch et al., 2008). The spore producing B. subtilis requires a Ca\(^{2+}\) ATPase, YloB, during spore formation but it is not required for growth of vegetative cells (Raeymaekers et al., 2002). Other P-type ATPases have also been characterised such as Pma1 from Synechocystis sp. and PacL from Synechococcus sp. (Geisler et al., 1993; Berkelman et al., 1994; Geisler et al., 1998).

Recent studies have identified a Ca\(^{2+}\) ATPase in L. monocytogenes, LMCA1. Faxén et al. (2011) cloned and over expressed the putative P-type ATPase in E. coli and identified that the formation of the phosphorylation intermediate was dependent on the presence of calcium. It was observed that this protein unlike some of the others described above had a lower affinity for calcium and ATP hydrolysis could also occur in the presence of Sr\(^{2+}\). This transporter is active between pH 6.5 -10 but has highest affinity for calcium at pH 9. Therefore at this pH, ATP hydrolysis was increased leading to an increase in transport activity. This ATPase was shown to exchange 1 Ca\(^{2+}\) (out) for 1 H\(^{+}\) (in) per molecule of ATP (Faxén et al., 2011). Another putative Ca\(^{2+}\) ATPase has been identified in L. monocytogenes and the structure of this protein has been crystallised. The crystallised structure of Lmo0818
suggests similarities to LMCA1 however, the exact function of this protein has not been elucidated (Hein et al., 2012). It is possible that if this bacterium has multiple Ca\textsuperscript{2+} ATPases, there may be redundancy associated when attempting mutational studies therefore making it hard to elucidate the function or mechanism of these proteins. Interestingly, Lmo0818 was disrupted in one of the transposon mutants constructed by Tiensuu et al. (2013). This transposon mutant also displayed other σ\textsuperscript{B} related phenotypes such as acid sensitivity suggesting its role in the regulation of σ\textsuperscript{B} (Dr C. O’Byrne, Personal communication).

1.11. Project Aims

As discussed above, L. monocytogenes is a real risk factor in the food processing industry. Gaining further insights into mechanisms underpinning its survival of harsh conditions presented in the food processing environment can provide information needed to prevent contamination by this pathogen. There are still gaps present in the literature accounting for how this bacterium can overcome different stresses encountered and whether persistent strains of the pathogen have specific traits enabling them to withstand and survive stresses encountered in the food processing industry. Novel mechanisms for controlling bacterial contamination in these environments could help to reduce the risk of this pathogen to consumers of at risk RTE products.

This project aimed to conduct a surveillance programme to monitor the occurrence rate of L. monocytogenes in Irish food processing facilities, specifically seafood facilities, over a 3 year period. The occurrence of the bacterium was established in both environmental and food samples from Irish seafood companies over 3 years and the rate of persistence was determined by molecular characterisation of strains isolated during the course of the project (Chapter 3).

Interesting isolates identified through the surveillance programme were characterised further using phenotypic assays to try pinpoint mechanisms that contribute to the survival and in some cases persistence of the isolates in the food processing industry. Amino acid sequences of proteins associated with stress response were also examined to explore the possibility that differences in these sequences may contribute to the survival of these strains. σ\textsuperscript{B} activity was also compared between the isolates to investigate if this could possibly impact survival (Chapter 4).
Visible light has been suggested as a possible control mechanism for bacterial contamination in food processing environments and hospital settings (Section 1.9.2). Here the effect of blue light, in the range of 460-470 nm, was investigated on the growth and survival of *L. monocytogenes*. The mechanism of inactivation was examined through the use of reactive oxygen scavengers. As it has recently been demonstrated that *L. monocytogenes* can sense blue light through the blue light sensor Lmo0799 (Ondrusch and Kreft, 2011), the mechanism behind light sensing was also examined (Chapter 5).

Finally, we sought to elucidate a mechanism for Lmo0818, a P-type ATPase, and investigate the part it plays in maintaining the physiology of *L. monocytogenes*. We attempted to determine the cationic substrate of this P-type ATPase. We also explored the possibility that lmo0818 was under the control of σB and examine the expression of Lmo0818 and Lmo0819 using Western Blots (Chapter 6). The overall aim of this study was to determine the prevalence of *L. monocytogenes* in the Irish food processing industry and investigate the role of the stress response in survival and persistence of *L. monocytogenes* in these facilities. We aim to provide insights into new methods of controlling the contamination and spread of *L. monocytogenes*. 
CHAPTER 2

Materials and Methods
2.1. **Bacterial strains, plasmids and culture conditions**

Bacterial strains and plasmids used in this study are listed in Table (2.1). *Listeria monocytogenes* permanent stocks were prepared by centrifuging 5 ml of an overnight culture and resuspending in 2 ml Brain Heart Infusion (BHI) (Lab M) broth supplemented with 7% DMSO. These stocks were stored at -80°C. Strains were streaked out onto BHI agar (Lab M) for *L. monocytogenes* or Luria-Bertani agar (LB) (Sigma Aldrich) for *Escherichia coli* and grown at 37°C overnight. Plates were stored 4°C. Where required, plates were supplemented with appropriate antibiotics. For overnight cultures, 25 ml of broth was inoculated with 1 single colony from a plate.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>COB Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes EGD-e</td>
<td>Wild-type</td>
<td>C. Gahan (UCC)</td>
<td>COB 261</td>
</tr>
<tr>
<td>EGD-e ΔsigB</td>
<td>ΔsigB</td>
<td>C. Gahan (UCC)</td>
<td>COB 262</td>
</tr>
<tr>
<td>EGD-e Δlm00818</td>
<td>Δlm00818</td>
<td>This Study</td>
<td>COB 720</td>
</tr>
<tr>
<td>EGD-e Δlm00819</td>
<td>Δlm00819</td>
<td>This Study</td>
<td>COB 721</td>
</tr>
<tr>
<td>EGD-e lm00818PsigB</td>
<td>Mutated internal σB dependent promoter sequence in lm00818</td>
<td>This Study</td>
<td>COB 722</td>
</tr>
<tr>
<td>EGD-e lm00818::Tn</td>
<td>Transposon insert in lm00818</td>
<td>J. Johansson (Umeå University)</td>
<td>COB 695</td>
</tr>
<tr>
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<td>Δlm00799</td>
<td>J. Johansson (Umeå University)</td>
<td>COB 644</td>
</tr>
<tr>
<td>EGD-e lm00799 C56A</td>
<td>Amino acid change at position 56 of Lmo0799</td>
<td>B. O’Donoghue</td>
<td>COB 611</td>
</tr>
<tr>
<td>EGD-e Δlm00799 pMK4::lm00799</td>
<td>Δlm00799 complemented with full copy of lm00799</td>
<td>J. Johansson (Umeå University)</td>
<td>COB 627</td>
</tr>
<tr>
<td>EGD-e pMK4::lm00799</td>
<td>Wild-type complemented with full copy of lm00799</td>
<td>J. Johansson (Umeå University)</td>
<td>COB 625</td>
</tr>
<tr>
<td>EGD-e PNFB::GFP</td>
<td>Constitutive GFP</td>
<td>J. Johansson (Umeå University)</td>
<td>COB 861</td>
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<tr>
<td>L. monocytogenes 10403S</td>
<td>Wild-type</td>
<td>K. Boor (Cornell University)</td>
<td>COB 46</td>
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<tr>
<td>10403S ΔsigB</td>
<td>ΔsigB</td>
<td>K. Boor (Cornell University)</td>
<td>COB 45</td>
</tr>
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<td>S. Leroy (Clermont Ferrand)</td>
<td>COB 420</td>
</tr>
<tr>
<td>L. monocytogenes ScottA</td>
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<td>Colworth laboratory, Unilever Plc.</td>
<td>COB 34</td>
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<td>This Study (Drain swab from FBO 1)</td>
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<td>This Study (Floor swab from FBO 5)</td>
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### Table 2.2 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKSV7</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, Cml&lt;sup&gt;r&lt;/sup&gt;, temperature sensitive shuttle vector</td>
<td>Smith and Youngman, 1992</td>
</tr>
<tr>
<td>pEX-A</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, shuttle vector</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>pMAD</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, Erm&lt;sup&gt;r&lt;/sup&gt;, temperature sensitive ori.</td>
<td>Arnaud, 2004</td>
</tr>
<tr>
<td>pKSV7::Δlmo0818</td>
<td>lmo0818 deletion cassette cloned into pKSV7</td>
<td>This Study</td>
</tr>
<tr>
<td>pKSV7::Δlmo0819</td>
<td>lmo0819 deletion cassette cloned into pKSV7</td>
<td>This Study</td>
</tr>
<tr>
<td>pKSV7::lmo0818PsigB</td>
<td>Insert with mutation in an internal σ&lt;sub&gt;B&lt;/sub&gt; dependent promoter in lmo0818 cloned into pKSV7</td>
<td>This Study</td>
</tr>
<tr>
<td>pKSV7-P&lt;sub&gt;2230&lt;/sub&gt;:egfp</td>
<td>The promoter of lmo2230 fused to egfp cloned into pKSV7</td>
<td>Utratna et al., 2012</td>
</tr>
<tr>
<td>pEX-A::Δlmo0818</td>
<td>lmo0818 deletion cassette cloned into pEX-A</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>pEX-A::Δlmo0819</td>
<td>lmo0819 deletion cassette cloned into pEX-A</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>pEX-A::lmo0818PsigB</td>
<td>Insert with mutation in an internal σ&lt;sub&gt;B&lt;/sub&gt; dependent promoter in lmo0818 cloned into pEX-A</td>
<td>MWG Eurofins</td>
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<td>pMAD::lmo0799C56A</td>
<td>Insert with amino acid change at position 56 of Lmo0799 cloned into pMAD</td>
<td>B. O‘Donoghue</td>
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<tr>
<td>pMK4::lmo0799</td>
<td>Full sized lmo0799 gene cloned into pMK4</td>
<td>J. Johansson</td>
</tr>
</tbody>
</table>

### 2.2. Culture media

All media, unless stated otherwise, was autoclaved for 15 min at 121°C using a Labo autoclave (Sanyo). Where autoclaving could not be used, filter sterilisation was carried out using a 0.22 µm syringe filter (Sparks) and syringe (Terumo).
2.2.1  **Brain Heart Infusion (BHI)**

BHI broth was prepared by adding 37 g of powder (Lab M) to 1000 ml of dH\textsubscript{2}O. For BHI agar, BHI broth was supplemented with 15 g l\textsuperscript{-1} of Agar No2 (Lab M).

2.2.2. **Leuri-Bertani (LB)**

Ten grams LB broth powder (Sigma Aldrich) was added to 1000 ml dH\textsubscript{2}O. To prepare agar, 15 g l\textsuperscript{-1} of Agar No 2 was added to LB broth.

2.2.3. **Marine agar**

Marine agar was prepared by adding 55.1 g of Marine agar powder (Difco) to 1000 ml dH\textsubscript{2}O.

2.2.4. **Fraser broth**

Fraser broth is an enrichment broth used for the isolation of *Listeria* spp. Fifty five grams of Fraser broth base (Lab M) was added 900 ml of dH\textsubscript{2}O for half Fraser broth or to 1000 ml of dH\textsubscript{2}O for full Fraser broth. Once autoclaved the media was cooled to 47°C before supplements were added. Half Fraser broth required one vial of X564 (Lab M) per 2.5 l of Fraser broth and full Fraser broth required one vial of X164 (Lab M) per 500 ml of Fraser broth.

2.2.5. **Harlequin™ *Listeria* chromogenic agar**

70.5 g of Harlequin™ *Listeria* Chromogenic agar powder (Lab M) was dissolved in 950 ml dH\textsubscript{2}O. Following autoclaving, the medium was allowed to cool to 47°C and two vials of reconstituted X072 (Lab M) supplement were added. The agar was swirled to mix. Two vials of X010 (Lab M) were then added and the bottle was inverted slowly to mix.
2.2.6. Proteose peptone yeast extract medium (PPY)

PPY was prepared by adding 20 g of proteose peptone (Oxoid) and 2.5 g of yeast extract to 1000 ml dH$_2$O.

2.2.7. SSP medium

SSP medium was made by adding 20 g proteose peptone, 1 g yeast extract, 2 g glucose and 33 µM of FeCl$_3$ to 1000 ml dH$_2$O.

2.2.8. Defined media (DM)

The defined media used here for *L. monocytogenes* was adapted from a method previously described by Amezaga *et al.* (1995). Stocks for the media were prepared as described below:

2.2.8.1. Salt solution (10X)

Solution was prepared by adding 79.9 g Dipotassium hydrogen Orthophosphate (K$_2$HPO$_4$), 31 g Sodium dihydrogen Orthophosphate (NaH$_2$PO$_4$.2H$_2$O) and 10 g Ammonium chloride (NH$_4$Cl) per 1000 ml molecular grade water (Sigma Aldrich). Solution was filter sterilised and stored at room temperature in the dark.

2.2.8.2. Magnesium sulphate solution (100X)

40 g Magnesium sulphate (MgSO$_4$.7H$_2$O) was added per 1000 ml molecular grade water. Solution was filter sterilised and stored at room temperature in the dark.

2.2.8.3. Ferric citrate (50X)

Per 1000 ml molecular grade H$_2$O, 5 g of Ferric citrate (FeC$_6$H$_5$O$_7$) was added and dissolved by applying heat in a water bath, mixing regularly. Following this it was filter sterilised and stored in the dark at room temperature.

2.2.8.4. Essential amino acid solution (100X)

For the amino acid solution, 10 g L-leucine, L-isoleucine, L-valine and L-methionine (Added last) and 20 g L-arginine monohydrochloride and L-histidine monohydrochloride monohydrate was dissolved in 1000 ml molecular grade H$_2$O. Each amino acid was
dissolved before the next was added. This solution was filter sterilised and stored in the dark at 4°C.

2.2.8.5. Cysteine and tryptophan solution (100X)

Ten grams of L-cysteine hydrochloride and L-tryptophan were added to 1000 ml molecular grade water. L-cysteine hydrochloride was fully dissolved before L-tryptophan was added. The solution was filter sterilised and stored in the dark at 4°C.

2.2.8.6. Glutamine solution (50X)

30 g L-glutamine was added per 1000 ml molecular grade water and was filter sterilised. It was stored at room temperature in the dark.

2.2.8.7. Vitamin solution (100X)

For the vitamin solution, 5 mg α-lipoic acid (D-L 6, 8 Thiotic acid) was added per 200 ml 70% ethanol. Four millilitres of this was added to another solution containing 10 mg biotin, 100 mg thiamine, 100 mg riboflavin and 250 ml ethanol (95%). This solution was mixed and brought to a final volume of 1000 ml with molecular grade H₂O. Finally it was filter sterilised and stored in the dark at 4°C.

2.2.8.8. Trace elements solution (100X)

This solution was made in two different ways. One contained all cations and the other contained no cations. The first solution was prepared by adding 6.75 g Sodium hydroxide (NaOH) and 13.5 g Nitrioloacetic acid (N(CH₂COOH)₃) per 800 ml molecular grade water. This was slowly added to a 160 ml solution which contained 0.55 g calcium chloride dihydrate (CaCl₂.2H₂O), 0.17 g zinc chloride (ZnCl₂), 0.059 g cupric chloride dihydrate (CuCl₂.2H₂O), 0.06 g cobaltous chloride 6-hydrate (CoCl₂.6H₂O) and 0.06 g sodium molybdate dihydrate (Na₂MoO₄.2H₂O) and was brought to a final volume of 1000 ml with molecular grade water before filter sterilising and storing in the dark at 4°C.

In the second solution, the 160 ml solution only contained 0.059 g cupric chloride dihydrate (CuCl₂.2H₂O) and 0.06 g sodium molybdate dihydrate (Na₂MoO₄.2H₂O). Again it was filter sterilised and stored in the dark at 4°C. If this solution was used, the medium was supplemented with the required concentrations of the absent cations.
2.2.8.9. Glucose solution

A 20% (w/v) solution of glucose was prepared by adding 200 g per 1000 ml molecular grade water. This was filter sterilised and stored at room temperature.

2.2.8.10. Individual cation solutions and EDTA

500 mM solutions of calcium chloride dehydrate (CaCl$_2$.2H$_2$O), magnesium sulphate (MgSO$_4$.7H$_2$O), zinc chloride (ZnCl$_2$), cobaltous chloride 6-hydrate (CoCl$_2$.6H$_2$O) and Ethylenediaminetetraacetic acid (EDTA) were prepared in molecular grade water and filter sterilised.

The stocks could be stored for up to 1 month but fresh DM was prepared from the stocks before an experiment was carried out. A working concentration of DM contained 810ml of molecular grade H$_2$O mixed with 100 ml salt solution, 10 ml magnesium sulphate solution, 20 ml ferric citrate, 10 ml amino acid solution, 10 ml cysteine and tryptophan solution, 20 ml glutamine solution, 10 ml vitamin solution and 10 ml of trace element solution. The DM was finally supplemented with 0.4% (v/v) glucose before again being filter sterilise prior to use.

2.2.9. Seawater

Seawater was obtained from Galway Bay, Ireland. This seawater was not treated prior to testing unless stated. Numbers of micro-organisms present in the seawater was tested before experiments were carried out, by diluting 1 ml of seawater tenfold to $10^{-5}$ in PBS. 10 µl was then spotted in triplicate onto Marine agar (Difco). The plates were incubated at room temperature for 1 week and the colony forming units (CFU) were determined.

2.3. Buffers, enzymes and antibiotics

2.3.1. Phosphate buffered saline (PBS)

One PBS tablet (Sigma Aldrich) was dissolved in 200 ml of dH$_2$O.
2.3.2. Page’s Amoeba saline solution (PAS)

PAS was prepared by making two separate stocks as per instruction from the Culture Collection of Amoeba and Protozoa (CCAP). Five millilitres of each stock were added to dH₂O and was brought to the final volume of 1000 ml.

2.3.2.1. Stock 1

This stock was made by adding 12 g Sodium chloride (NaCl₂), 0.40 g Magnesium sulphate (MgSO₄·7H₂O), 0.60 g Calcium chloride (CaCl₂·6H₂O) to 500 ml of dH₂O.

2.3.2.2. Stock 2

Stock 2 contained 14.20 g Sodium dihydrogen Orthophosphate (NaH₂PO₄·2H₂O) and 13.60 g Dipotassium hydrogen Orthophosphate (K₂HPO₄) in 500 ml dH₂O.

2.3.3. Lysozyme

Lysozyme was prepared by dissolving 10 mg ml⁻¹ of lysozyme powder (Sigma Aldrich) in dH₂O. The solution was filter sterilised and stored at -20°C.

2.3.4. Ampicillin

Ampicillin (Amp) was prepared by dissolving 50 mg ml⁻¹ ampicillin sodium salts (Sigma Aldrich) in dH₂O. The solution was filter sterilised and stored at -20°C.

2.3.5. Chloramphenicol

Chloramphenicol (Cml) was prepared by adding 50 mg ml⁻¹ chloramphenicol (Sigma Aldrich) to ethanol (70%) and stored at -20°C.

2.3.6. Erythromycin

Erythromycin (Erm) was made by adding 10 mg ml⁻¹ erythromycin (Sigma Aldrich) to ethanol (70%) and stored at -20°C.
2.3.7. Penicillin G

Penicillin G was prepared by adding 5 mg ml\(^{-1}\) of penicillin G (Sigma Aldrich) to dH\(_2\)O. The solution was filter sterilised and stored at -20°C.

2.4. Light apparatus

2.4.1. Blue light apparatus for illumination of agar plates

A high-power mounted 470 nm LED (Thorlabs, model M470L2) was the light source (designated light setup 1) used to investigate the effects of blue light across the surface of an agar plate. An aspheric condenser lens (Ø 75 mm) was used to create a uniform distribution of light across a circular area of 7 cm in diameter on the agar plate. An irradiance map (Fig. 2.1.) was created using an optical power sensor (Thorlabs, model PM121D). This setup produced 1.5-2.0 mW cm\(^{-2}\), which could be increased to an irradiance of 8.0 mW cm\(^{-2}\) by increasing the current using a T-cube LED driver (LEDD1B, ThorLabs). The lower irradiance setting was used for growth inhibition experiments while the higher setting was used for survival experiments.

![Light apparatus with variable light intensity. Light setup 1 consists of a high-power mounted 470 nm LED and an aspheric condenser lens (Ø 75mm) to create a uniform distribution of light across a circular area of 7 cm in diameter.](image)

2.4.2. Blue light apparatus for illumination of 96-well microtitre plates

An alternative setup (designated light setup 2) was used to test the effects of light across the area of a 96-well microtitre plate containing liquid medium. This setup was composed of 80 blue (460 nm) 10 mm prewired LEDs (Phenoptix) arranged in a 10 x 8 array, measuring
16 x 13.5 cm. A diffuser membrane was placed below the lights to create a uniform distribution of light across the area of the plate which was confirmed by the generation of an irradiance map. The lights were fixed in position approximately 16 cm above the 96-well plate.

**Figure 2.2. Light apparatus for 96-well microtitre plates.** Light setup 2 consists of 80 blue LEDs arranged in a 10 x 8 array. The measurements of the array were 16 x 13.5 cm. A diffuser membrane was added below the array to help achieve a uniform distribution of light across the area of a 96-well plate which was placed 16 cm below the lights.

### 2.4.3. UV light apparatus

A UV light setup was used to test the effect of UVA light on cells. This consisted of a high power mounted 385 nm LED which required an aspheric condenser lens (Ø 55 mm) to create a uniform distribution of light. This setup provided the maximum power density centered on the middle 9 wells of a 96 well microtitre plate. The light was placed 6 cm above the plate for optimum exposure. During the experiments for which this setup was used, the power density was set to deliver 6.7 mW cm\(^{-2}\) to the plate.
2.5. Phenotypic assays

2.5.1. Inhibitory effects of various stresses on growth on solid agar

Overnight cultures were grown with aeration for 18 h at 37°C before use. Cultures were standardised to OD$_{600nm}$ = 1.0 and then serially diluted tenfold to $10^{-8}$ in PBS. Four microliters of each dilution was inoculated on BHI agar. Plates were incubated at 30°C for 24 h and plates were imaged using a CCD camera (G:box, Syngene) and imaging software (Syngene) using the white light filter. Depending on the stress being tested, the plates were treated as stated below.

2.5.1.1. Blue light assays

The plates were exposed to 470 nm blue light (light setup 1) at 1.5-2.0 mW cm$^{-2}$ or incubated in the dark (plates were wrapped in aluminium foil) at 30°C for 24 h. Agar plates were incubated with the inoculated surface facing downwards and illuminated with the blue light source from above (see Fig. 2.1.) The medium and the plastic of the plate reduced the transmittance of light by approximately 50%.

2.5.1.2. Reactive oxygen species scavengers

For experiments investigating the involvement of ROS in growth inhibition by light, 20 mM dimethylthiourea (Sigma Aldrich) was included during medium preparation or 100 µl of 125 U ml$^{-1}$ catalase (Sigma Aldrich) was spread on the surface of the agar plate. The plates were then treated as described for the blue light assay above (Section 2.5.2.1.).

2.5.1.3. Salt assays

When testing the stress NaCl exerted on the bacteria, 1 M NaCl (58.44 g l$^{-1}$; Sigma Aldrich) was added to the media during preparation. The plates were wrapped in aluminium foil prior to incubation to prevent exposure to light.

2.5.2. Blue light assays in liquid culture

Overnight cultures were grown in BHI for 18 h shaking at 37°C. These were diluted in fresh medium to an OD$_{600nm}$ = 0.05. This suspension was then further serially diluted in BHI to a final dilution of $10^6$, with respect to the suspension at OD$_{600nm}$ = 0.05. Two hundred microliters of each dilution was then added to two 96-well microtitre plates. One plate was
wrapped in aluminium foil as a dark control, while the other was placed under the light apparatus (light setup 2; Fig 2.2), which produced blue light at 460 nm with a power density of 1.5-2.0 mW cm$^{-2}$ (Fig. 2.2.). These plates were incubated at 30°C for 14 h and growth was monitored by recording the OD$_{600}$ nm hourly on a Sunrise–Tecan plate reader. The light transmittance through the plastic lid on top of the plate and medium was approximately 68% with this setup. To take OD readings, plates were removed from the light apparatus and incubator and were shaken for 10 s immediately prior to each reading. To avoid taking readings throughout the night a second set of plates was later set up using the same overnights. These were incubated as before in the presence of light or dark for 15 h overnight without taking readings. The OD$_{600}$ nm was recorded at the 15 h time point, and subsequently every hour until 24 h was reached, allowing a full growth curve to be generated from the two sets of data. Lag phase was defined as the time taken to reach an OD$_{600}$ nm value of 0.1 and this was derived directly from the growth curves. For experiments investigating the involvement of ROS in growth inhibition by light, 20 mM Dimethylthiourea (DMTU) or 125 U ml$^{-1}$ catalase was added to the medium prior to the experiment.

2.5.3. Ring phenotype and motility assays

For the ring phenotype assays overnight cultures were standardised to OD$_{600nm}$ = 2.0 and a 2 µl aliquot was inoculated onto the centre of a semi-solid BHI agar (0.3% agar w/v). Plates were exposed to five consecutive 12 hour cycles of light (from white fluorescent bulbs producing a power density of approximately 0.1-0.2 mW cm$^{-2}$) and dark (wrapped in aluminium foil) at 30°C. For motility assays, semi-solid BHI agar plates were inoculated in the same way and then incubated in the continuous light (from white fluorescent bulbs producing a power density of approximately 0.1-0.2 mW cm$^{-2}$) or dark (wrapped in aluminium foil) at 30°C. Colony diameter (mm) was measured after a 60 h incubation period. Plates were imaged using a CCD camera (G:box, Syngene) and imaging software (Syngene) using the white light filter.

2.5.4. Seawater survival assay under ambient conditions

Overnight cultures were incubated at 37°C for 18 h shaking. One millilitre of the overnight cultures was centrifuged at 6800 RCF for 6 min. Cells were washed twice in PBS. Flasks containing 50 ml of live seawater or filtered seawater were inoculated with a 1 in 10 dilution
of the bacterial culture. This was carried out in triplicate. For determining the survival of
*L. monocytogenes* at 16°C, flasks were wrapped in aluminium foil and incubated at 16°C for
25 days. For outdoor experiments, flasks were incubated outside under ambient conditions.
A dark control was also set up whereby the flasks were covered with aluminium foil to stop
exposure to light but were also incubated outside exposed to ambient temperature. At each
time point, 1 ml of sample was taken from each flask the sample was diluted tenfold in PBS
to $10^{-5}$. 10 µl of each dilution was spotted in triplicate on BHI agar and plates were incubated
inverted for 24 h at 37°C. Colonies were counted to determine numbers of CFU ml$^{-1}$ in the
seawater. Temperature data was obtained from [www.met.ie](http://www.met.ie) and irradiance data from
[www.iruse.ie](http://www.iruse.ie), whose instruments are located on the NUI Galway campus.

### 2.5.5. Survival assays using UV or blue light

Cultures were incubated overnight for 18 h shaking. 1 ml of the culture was centrifuged at
6800 RCF for 6 min. Bacterial pellets were washed twice in PBS. For seawater experiments,
5 ml of live seawater was inoculated with a 1 in 10 dilution of bacterial cells. 200 µl of the
seawater was added to the centre 9 wells of a 96 microtitre plate. The plate was exposed to
either UV light or blue light. Both light setups were set to deliver a power density of 6.7 mW
cm$^{-1}$ at 16°C. For survival in PBS, samples were resuspended in PBS and exposed to blue
light at a power density of 8 mW cm$^{-2}$ at 30°C. A dark control was also prepared similarly
and wrapped in aluminium foil. At each time point, 40 µl was taken and serially diluted to
$10^{-6}$ in PBS. Ten microliters of each dilution was spotted in triplicate on BHI agar. The
plates were incubated inverted at 37°C for 24 h before the CFU ml$^{-1}$ could be determined.

### 2.5.6. Acid tolerance test

Overnight cultures were grown with shaking at 37°C for 18 h until they had reached
stationary phase. 1 ml of the cultures was added to an Eppendorf and centrifuged at 6800
RCF for 6 min. The bacterial pellet was resuspended in BHI acidified to pH 2.5 or in BHI
broth as a control. The pH of the acidified broth was reduced by adding 3 M HCl and
checked using a Mettler Toledo SevenEasy pH meter. At each specific time point, 60 µl was
taken from each sample and serially diluted in PBS. Spot plates were carried out as
discussed in previous sections on BHI agar. Plates were incubated for 24-48 h at 37°C. The
CFU ml$^{-1}$ was determined from the colony counts.
2.5.7. Biofilm assays

Biofilm assays were adapted from Feehily (2014). An overnight culture of *L. monocytogenes* was incubated at 37°C for 18 h. 1 ml of the overnight was centrifuged at 8000 RCF for 6 min. The bacterial pellet was washed once in PBS and finally resuspended in 1 ml of PBS. 7 µl of the bacterial suspension was added to 7 ml DM supplemented with 0.4% (v/v) glucose. 200 µl of this suspension was added to a flat bottom 96 well microtitre plate. Six technical repeats were added to the plate for each strain. The outside wells had no culture added to them. Instead, 200 µl of sterile media was added to these wells. The plates were incubated at the appropriate temperature for the experiment (37°C, 30°C or 16°C) for the specified time (24 h, 48 h or 1 week). After incubation the media was removed carefully from each well using a pipette and each well was washed 3 times with 200 µl PBS. The plates were allowed to dry at 40°C for 45 min – 1 h. Once dry 150 µl of 1% (w/v) crystal violet (Sigma Aldrich) was added to each well and the plate was incubated at 37°C for 30 min. The crystal violet was carefully removed from the wells and the plate was washed twice more with PBS. Finally, the plate was rinsed with dH₂O by allowing the water to gently trickle over the plate. 160 µl of ethanol (95%) was added to the plate and it was incubated for 30 min shaking at 30 rpm at room temperature. The OD₆₀₀nm was recorded and used as an indication of biofilm levels.

2.5.8. Protozoal assays

To prepare *L. monocytogenes* for co-culture experiments, overnight cultures of *L. monocytogenes* were grown at 37°C for 18 h in BHI broth shaking. From the overnight, 1.5 ml was centrifuged in a 15 ml tube at 9000 RCF for 10 min. The pellet was washed in PBS three times before it was finally resuspended in 1 ml PAS (Section 2.3.2). The OD₆₀₀nm was equalised to 0.5 to give approximately 10⁸ CFU ml⁻¹. From here the culture was further diluted 1:10 to give the desired concentration of cells (10⁷, 10⁶, 10⁵, 10⁴ CFU ml⁻¹)

For the protozoa, *Tetrahymena pyriformis* was used in this study. Cultivation of *T. pyriformis* was carried out as per instructions from the Culture Collection of Amoeba and Protozoa (CCAP). Cultures of *T. pyriformis* were grown at 28°C for approximately 3 days before use in either PPY or SSP (Section 2.2.6 and Section 2.2.7). Five millilitres of the culture was centrifuged at 3000 RCF for 3 min in a 15 ml tube. The protozoal pellet was washed twice in PAS before being resuspended in a final volume of 1 ml PAS. Twenty microliters of this suspension was placed at -20°C for 5 min to slow down the movement of
the protozoa. Without this step their movement was too rapid to achieve an accurate count. Seven microliters was then added to a haemocytometer to count the number of protozoa present per ml. From this calculation, the volume required for a final concentration of 100 protozoa in the co-culture was determined.

Finally, the various concentrations of *L. monocytogenes* were added with the 100 protozoa and incubated at 28°C for the duration of the experiment. At desired time points, samples were taken and serially diluted in PBS. Ten microliters of each dilution was plated in triplicate onto BHI agar and plates were incubated for 24 h at 37°C. Colonies were counted and the CFU ml⁻¹ was determined allowing a determination of loss of viability over time in the presence of *T. pyriformis*.

### 2.6. Preparation and transformation of electrocompetent bacterial cells

#### 2.6.1. *E.coli* Top 10

A culture of *E. coli* Top10 was incubated overnight at 37°C for 18 h. A 1:100 dilution of the overnight culture was added into 500 ml fresh LB broth. This was incubated for 3-5 h until the OD₆₀₀nm = 0.35 – 0.40. The culture was cooled for 10 min on ice. It was then split into 10, 50 ml centrifuged tubes (Abdos). The cultures were centrifuged for 15 min at 2500 RCF at 4°C. The supernatant was discarded and the pellets were combined into two 50 ml tubes. Each pellet was washed 4 times in 25 ml ice-cold 10% (v/v) glycerol (Sigma Aldrich) and centrifuged in between washes at 2500 RCF for 15 min at 4°C. The pellets were resuspended and combined in a final volume of 1 ml. This suspension was centrifuged again as before and the pellet was resuspended in 500 µl ice-cold 10% (v/v) glycerol solution. Fifty microliters of cells were aliquoted out into pre-frozen, pre-labelled Eppendorf tubes and these were immediately stored at -80°C.

#### 2.6.2. *L. monocytogenes* EGD-e

For making electrocompetent EGD-e cells, an overnight culture was incubated with shaking at 37°C for 18 h in BHI broth. This was diluted 1:100 into 250 ml fresh BHI broth which contained 500 mM sucrose (Sigma Aldrich) and incubated at 37°C with shaking until the OD₆₀₀nm = 0.2-0.25. Into the culture, 10 µg ml⁻¹ of ampicillin was added and it was further
incubated for 2 h at 37°C. The culture was chilled on ice for 10 min and was split into five 50 ml tubes, which had been pre-chilled. From here all work was carried out on ice. The tubes were centrifuged at 5000 RCF for 10 min at 4°C. After the supernatant was discarded, each pellet was washed in 50 ml of sterile sucrose-glycerol wash buffer (SGWB). This buffer was composed of 10% (v/v) glycerol and 500 mM sucrose. The pH was adjusted to pH 7 with 2 M NaOH. The cell suspensions were again centrifuged at 5000 RCF for 10 min at 4°C and the supernatant was discarded. Each pellet was then resuspended in 35 ml ice-cold SGWB and were centrifuged again. Finally the pellets were resuspended in 10 ml ice – cold SGWB and were combined in a 250 ml flask. Into this suspension, 10 µg ml⁻¹ lysozyme was added. The culture was incubated at 37°C for 20 min and then immediately centrifuged at 3000 RCF for 10 min at 4°C. The pellet was washed in 20 ml SGWB and was re-centrifuged again as before. Finally the pellet was resuspended in 2.5 ml ice-cold SGWB. The suspension was aliquoted out into 50 µl volumes in 1.5 ml Eppendorf tubes that had been pre-frozen and pre-labelled. The tubes were immediately stored at -80°C.

2.6.3. L. monocytogenes 10403S and food isolates isolated in this study

For L. monocytogenes strain 10403S, a different method is required to achieve optimal transformation efficiency as it is not as competent as EGD-e. This method was also used for the food isolates isolated in this study as they have been classified (Chapter 4) as being more closely related to 10403S than EGD-e. An overnight culture was incubated with shaking at 37°C for 18 h in BHI broth. This was diluted 1:100 into 250 ml fresh BHI broth which contained 500 mM sucrose (Sigma Aldrich) and incubated at 37°C with shaking until the 0D₆₀₀nm = 0.2. Once this OD was reached, 10 µg ml⁻¹ of penicillin G was added to the culture and it was incubated for a further 2 h. The culture was divided into five 50 ml centrifuge tubes, and centrifuged for 10 min at 5000 RCF at 4°C. From here, all work was carried out on ice. Each pellet was washed with 40 ml ice-cold transformation buffer containing 500 mM sucrose and 1 mM HEPES (Fluka) and centrifuged at 5000 RCF for 10 min at 4°C. The supernatant was discarded and the pellets were resuspended in 10 ml ice-cold transformation buffer and were combined into one tube before being centrifuged again as before. One final wash was carried out in 15 ml ice-cold transformation buffer and the culture was centrifuged again. Finally the pellet was resuspended in 500 µl ice-cold transformation buffer with 10% (v/v) glycerol. The cells were aliquoted out into 50 µl volumes into pre-frozen Eppendorf tubes. Cells were immediately stored at -80°C until required for use.
2.6.4. Transformation of plasmid DNA into bacterial strains

Once the cells had been made electrocompetent, it was then possible to transform purified plasmid DNA (Section 2.8.3) into the strain. For transformations, electrocompetent cells were first thawed slowly on ice. One microliter of plasmid (~100 ng) was added to the tube and mixed gently with the pipette tip. This was incubated on ice for 10 min. The cells were then transferred into a pre-chilled 0.2 cm electroporation cuvette (Ingenio) carefully making sure there were no air bubbles at the bottom of the cuvette. The cuvette was exposed to a voltage of 2.1-2.5 kV at 200 Ω and 25 μF in a GenePulser (BioRad), which produced typical at time constants ranging between 4.5-4.8 ms. Immediately following this the cells were resuspended in either BHI supplemented with 500 mM sucrose (for L. monocytogenes) or SOC media (for E. coli). The cells were allowed to recover at 37°C for 1.5 h before 20 µl, 50 µl and 100 µl of the cells were spread on BHI or LB agar plates supplemented with the selective antibiotic for the plasmid. A negative control was also set up where sterile dH2O was used instead of DNA. Plates were incubated at 30°C for 24-48 h. Colonies were screened for positive transformants by PCR using primers specific to the plasmid (Table 2.7).

2.7. Protein methods

2.7.1. Protein extractions

Bacterial cultures were grown in either 25 ml BHI broth or in DM supplemented with 0.4% (v/v) glucose. For cultures grown in BHI, cells were grown for 16 h. For cultures in DM, samples were grown overnight first and then to exponential phase (OD600nm = 0.8) in 50 ml tubes instead of glassware to limit excess cations being added to the media. Once this OD was reached, samples were resubbed into fresh DM three times before protein extractions were carried out on the cells. Once cells were removed from the incubator, 10 µg ml⁻¹ chloramphenicol was added immediately to each culture. Samples were transferred to 50 ml tubes and centrifuged for 15 min at 9000 RCF at 4°C. The supernatant was discarded and each pellet was resuspended in 2 ml sonication buffer. This buffer contained 10 mM Tris-HCl, 0.1 mM EDTA and 5 mM MgCl₂ and the pH was adjusted to pH 8 using 2 M NaOH. Two mg ml⁻¹ lysozyme was added to each sample and they were incubated at 37°C for 30 min. The samples were centrifuged again at 4°C for 15 min at 9000 RCF. Each pellet was resuspended in 0.5 ml sonication buffer containing protease inhibitor (1% v/v). The
suspension was then added to a 2 ml cryo-vial that contained 0.75 ml zirconia beads (0.5 ml 0.1 mm beads and 0.25 ml 0.5 mm beads poured directly into the vials). Samples were bead-beated for 10 min alternating between 30 s bead beating and 30 s on ice. Finally samples were centrifuged at 13000 RCF for 30 min. The supernatant was removed and added to a fresh Eppendorf tube and samples were stored at -20°C. The concentration of protein extracted was determined using the Bio-Rad DC protein assay (Bio-Rad) in accordance with the manufacturer’s protocol. Bovine serum albumin (BSA) was used for standards (0 - 1.5 mg ml\textsuperscript{-1}).

2.7.2. SDS – PAGE

2-D gels were used to separate the proteins after extraction and the method was adapted from Abram (2007). All the components required for preparing the gels were kept at 4°C with the exception of the 10% (w/v) SDS solution and 10% (w/v) ammonium persulphate (APS; Sigma Aldrich). The SDS was stored at room temperature and the APS was made up fresh each day. The gel apparatus was set up as per manufacturer’s instructions prior to preparing the gels. Two separating gels were made for 0.75 mm plates according to Table 2.3.

Table 2.3 Components required for separating gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Separating gel (7.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH\textsubscript{2}O</td>
<td>4.85 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>30% Acrylamide bis-acrylamide</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

This recipe was adjusted for 10% or 12% acrylamide gels where required. The gels were poured and dH\textsubscript{2}O was added to the top to exclude any bubbles that may have formed. This gel was allowed to polymerise for 40 min after which the dH\textsubscript{2}O was removed from the plates with absorbent tissue paper.

Table 2.4 Components required for stacking gels

<table>
<thead>
<tr>
<th>Component</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH\textsubscript{2}O</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>30% Acrylamide bis-acrylamide</td>
<td>665 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>
Next the stacking gel was prepared using the amounts specified in Table 2.4. This gel was added to the top of the separating gel and immediately afterwards a comb was added between the plates to define wells in the gel. The gel was allowed to polymerise for 20 min. Finally, the gels were transferred into the running tank. A 10X solution of running buffer containing 30.3 g l⁻¹ Tris base, 144 g l⁻¹ glycine and 10 g l⁻¹ SDS, was diluted to give a 1X solution. The buffer was added to the tank ensuring that the electrodes were covered. Either 10 or 15 µl of each sample was loaded into the gel depending on which comb was used. Five microliters of a prestained protein ladder (PageRuler prestained protein ladder 10-180 kDa; Thermo Scientific) was also loaded to the gel. The gel was run at 25 mA (per gel) and 150 V for 1 h. Following this gels were either stained with GelCode® Blue staining reagent (Pierce) for 24 h or were prepared for Western blot.

2.7.3. Western blotting

The method used for the transfer of proteins onto an immobilised membrane was a semi-dry method using PVDF membrane. Following the completion of the SDS-PAGE (Section 2.7.2.), the gels were removed from the plates and rinsed first with dH₂O before being submerged in 10 ml of transfer buffer for 5 min. The transfer buffer contained 2.8 g l⁻¹ Tris Base, 11.3 g l⁻¹ glycine and 200 ml methanol (100%). The final volume was brought to 1000 ml with dH₂O. During this incubation, the PVDF membrane was charged by placing it in methanol (100%) for 30 s. It was then transferred into 10 ml transfer buffer for 5 min. Following this a transfer stack was assembled by first placing a sheet of Mini Trans-Blot® Filter paper (BioRad, USA) pre-soaked in transfer buffer onto the anode plate. The PVDF membrane was placed on top of the filter paper and the gel on top of the filter. The stack was finished by placing another pre-soaked sheet of filter paper on the top. The stack was rolled with a 5 ml serological pipette (Sarstedt) to remove air bubbles. The cathode plate was placed carefully on top of the apparatus taking care not to disrupt the stack. The transfer was run at 5 V at 100 mA for 1 h. Once the transfer was complete, the membrane was removed and was added to 10 ml TBST which contained 1% (v/v) tween. The membrane was washed for 10 min at 70 oscillations per min using a rocker (Stuart See-Saw Rocker SSL4). To block the membrane, 5% skimmed milk was prepared in 1% TBST. The membrane was blocked in 15 ml of this solution for 1 h at 30 oscillations per min. Following this, the membrane was washed three times in 1% TBST at 70 oscillations per min for 10 min each. The primary antibody was diluted 1:5000 for Anti-Lmo0818 (Rabbit polyclonal IgG, GenScript) or
1:3750 for Anti-Lmo0819 (Rabbit polyclonal IgG, GenScript) in 5% (w/v) skimmed milk prepared with 1% TBST. The membrane was incubated overnight with the primary antibody for 16 h at 4°C at 30 oscillations per min. After incubation, the primary antibody was removed and the membrane was washed in 1% TBST for 10 min at 70 oscillations per min. This was repeated 3 times. The secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology) was diluted 1:5000 into 5% (w/w) skimmed milk again made with 1% TBST. Fifteen millilitres of this was added to the membrane and it was incubated at room temperature for 1 h at 30 oscillations per min. After incubation, the membrane was again washed three times in 1% TBST. The membrane was placed between two pieces of acetate for the detection process. To detect the protein signal, Amersham ECL Western Blotting Detection Kit was used. The components of this were mixed in a 1:1 ratio and 400 µl of this mix was applied to each membrane within the acetate. The acetate was placed into a press and a piece of x-ray film was placed over the blot before closing the press. The blot was exposed for different time periods up to 1 h for effective detection. The x-ray film was developed to show the expression levels of detected proteins.

2.8. Molecular techniques

2.8.1. Polymerase chain reaction (PCR)

2.8.1.1. Routine and high fidelity PCR

PCR was used regularly for routine screening of strains. For the template DNA, one bacterial colony was added to 250 µl of sterile dH₂O and vortexed to disperse the cells. From stock solutions of 100 mM dATP, dTTP, dGTP and dCTP, a 10 mM solution was prepared in sterile dH₂O. Primers were obtained from MWG Eurofins and were rehydrated with sterile dH₂O to 100 µM concentration as per instructions provided by the company. For a working concentration of primers, they were diluted to 25 µM in sterile dH₂O unless stated otherwise. A PCR master mix was prepared as per Table 2.5 and 1 µl of the DNA template was added to bring the final volume to 25 µl per reaction.
Table 2.5 Components for routine PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Master mix</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>16.25 µl</td>
<td>Bioline</td>
</tr>
<tr>
<td>Reaction Buffer</td>
<td>5 µl</td>
<td>Bioline</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>0.5 µl</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Primer A (25 µM)</td>
<td>1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer B (25 µM)</td>
<td>1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>My Taq™ DNA Polymerase</td>
<td>0.25 µl</td>
<td>Bioline</td>
</tr>
<tr>
<td>Total</td>
<td>24 µl</td>
<td></td>
</tr>
</tbody>
</table>

PCR reactions were run in a thermocycler (MWG Biotech Primus) with the conditions specified in Table 2.6, unless indicated otherwise. The steps shown in italics ran for 30 cycles.

Table 2.6 Conditions used for PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturing step</td>
<td>94°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>53 -56.2°C</td>
<td>1 min</td>
</tr>
</tbody>
</table>
| Elongation         | 72°C        | 1-3 min  
| Final Elongation   | 72°C        | 7 min|

*varied depending on primers used.

For PCRs requiring high fidelity amplification such as PCRs for cloning or preparation for sequencing, Q5® High-Fidelity DNA polymerase was used and molecular grade water (Ambion) was used in the master mix. These reactions were run similarly to routine PCR but they required 98°C rather than 94°C for denaturation.
### Table 2.7. Primers used in this study

<table>
<thead>
<tr>
<th>Primer (COB #)</th>
<th>Primer Sequence (5’-3’)</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>395</td>
<td>CAGGAAACAGCTATGAC</td>
<td>For M13</td>
<td>Eurofins Genomic</td>
</tr>
<tr>
<td>396</td>
<td>GAAAAACGGCCGACAG</td>
<td>Rev M13</td>
<td>Eurofins Genomic</td>
</tr>
<tr>
<td>770</td>
<td>AGGGCTTCAAGGACTTACCC</td>
<td>For lmo0737</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>771</td>
<td>AGCATTTCTGCTTGCCCATT</td>
<td>Rev lmo0737</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>772</td>
<td>AGGGGTCTTAAATCTGGAA</td>
<td>For lmo1118</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>773</td>
<td>CGGCTTGTGGCATCTTA</td>
<td>Rev lmo1118</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>774</td>
<td>AGCAAAATGCAAAACTCGT</td>
<td>For ORF2819</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>775</td>
<td>GATCACTAAAGCCCTCCATTG</td>
<td>Rev ORF2819</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>776</td>
<td>AGTGGACAATTGATGTTGTA</td>
<td>For ORF2110</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>777</td>
<td>CATCCATGCCCTTTGGAC</td>
<td>Rev ORF2110</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>778</td>
<td>GCTGAAGAGATGGCGAAGAAG</td>
<td>For Prs</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>779</td>
<td>CAAAGAACCTGGATTTTGGCG</td>
<td>Rev Prs</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>848</td>
<td>ATAAAGGCACAAGCTTCG</td>
<td>For sigB</td>
<td>This study</td>
</tr>
<tr>
<td>849</td>
<td>TTATGGCGTCAACAGTGG</td>
<td>Rev sigB</td>
<td>This study</td>
</tr>
<tr>
<td>872</td>
<td>CGACTATGCATCAATCGC</td>
<td>For flanking</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δlmo0818</td>
<td></td>
</tr>
<tr>
<td>873</td>
<td>CCAAAACATACGGAAGCA</td>
<td>Rev flanking</td>
<td>Δlmo0818</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δlmo0818</td>
<td></td>
</tr>
<tr>
<td>874</td>
<td>GCATCGCCATCAAAATTGTTC</td>
<td>For flanking</td>
<td>Δlmo0818</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δlmo0818</td>
<td></td>
</tr>
<tr>
<td>875</td>
<td>GAGAGCTAAGCCATCTCC</td>
<td>For flanking</td>
<td>Δlmo0819</td>
</tr>
<tr>
<td></td>
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<td>Δlmo0819</td>
<td></td>
</tr>
<tr>
<td>876</td>
<td>GCTGTCCCAACACCATGAC</td>
<td>Rev flanking</td>
<td>Δlmo0819</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δlmo0819</td>
<td></td>
</tr>
<tr>
<td>877</td>
<td>TACTAGCGGCGAGGAGGCA</td>
<td>For on</td>
<td>Δlmo0818ΔPsigB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δlmo0818</td>
<td></td>
</tr>
<tr>
<td>878</td>
<td>TACGCCCTCTTTTGAATG</td>
<td>Rev flanking</td>
<td>Δlmo0818</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δlmo0818</td>
<td></td>
</tr>
<tr>
<td>879</td>
<td>CATCAGGTGCTTTGGAAGC</td>
<td>For rsbU</td>
<td>This study</td>
</tr>
<tr>
<td>880</td>
<td>GCGCTGCTATAATTCTGTTAAC</td>
<td>Rev rsbU</td>
<td>This study</td>
</tr>
</tbody>
</table>

### 2.8.1.2. Multiplex PCR

For multiplex PCRs used for characterisation of food isolates, multiple primers were used to first confirm the presence of *L. monocytogenes* and then to further serogroup the isolates (Doumith et al., 2004). For these reactions the DNA template was prepared as before but the master mix differed. The requirements for this PCR are found in Table 2.8.
Table 2.8 Components required for multiplex PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Master mix</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>16.75 µl</td>
<td></td>
</tr>
<tr>
<td>Reaction Buffer</td>
<td>5 µl</td>
<td>Bioline</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>0.5 µl</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Primer COB 770 <em>iso0737</em> (50 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 771 <em>iso0737</em> (50 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 772 <em>iso1118</em> (50 µM)</td>
<td>0.35 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 773 <em>iso1118</em> (50 µM)</td>
<td>0.35 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 774 ORF2819 (50 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 775 ORF 2819 (50 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 776 ORF2110 (50 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 777 ORF2110 (50 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 778 <em>prs</em> (10 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>Primer COB 779 <em>prs</em> (10 µM)</td>
<td>0.1 µl</td>
<td>MWG Eurofins</td>
</tr>
<tr>
<td>My Taq™ DNA Polymerase</td>
<td>0.25 µl</td>
<td>Bioline</td>
</tr>
<tr>
<td>Total</td>
<td>24 µl</td>
<td></td>
</tr>
</tbody>
</table>

The conditions for this PCR were slightly different from routine PCR and are stated in Table 2.9. Here the steps shown in italics were run for 35 cycles.

Table 2.9 Conditions used for multiplex PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturing step</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94°C</td>
<td>0.4 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>53°C</td>
<td>1.15 min</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>1.15 min</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

2.8.2. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and subsequently visualise DNA. A 1% (w/v) or 2% (w/v) (for multiplex PCRs) was made by combining agarose powder (Sigma Aldrich) with 1X TAE buffer and boiling for 1 min in a microwave (Panasonic microwave). The gel cast used varied in volume from 50 ml to 150 ml. A 10X solution of TAE buffer was prepared by combining 40 mM Tris base, 0.114% (v/v) glacial acetic acid and 1 mM EDTA in dH₂O before adjusting the pH to 8.0 with 2M NaOH. This was diluted to 1X TAE in dH₂O before use. Once the gel had cooled sufficiently, SYBR Safe (Invitrogen) was added at a final concentration of 0.01% (v/v), to stain the gel, before pouring it into a gel cast. The gel was allowed to set for 30 min before it was placed into a gel electrophoresis tank and covered with 1X TAE. DNA samples were mixed with 5 µl of loading dye before being added to the gel. Loading dye was prepared by dissolving 40% (w/v) sucrose and 0.25% (w/v) of Bromophenol Blue (Sigma Aldrich) in TE buffer (10 mM Tris-HCl and 1mM EDTA prepared in dH₂O adjusted to pH 8.0). Five microliters of Hyperladder I (Bioline)
was also added to one lane of each gel as a molecular weight marker. This ladder ranged in band sizes from 200 bp to 10037 bp and also displayed band concentrations of 20 ng to 100 ng / band. The gel was run for 1 h at 100V (Consort E132). DNA Gels were imaged using a CCD camera (G:box, Syngene) and imaging software (Syngene) using the SybrGold filter (UV light).

### 2.8.3. Plasmid extraction

One colony of *E. coli* Top10 was inoculated into 5 ml of LB broth supplemented with the appropriate antibiotic required to maintain selection for the specific plasmid. The culture was incubated at 37°C for 16 h. The culture was then centrifuged for 3 min at 6800 RCF. The supernatant was discarded and the pellet was put at -80°C for 2 h prior to plasmid extraction. The plasmid was extracted from the pellet following the instructions with the GenElute Plasmid Miniprep Kit (Sigma Aldrich). The Plasmid DNA was eluted into 30 µl of sterile dH₂O before storing at -20°C.

### 2.8.4. Gel extraction

To extract a specific DNA amplicon from a gel, the PCR product was first run on a 1% agarose gel. A GenElute Gel Extraction Kit (Sigma Aldrich) was used to extract the DNA from the gel as per instructions provided with the kit. The DNA was eluted in 30 µl molecular grade dH₂O (Ambion).

### 2.8.5. PCR product purification

To purify a PCR product or a DNA amplicon from an agarose gel slice, a QIAquick PCR Purification Kit (Qiagen) was used. The manufacturer’s protocol was followed and DNA was eluted into 30 µl of molecular grade dH₂O. To check the concentration of the purified product, the product was run on a 1% agarose gel and compared with the bands of Hyperladder I. Samples were stored at -20°C until required for use.
2.8.6. Genomic DNA extraction and whole genome sequencing (WGS)

Genomic DNA extractions from *L. monocytogenes* were carried out using DNeasy® Blood & Tissue Kit (Qiagen). One colony of desired strain was inoculated into 5 ml of appropriate broth supplemented with an antibiotic if required. The culture was grown overnight for 16 h at 37°C. Two millilitres of the overnight culture was centrifuged at 5000 RCF for 10 min. For *L. monocytogenes* a lysis step was required using and enzymatic lysis buffer composed of 20 mM Tris-HCl (pH 8.0), 2 mM EDTA and 1.2% (v/v) Triton X-100. Lysozyme (20 mg ml⁻¹) was added to the buffer immediately before use. The pellet was resuspended in 180 µl enzymatic lysis buffer and incubated at 37°C for 30 min. Following this incubation step, 25 µl of proteinase K and 200 µl buffer AL (without ethanol) was added to the sample and vortexed to mix. The sample was incubated at 56°C for 30 min. Following this, 200 µl ethanol (95%) was added to the sample and vortexed. From this point the protocol was carried out as per the manufacturer’s guidelines. Samples were eluted in 100 µl of molecular grade dH₂O and stored at -20°C. The concentration of genomic DNA was determined using a Quibit™ Fluorometer (Invitrogen). For sequencing the company required 30 ng of genomic DNA in a volume of 30 – 100 µl molecular grade water. The integrity was checked by running the DNA sample on a 0.75% agarose gel (Section 2.8.2). Genomic DNA samples were sent by overnight courier on ice packs to MicrobesNG for whole genome sequencing (WGS). This company performs all their sequencing using MiSeq and HiSeq 2500 platforms. They perform de novo assembly of the reads using SPAdes and return both the assembled and annotated genome and the raw data. The sequences were visualised using the programme Artemis (Rutherford et al., 2000).

2.8.7. Restriction enzyme digestion

All plasmid digestions were carried out using FastDigest enzymes (Thermo Fisher Scientific). To 12 µl of nuclease free water, 4 µl of plasmid, 2 µl 10x FastDigest buffer and 2 µl of FastDigest enzyme was added. Samples were incubated for 2.5 h at 37°C and the enzyme was then denatured at 65°C for 15 min. Two microliters of the sample was run on a 1% agarose gel to assess the outcome of the digestion.
2.8.8. Phosphatase treatment

To stop the re-ligation of the plasmid to itself, the cut plasmid was treated with Shrimp Alkaline Phosphatase (SAP; Roche) prior to ligation with insert DNA. Following digestion of the plasmid, 1 µl of SAP was added to the digested plasmid. This was incubated at 37°C for 30 min before the enzyme was inactivated by incubating at 65°C for 15 min.

2.8.9. Ligations

For DNA ligations, purified insert and vector were incubated together in 3:1, 2:1 and 1:1 ratio. The ratio was calculated using the following equation.

\[
ng \text{ (insert)} = \frac{ng \text{ (vector)} \times kb \text{ (insert)}}{kb \text{ vector}} \times \frac{ratio\text{(insert)}}{ratio\text{(vector)}}
\]

Ligations were prepared as follows. Two microliters of insert was mixed with 2 µl 10X T4 Ligase Buffer (Thermo Scientific), 1 µl of T4 Ligase (Thermo Scientific), the required volume of vector. The reaction mix was made up to a final volume of 20 µl with molecular grade water. Finally, the reaction was incubated for 16 h at 16°C and stored at -20°C until needed.

2.9. Generation of Mutants

2.9.1. Construction of Δlmo0818, Δlmo0819 and lmo0818ΔPsigB

For the generation of the mutants created during this study, deletion cassettes were designed for each of the mutants required. Each of the cassettes had an EcoRI restriction digestion site added to the 5’ and 3’ ends of the sequence. Sequences of the genes were acquired from the ListiList database (http://genolist.pasteur.fr/ListiList/). The cassettes for Δlmo0818 and Δlmo0819 were designed to include 12 bp of the deleted gene together with 294 bp flanking regions creating deletion cassettes 600 bp in size. lmo0818ΔPsigB was designed slightly differently. This cassette consisted of 39 bp about two thirds the way through the lmo0818 gene with 129 bp upstream and 132 bp downstream of this region. The 39 bp region contained a σB-dependent promoter, of which 5 bp of this sequence were mutated. Mutations were silent so as not to change the amino acid sequence of the Lmo0818 protein. The final
size of this cassette was 300 bp. These sequences were sent to MWG Eurofins for synthesis. When constructed, they came back cloned into the plasmid pEX-A2 (Table 2.2). A plasmid digest was carried out using EcoRI (Section 2.8.7) and the fragment required was excised from the gel and purified as described in Section 2.8.4.

2.9.1.1. Generation of pKSV7::insert

pKSV7 is the shuttle vector that was used to generate all mutants in this study. pKSV7 contains a temperature-sensitive origin of replication and this helps to facilitate selection of homologous recombination events between the deletion cassette and the regions upstream or downstream of the target gene when grown at higher (non-permissive) temperatures. This plasmid was prepared and purified from the strain E. coli DH5α pKSV7 as discussed in Section 2.8.3. The plasmid was digested with EcoRI (Section 2.8.7), and treated with SAP to stop self-ligation (Section 2.8.8). The purified insert carrying the mutation was then ligated into pKSV7 as outlined in Section 2.8.9. The newly ligated plasmid was subsequently transformed into E.coli Top 10 cells to propagate the plasmid (Section 2.6.4). After confirming by PCR using M13 primers (Table 2.7), that the transformation had been successful, pKSV7::insert was purified from E. coli and subsequently transformed into L. monocytogenes EGD-e.

2.9.1.2. Integration

The success of the transformation was again confirmed by PCR using primers specific to the plasmid (Table 2.7). Once candidates were identified they were inoculated into 5 ml BHI broth with 10 µg ml⁻¹ of chloramphenicol. These cultures were grown at 42°C in a water bath for 24-48 h. The cultures were re-cultured 2-3 times until steady growth of the cultures was apparent. This indicated that integration of the plasmid into the chromosome may have occurred. Following this 100 µl of the cultures was serially diluted in PBS and 100 µl of each dilution was plated onto BHI agar supplemented with 10 µg ml⁻¹ of chloramphenicol. To confirm integration of the plasmid, a PCR was carried out using different combinations of primers that were specific to either the chromosome or the plasmid (Fig. 2.3).
Figure 2.3 Schematic representation of how integration of the constructed insert can occur during growth at 42°C, either upstream (a) or downstream (b) of the Wild-type gene. Outside primers (OP1 and OP2) are designed to the chromosome outside of the mutated region, while M13F and M13R are primers specific to pKSV7. These are indicated by the angled arrows and enable PCR to determine which type of integration has occurred. pKSV7::IN denotes the plasmid pKSV7 with the inserted mutation cassette (IN). The square and the circle on the plasmid represent the antibiotic cassette and the temperature sensitive origin of replication respectively.

2.9.1.3. Passaging and mutant confirmation

Once integration was confirmed, the next step was to remove the plasmid from the host chromosome by another recombination step. During this step, either the insert carrying the mutation or the wild-type gene could be removed, depending on which recombination event occurred. Confirmed integrants, were inoculated into 20 ml of BHI broth without antibiotic selection, and grown at 30°C. The culture was passaged every 12 h into fresh media. After the third passage, samples were taken at all subsequent passages and serially diluted in PBS before being spread on BHI agar plates and incubated at 37°C for 24 h. To confirm the excision of pKSV7 from the strain, colonies were streaked onto replica grid plates with and without 10 μg ml⁻¹ chloramphenicol and incubated at 37°C for another 24 h. If a colony
failed to grow on the agar plate containing chloramphenicol, this suggested that the plasmid had been excised from the strain. To confirm that a mutant had been successfully generated, a final PCR was carried out with primers specific to the regions on the chromosome located just outside the deleted region (Fig 2.4). Permanent stocks of each mutant strain were prepared as described in Section 2.1 and these were stored at -80°C until required for further study.

**Fig 2.4 Schematic representation of the excision of pKSV7 by a second recombination step.** After integration, the plasmid must then be removed from the host through a second homologous recombination step. This occurs via growth at 30°C and can occur in two ways. (a) occurs when the plasmid is excised with the Wild-type gene leaving the constructed insert in the chromosome (c). Whilst (b) results in the excision of the Wild-type gene with the constructed insert leading to reversion to the Wild-type genotype (d). Dotted lines indicate the plasmid excision and how it can occur taking either the wild-type gene or mutation cassette and the plasmid is as shown in Figure 2.3.
2.10. *L. monocytogenes* sampling programme

2.10.1. Analysis sites and Food Business Operators (FBO)

Over three years, from April 2013 to December 2015, 10 food processing facilities submitted samples for detection of *L. monocytogenes*. At NUIG, all samples were received from seafood producers. Sampling was also carried out on other sites as part of a nationwide study. These laboratories were Teagasc Food Research Centre Moorepark (TFRCM), University College Dublin (UCD) or University of Limerick (UL). Overall there were 54 food producers that took part in the nationwide study. The majority of these food producers (51) produce ready-to-eat foods. Sample kits were sent to the food producers; each consisting of a polystyrene box (DS Smith, UK), six pre-moistened 3M swabs (Technopath, Ireland), a sterile liquid container (VWR, Ireland), two sterile bags (VWR, Ireland), two cable ties and two ice packs.

Each food producer submitted samples every two months generally consisting of a sample set of six environmental swabs and two food samples. Food processors were given detailed instructions on how to sample and were requested to swab from a drain, a shelf and the floor (an area of approximately 1 m²). Processors were free to choose the location of the remaining swabs, depending on the layout and design of the particular facility. Food samples were requested to be at the point of leaving the facility and were tested following their best-before-date to avoid “Recalls” occurring within the company, which may otherwise have caused producers to withdraw from the project. Liquid samples could also be sent if the producer wished to test brine, water, milk etc. Following sampling, the sample kit was sent by overnight courier to the appropriate laboratory for testing. Sampling at NUIG was conducted on samples from Seafood facilities. Food samples included whole crab, crab meat, BBQ smoked salmon, cold smoked salmon, mussels, clams and lobsters.

2.10.2. Sample analysis

Analysis of samples for the presence of *L. monocytogenes* was performed according to the ISO 11290-1 method, except that only one chromogenic agar was used for the initial isolation (Leong *et al.*, 2014). This method involves two enrichment steps followed by isolation using chromogenic agar plates. When samples were received, they were treated slightly different depending on the type of samples. For environmental swabs, 100 ml of half Fraser broth was added directly into the pouches containing the swabs. Often producers left
the sticks still attached to the swabs so these were also removed if necessary. Food samples, which were of products such as smoked salmon or crab meat were weighed out aseptically in approximately 25 g samples. Whole crabs were difficult to weigh or crack open and therefore the whole crab was added directly to the stomacher bag but was not stomached. For all other food samples, 150 ml of half Fraser broth was added to the sample in a stomacher bag (Seward) and the sample was stomached for 4 min. The sample was then transferred into a 250 ml bottle that had extra half Fraser broth to bring the final volume to 225 ml. Twenty five millilitres of a liquid sample was added to 225 ml of Half Fraser broth in a 250 ml bottle to bring the final volume to 250 ml. The first enrichment in half Fraser broth was incubated for 24 h at 30°C. Following this, 100 µl of half Fraser broth from each of the samples was added to 10 ml full Fraser broth. These were incubated at 37°C for 48 h. Ten microliters of each of the half Fraser broth samples were also inoculated onto a Harlequin chromogenic agar plate. Plates were incubated at 37°C for 48 h before being examined for colonies indicative of L. monocytogenes (blue/green colonies with a halo surrounding them). Following the 2nd enrichment in full Fraser broth, 20 µl of each sample was spread onto a Harlequin chromogenic agar plate. Again these were grown at 37°C for 48 h. Following identification of positive samples, two presumptive positive colonies from each positive plate were streaked onto BHI agar and grown at 37°C for 24 h to purify the culture. Up to four isolates were retained from each positive sample, two from each of the positive enrichment steps. Following purification, permanent stocks were prepared as discussed in Section 2.1. and stored at -80°C for further analysis.

2.10.3. Confirmation and serogrouping of isolates

To confirm and serogroup samples, a multiplex PCR was carried out as described in Section 2.8.1.2. This method was adapted from that described by Doumith et al., 2004.

2.10.4. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to the International Standard PulseNet protocol (PulseNetUSA, 2009) with the restriction enzymes SgsI (formerly AscI) and ApaI, in two separate experiments. Prior to starting the experiment, 1% (w/v) Seakem Gold agarose gel (Lonza) was prepared. This was performed by placing 1.5 ml of 20% (w/v) SDS into a water bath to be pre-warmed at 56°C. To 25 ml of TE buffer, 0.25 g of Seakem Gold agarose powder was added. This was microwaved swirling every 10 s until boiling and fully
dissolved. The agarose was placed into a water bath at 56°C to cool to the appropriate
temperature before preparing the plugs. Just before use the 20% SDS solution was added to
the molten agarose.

Freezer stocks for each of the positive isolates, identified as described in Section 2.10.2.,
were streaked onto BHI agar plates and incubated at 37°C for 24 h. Culture densities were
equalised using a spectrophotometer (Spectronic 20 Genesys) to OD_{600nm} = 1. Four hundred
microliters of this cell suspension was added to a 1.5 ml Eppendorf tube. To this 20 µl of a
20 mg ml^{-1} stock solution of lysozyme was added to the suspension and incubated at 56°C
for 20 min. In the meantime, the plug moulds were assembled according to the
manufacturer’s guidelines. Following the incubation of the sample, the tube was removed
from the water bath and 20 µl of proteinase K (20 mg ml^{-1} stock) was added to the
suspension. The agarose was removed from the water bath and put in a small bowl of warm
water to keep the agarose molten. Four hundred microliters of agarose was added to the cell
suspension and was quickly mixed by pipetting. This mix was then added to the plug mould,
making two plugs for each sample. The plugs were placed in the fridge for 15 min to set.
The plugs were removed from the moulds once they had set and the two plugs for each
sample were added to a 50 ml tube containing 5ml cell lysis buffer (1 M Tris (pH 8.0), 0.5
M EDTA (pH 8.0), 5% Sarcosyl (N-Lauroylsarcosine, Sodium salt), diluted in dH2O) and 25
µl of 20 mg ml^{-1} proteinase K. Lysis was carried out in a shaking water bath at 54°C for 2 h.
Following lysis, the caps of the 50 ml tubes were removed and replaced with plug caps. The
cell lysis buffer was poured out of the tubes and the plugs were washed at 50°C in a shaking
water bath, twice with 10 ml dH2O pre-warmed to 50°C and four times with 10 ml preheated
(50°C) TE buffer for 15 min each. After the final wash, plugs were removed from the 50 ml
tubes and stored at 4°C in 3 ml TE buffer until required.

For digestion of the genomic DNA retained in the plugs, Sgs1 and ApaI were used in
separate experiments. XbaI was used for digestion of Salmonella braenderup plugs, which
were run in three wells on each gel as standards. The restriction digests were prepared as
outlined in Table 2.10.
The plugs were removed from the TE buffer and sliced with a clean scalpel to approximately 2.0-2.5 mm. Any excess TE buffer was soaked up using absorbent tissue. The plugs were added into a 1.5 ml Eppendorf tube and 200 µl of the appropriate restriction enzyme mix was added to each plug, making sure they were fully immersed in the liquid. Digestions were incubated for 2.5 h at 25°C for *ApaI* or at 37°C for *SgsI* or *XbaI*. During this incubation a 1% SeaKem Gold agarose was prepared using 100ml 0.5X TBE (Sigma) for a 10 well gel. This was kept at 56°C until the gel was ready to pour. The chamber was prepared before the gel was cast. For this the frame of the gel was placed into the tank and 2.4 l of 0.5X TBE was added. The temperature of the tank was set to 15°C and allowed to cool. The gel apparatus was cleaned and assembled as per manufacturer’s instructions. To stop the restriction digest, the enzyme solution was carefully removed with a pipette and 200 µl of 0.5X TBE buffer was added and incubated at room temperature for 5 min. After incubation, the plugs were removed from the tubes and placed on the bottom of the teeth of the gel comb. *S. braenderup* plug digests were placed in wells 1, 5 and 10 as standards. Each plug was carefully lined up with the bottom and any excess TBE was removed with tissue before being left to dry on the comb for 10 min. The comb was lined up with the cast and the agarose was carefully poured into the cast so that it covered all the plugs on the comb keeping approximately 2 ml of the agarose for later. The gel was allowed to solidify for 30 min. Once the gel had set, the remaining 2 ml of the agarose was added into each well to seal the gel. This was allowed to set for 5 min. The gel was then removed from the cast and carefully placed into the frame in the electrophoresis chamber and allowed to cool for 10

---

**Table 2.10 Components for restriction digests for PFGE**

<table>
<thead>
<tr>
<th>Component</th>
<th>µl/7 Plug slices + 1 additional test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile dH₂O</td>
<td>1384 µl</td>
</tr>
<tr>
<td>A Buffer (Roche)</td>
<td>160 µl</td>
</tr>
<tr>
<td>BSA (10 mg ml⁻¹; Roche)</td>
<td>16 µl</td>
</tr>
<tr>
<td><em>ApaI</em> (10 U µl⁻¹; Roche)</td>
<td>40 µl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1600 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>µl/7 Plug slices + 1 additional test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile dH₂O</td>
<td>1408 µl</td>
</tr>
<tr>
<td>Cut smart buffer (NEB)</td>
<td>160 µl</td>
</tr>
<tr>
<td>BSA (Roche)</td>
<td>16 µl</td>
</tr>
<tr>
<td><em>SgsI</em> (10 U µl⁻¹; NEB)</td>
<td>16 µl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1600 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>µl/7 Plug slices + 1 additional test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile dH₂O</td>
<td>700 µl</td>
</tr>
<tr>
<td>H Buffer (Roche)</td>
<td>80 µl</td>
</tr>
<tr>
<td><em>XbaI</em> (10 U µl⁻¹; Roche)</td>
<td>20 µl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>800 µl</td>
</tr>
</tbody>
</table>
min. The gel was run using a BioRad CHEF Mapper for 19 h at 15°C at 6 V with a switch angle of 120°. The initial and final switch times were set to 4 s and 40 s respectively to help increase the mobility of the fragments. The sizes of the fragments were programmed to range from 49 kb (low MW) to 450 kb (high MW).

The gel was stained using ethidium bromide. Forty microliters of a 10 mg ml⁻¹ ethidium bromide stock was added to 400 ml of dH₂O. The gel was added to this solution and rocked gently for 40 min. Following this the gel was washed twice with 500 ml dH₂O for 45 min each before being imaged using BioRad Gel Doc 2000 imaging system. Isolate similarity dendrograms were generated using Bionumerics version 7.5 software (Applied Maths, Belgium), using the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimization settings of 1%. Comparisons with pulsotypes from other countries were made using BioNumerics ‘bundles’.

2.11. Microscopy

2.11.1. EGFP visualisation to determine SigB activity

All of the food isolates were transformed with the pKSV7-P<sub>lmo2230</sub>::<sup>e</sup>gfp reporter plasmid (Utratna <i>et al.</i>, 2012) as described in Section 2.6.4. On this plasmid the enhanced Green Fluorescent Protein (eGFP) was fused with the promoter of <i>lmo2230</i>, which is a gene known to be strongly dependent on SigB transcription (Utratna <i>et al.</i>, 2011). Therefore this plasmid when transformed into strains can be used to give a good indication of σ<sup>B</sup> activation in response to a particular stress using the fluorescence produced by eGFP.

For this experiment overnight cultures were grown up in BHI with 10 mg ml⁻¹ chloramphenicol at 37°C for 16 h. The OD of the overnight cultures were equalised to OD<sub>600nm</sub> = 0.6 in 1 ml BHI, supplemented with 10 µg ml⁻¹ chloramphenicol in a 15 ml tube that had been wrapped in aluminium foil to stop additional exposure to light as the eGFP signal can be bleached by light exposure. To fix the eGFP signal, 1 ml of a 1:1 ethanol: methanol mix was added to the cells and the mixture incubated at -20°C for 20 min. The culture was centrifuged at 9000 RCF for 10 min at 4°C. Once the supernatant was discarded, the pellet was resuspended in 1 ml PBS and stored at -20°C for up to 1 month or until use. To image the cells, 5 ml of the fixed suspension was placed on a microscope slide with a cover slip placed on top. Cells were viewed using a GFP filter on a fluorescence microscope (Leica DMI 3000 B) at a magnification of 40x. For each sample, five fields were captured in
both bright field and under fluorescence. The experiment was repeated on three independent biological repeats.

Images were analysed using Image J. Images were captured using the same parameters across the three replicate experiments, which was important for subsequent comparison of fluorescence levels. After each frame was imported into Image J, the background noise was corrected by subtracting the background (Process>Background subtraction). This is carried out using a rolling ball algorithm. The rolling ball radius was set to 5.0 pixels. The threshold of the image was adjusted (Image> Adjust> Threshold) to distinguish objects that were outside the limits of the threshold. These pixels turned white while the rest of the image remained black. The algorithm used to define the threshold was left at default so as to limit user bias during this step. A binary image of this was made once the threshold was defined (Process> Binary> Make Binary). To eliminate the possibility of overlapping cells, a watershed algorithm was applied to separate overlapping elements (Process> Binary> Watershed). Lastly, before analysing the number of particles, the image was despeckled to remove the small speckly particles in the background (Process> Noise> Despeckle). Finally the number of particles were analysed (Analyze> Analyze particles) and a summary box of the results opened. The results could then be copied and pasted into a new excel file for further analysis.

2.12. Bioinformatics

All genomic sequences were firstly viewed using Artemis (Rutherford et al., 2000). Fasta sequences of desired genes were extracted from the whole genome through this browser and transferred to a notepad file. From here they were transferred into Geneious version 10.0.5 (http://www.geneious.com, Kearse et al., 2012). Multiple sequence alignments were performed using this software to identify mutations and compare the similarity between both the nucleotide sequences and the protein sequences.
2.13. Statistical analysis

Statistical analysis of experiments was carried out using GraphPad Prism 6.01. Data was transformed (log10) when necessary and the appropriate test was used to determine the significance of the result. $P$ value equal to or less than 0.05 was considered significant. Examples of tests used include Student’s $t$-test and a one-way Anova.
CHAPTER 3

A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland

Note. The majority of this chapter has recently been published in the joint author paper ‘A 3-year multi-food study of the presence of *Listeria monocytogenes* in 54 small food businesses in Ireland’ (Leong et al., 2017, International Journal of Food Microbiology). The author contribution included sample analysis, serogrouping and PFGE analysis conducted at NUIG and helped with producing some of the figures along with contributing to the writing and editing of the paper.
3.1. Introduction

*Listeria monocytogenes* is an opportunistic pathogen and it is the aetiiological agent responsible for listeriosis cases in humans and a variety of animals. Human listeriosis is linked to the consumption of contaminated food and generally affects pregnant women and immunocompromised individuals, including newborns and elderly people (Scallan *et al.*, 2011). Listeriosis in adults is often manifested as a mild gastroenteritis and in some cases it can lead to more severe symptoms, which produce life-threatening illnesses, including endocarditis, encephalitis or meningitis, and severe sepsis (Vázquez-Boland *et al.*, 2001; Roberts and Wiedmann, 2003). The incidence of human listeriosis is relatively low, however, over the last few years (2008–2014) the number of recorded cases in Europe has significantly increased (EFSA, 2015). Furthermore, those infected by *L. monocytogenes* suffer a mortality rate of 20-30% (Silk *et al.*, 2012), the third highest among all foodborne pathogens (Goulet *et al.*, 2013).

As a foodborne pathogenic bacterium, in addition to being a public health problem, *L. monocytogenes* is of greatest concern to the ready-to-eat (RTE) food industry as there is no cooking or other microbial inactivation steps between production and consumption. As *L. monocytogenes* is a psychrotrophic facultative anaerobe, its occurrence in RTE refrigerated foods is of particular importance, particularly in the elderly population where a three-fold increase in listeriosis has been reported in the UK since the 1990s (Gillespie *et al.*, 2006). It is ubiquitously found in a variety of environments, such as soil, water, animals and humans, and is therefore very difficult to eliminate from the food processing environment. Thus, preventing cross-contamination from the processing environment to food is essential in RTE processing facilities. Regulatory compliance for the RTE food industry is challenging. Testing for *L. monocytogenes* may be expensive for some companies and results can cause product recalls and withdrawals (Gandhi and Chikindas, 2007), but are necessary from a public health perspective.

*L. monocytogenes* can be found in raw products and RTE foods, such as delicatessen meats, soft cheeses or smoked fish (Jensen *et al.*, 2016). Due to its psychrotrophic nature, RTE foods stored at low temperatures are particularly vulnerable to the possibility of growth, and its ability to survive and grow in the presence of food preservation systems, such as low pH and high salt concentrations (Ryan *et al.*, 2008) increase the risks. Any level of contamination could cause a problem if *L. monocytogenes* is able to survive and grow and therefore strict microbiological criteria are applied. In the European Union (EU), in food products intended for infants and for special medical purposes the absence of *L. monocytogenes* in 5 x 25 g of
product is required. For foods capable of supporting growth of *L. monocytogenes*, the food business operator (FBO) must demonstrate (for example, by a challenge study) that the numbers will not exceed 100 CFU/g during the shelf-life of the food. If such data is not available, absence in 5 x 25 g is required. For RTE products not capable of supporting growth of *L. monocytogenes*, the numbers must not exceed 100 CFU/g during shelf-life (EU, 2005). In the United States of America, absence of *L. monocytogenes* is required in all cases, even in food processing environments (FDA, 2008). In Canada, Australia and New Zealand the regulations are similar to those in the EU (HealthCanada, 2011; FSANZ, 2014). Samples positive for *L. monocytogenes* have been reported at retail in fish products, soft, semi-soft and hard cheeses, RTE meat and fresh cut vegetable products (Luber et al., 2011; EFSA, 2015). Indeed, in 2014 the European Food Safety Authority reported the non-compliance of RTE foods at processing and retail, and the proportion of non-compliant units at processing level was considerably higher than at retail (EFSA, 2015).

RTE food processing environments are recognised as an important source of *L. monocytogenes* contamination (Tompkin, 2002). Therefore, it is important for food businesses to have an in situ surveillance programme to monitor and control routes of contamination and cross-contamination in order to limit the risk of *L. monocytogenes* in the final product. Such environmental monitoring programmes are mandatory in the USA (USFDA, 2003) and are recommended in the EU (EU, 2005). These approaches play a crucial role in monitoring, facilitating the identification and tracking of *L. monocytogenes* along the food chain and within food processing facilities, and can have an impact on avoiding cross-contamination to food (Lappi et al., 2004). Whole genome sequencing (WGS) of isolates from such monitoring programmes may facilitate studies on isolate characterisation (Stasiewicz et al., 2015).

Persistence of *L. monocytogenes* in food processing facilities, generally regarded as the repeated isolation of strains with indistinguishable PFGE profiles at intervals of 6 or more months apart (Leong et al., 2014), is of particular relevance. Pathogen monitoring programmes using molecular sub-typing techniques (e.g. PFGE or WGS) may be helpful in identifying persistent isolates within food processing facilities (Fox et al., 2015). The aim of this study was to assess the occurrence and persistence of *L. monocytogenes* in 54 Irish food processing facilities over a three-year period (March 2013 to December 2015), through regular monitoring of ready-to-eat foods and processing environments, followed by the molecular characterization of the *L. monocytogenes* strains isolated, whilst making FBOs more aware of the issues relating to the organism. The isolates obtained were compared to
other food processing and clinical isolates. The application of this approach is discussed as a means of improving food safety in the processing environment and protecting public health.

3.2. Results

3.2.1. Isolation and characterisation of L. monocytogenes food and environmental isolates at NUIG

During the course of this 3 year project, 886 samples were tested at NUIG over 18 different sampling dates. All samples tested at NUIG were from the seafood sector. The study started with ten companies in 2013 but was reduced to seven by the end of the study. Of all samples tested, 1.67% (15 samples) were positive for L. monocytogenes (Table 3.1). Thirteen of these were isolated from environmental swab samples and two were isolated from food products – smoked salmon and crab meat. 2014 had the lowest incidence rate with only two samples isolated during this period compared to five isolated in 2013 and seven in 2015. These samples were serogrouped into two groups, with 14 being in the 1/2a serogroup and one was 4b. To further characterise these strains PFGE was carried out on the isolates (Section 2.10.4). For each positive sample up to four colonies had been retained, two from the first enrichment and two from the second. Each of these were characterised by PFGE. It was found that the 15 positive samples were characterised by 11 different PFGE pulsotypes. For one sample, two different pulsotypes were detected from the two different enrichment steps. Pulsotype P64 was shown to persist in NUIG samples but all others were not considered to be persistent although they were persistent in the wider study (Table 3.1)
Table 3.1 Summary of environmental samples and food samples analysed at NUIG from 2013 – 2015. Asterix indicate that the pulsotype was persistent during the course of the whole study. Plus symbol (+) indicates that pulsotype was persistent in samples taken by NUIG

<table>
<thead>
<tr>
<th>Month</th>
<th>Year</th>
<th># Negatives</th>
<th># Positives</th>
<th>Source</th>
<th>Serogroup</th>
<th>Pulsotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>2013</td>
<td>62</td>
<td>0</td>
<td></td>
<td>1/2a -3a</td>
<td>P64*</td>
</tr>
<tr>
<td>June</td>
<td>2013</td>
<td>53</td>
<td>1</td>
<td>Drain swab</td>
<td>1/2a -3a</td>
<td>P64*</td>
</tr>
<tr>
<td>August</td>
<td>2013</td>
<td>52</td>
<td>2</td>
<td>Drain swab</td>
<td>1/2a -3a</td>
<td>P45*</td>
</tr>
<tr>
<td>October</td>
<td>2013</td>
<td>51</td>
<td>3</td>
<td>Drain swab</td>
<td>1/2a -3a</td>
<td>P61*</td>
</tr>
<tr>
<td>December</td>
<td>2013</td>
<td>45</td>
<td>0</td>
<td></td>
<td>1/2a -3a</td>
<td>P30</td>
</tr>
<tr>
<td>February</td>
<td>2014</td>
<td>43</td>
<td>1</td>
<td>Smoked salmon</td>
<td>1/2a -3a</td>
<td>P61*</td>
</tr>
<tr>
<td>April</td>
<td>2014</td>
<td>62</td>
<td>0</td>
<td></td>
<td>1/2a -3a</td>
<td>P61*, P74</td>
</tr>
<tr>
<td>June</td>
<td>2014</td>
<td>53</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>August</td>
<td>2014</td>
<td>54</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>October</td>
<td>2014</td>
<td>52</td>
<td>1</td>
<td>Floor swab</td>
<td>1/2a -3a</td>
<td>P64*, P74</td>
</tr>
<tr>
<td>December</td>
<td>2014</td>
<td>45</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>2015</td>
<td>51</td>
<td>1</td>
<td>Floor swab</td>
<td>1/2a -3a</td>
<td>P64*</td>
</tr>
<tr>
<td>April</td>
<td>2015</td>
<td>44</td>
<td>2</td>
<td>Drain swab</td>
<td>1/2a -3a</td>
<td>P44*</td>
</tr>
<tr>
<td>June</td>
<td>2015</td>
<td>52</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>August</td>
<td>2015</td>
<td>53</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>October</td>
<td>2015</td>
<td>54</td>
<td>3</td>
<td>Belt swab</td>
<td>1/2a -3a</td>
<td>P59*</td>
</tr>
<tr>
<td>December</td>
<td>2015</td>
<td>45</td>
<td>1</td>
<td>Pooled water swab</td>
<td>1/2a -3a</td>
<td>P6*</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>871</td>
<td>15</td>
<td></td>
<td>14 - 1/2a -3a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 Food samples</td>
<td>1 - 4b – 4d – 4e</td>
<td></td>
</tr>
</tbody>
</table>
3.2.2. \textit{L. monocytogenes} overall occurrence

In total, 5869 samples from 54 food processing facilities were analysed for the presence of \textit{L. monocytogenes} from 2013 to 2015. This included 4667 processing environment samples and 1202 food samples (Table 3.2). The average number of samples submitted by each food processing facility was 108.7 (Standard Deviation 29.6). Ten food processing facilities maintained a 0\% \textit{L. monocytogenes} prevalence over the three years of sampling; these included one dairy facility, three meat facilities and six seafood facilities. Thirty-two food processing facilities had an overall occurrence between 0 and 5\%, seven between 5 and 10\% and five between 10 and 20\% (Appendix 1).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
 & 2013 & 2014 & 2015 & Total \\
\hline
No of samples taken & 1696 & 2102 & 2071 & 5869 \\
No. of positive samples & 81 & 78 & 67 & 226 \\
\% of positive samples & 4.78\% & 3.71\% & 3.24\% & 3.85\% \\
No. of environmental samples taken & 1345 & 1654 & 1668 & 4667 \\
No. of positive environmental samples & 63 & 57 & 56 & 176 \\
\% of positive environmental samples & 4.68\% & 3.45\% & 3.36\% & 3.77\% \\
No. of food samples taken & 351 & 448 & 403 & 1202 \\
No. of positive food samples & 18 & 21 & 11 & 50 \\
\% of positive food samples & 5.13\% & 4.73\% & 2.73\% & 4.17\% \\
\hline
\end{tabular}
\caption{Summary of results of the annual occurrence of \textit{L. monocytogenes} in processing environments and food obtained from 54 food businesses in Ireland.}
\end{table}

Scatter plots in Figure 3.1 show the annual occurrence of \textit{L. monocytogenes} in processing facilities in the four different food sectors. In the dairy and meat sectors, the average value and the amount of variation decreased over time. In the seafood sector, the occurrence was relatively low and apart from one facility in 2015, there was little difference between 2013 and 2015, although there was a decrease in 2014. There were a low number of vegetable processing facilities involved, but the occurrence was relatively high. The number of facilities with zero occurrences increased over time. The overall prevalence of \textit{L. monocytogenes} decreased from 4.8\% in 2013 to 3.7\% in 2014 and 3.2\% in 2015. The prevalence of \textit{L. monocytogenes} in food samples decreased from 5.1\% in 2013 to 4.7\% in 2014 and 2.7\% in 2015 (Table 3.2). Overall, 29 companies showed a decrease in occurrence between 2013 and 2015 and 13 showed an increase in occurrence from 2013 to 2015.
Figure 3.1. Scatter plots of the annual occurrence of *L. monocytogenes* in processing facilities in the four different food sectors. The line in each plot is the average. Each symbol represents a facility, with different symbols for each year.

There was an uneven geographical distribution of participants in the surveillance programme across the country (Appendix 2). Considering this limitation, no geographical differences could be determined in the distribution of *L. monocytogenes*. Any differences observed could be due to the different number of samples received from the different locations. Additionally, no seasonal difference was observed in the occurrence of *L. monocytogenes* over the three years (data not shown).

Different food sectors had differing rates of occurrence in samples (p<0.05). Including food and processing environment samples, the industry with the lowest prevalence was the seafood industry, in which 1.73% of 1621 samples were positive for *L. monocytogenes*. The dairy industry had 3.7% *L. monocytogenes* positives from 1920 samples and the meat industry had 4.28% *L. monocytogenes* positives from 1681 samples. The highest processing environment prevalence occurred in the vegetable industry with 9.5% of 474 samples positive for *L. monocytogenes* as opposed to 4.1% in both the dairy and meat environmental samples and 1.6% in the seafood environmental samples. Positive food samples were obtained from all food sectors (Appendix 1, Table 3.3).
Table 3.3. Breakdown of the occurrence of *L. monocytogenes* in processing environments and food by food sector obtained from 54 food businesses in Ireland over three years

<table>
<thead>
<tr>
<th>Food Category</th>
<th>No. Process Environment Samples</th>
<th>% Positive</th>
<th>No. Food Samples</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy</td>
<td>1512</td>
<td>4.2</td>
<td>408</td>
<td>2.2</td>
</tr>
<tr>
<td>Meat</td>
<td>1332</td>
<td>3.5</td>
<td>349</td>
<td>7.5</td>
</tr>
<tr>
<td>Seafood</td>
<td>1349</td>
<td>1.6</td>
<td>272</td>
<td>1.8</td>
</tr>
<tr>
<td>Vegetables</td>
<td>474</td>
<td>9.5</td>
<td>173</td>
<td>5.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4667</strong></td>
<td><strong>3.8</strong></td>
<td><strong>1202</strong></td>
<td><strong>4.2</strong></td>
</tr>
</tbody>
</table>

3.2.3. Selection of isolates for characterisation

Ten percent of the positive samples yielded more than one PFGE pulsotype. When all four isolates from the same sample belonged to the same PFGE pulsotype, only one isolate was carried forward for further study. If differing pulsotypes were seen from the same positive sample, a representative isolate of each pulsotype was carried forward. Only isolates which were confirmed as *L. monocytogenes* by multiplex PCR were retained for further study. This approach yielded 255 isolates from 226 positive samples.

3.2.4. Serogrouping and serotyping

Multiplex PCR was performed to serogroup all 255 isolates (Section 2.10.3 & Section 2.8.1.2), resulting in 43.9% of isolates in the 1/2a-3a serogroup, 27.5% of the isolates in the 4b-4d-4e serogroup, 16.1% of the isolates in the 1/2b-3b-7 serogroup and 12.2% of the isolates in the 1/2c-3c serogroup (Table 3.4). Serotyping was also performed on 110 of these isolates; all isolates in each serogroup belonged to a single serotype (Table 3.4). The serotypes 4b and 4e cannot currently be differentiated with the available antisera. All isolates, except one untypeable isolate, belonged to lineage I (111 isolates) or lineage II (143 isolates) (Orsi *et al.*, 2011).
Table 3.4. Serogroup/serotype testing of the *L. monocytogenes* isolates obtained from a 3-year surveillance programme of processing environments and food obtained from 54 food businesses in Ireland. Serogroup testing was carried out according to Doumith *et al.* (2004). Serotype testing was conducted using antisera from Denka Seiken UK Ltd, Coventry, UK.

<table>
<thead>
<tr>
<th>Serogroup testing</th>
<th>Serotype testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serogroup</td>
<td>No. of isolates</td>
</tr>
<tr>
<td>1/2a-3a</td>
<td>112</td>
</tr>
<tr>
<td>1/2b-3b-7</td>
<td>41</td>
</tr>
<tr>
<td>1/2c-3c</td>
<td>31</td>
</tr>
<tr>
<td>4b-4d-4e</td>
<td>70</td>
</tr>
<tr>
<td>Untypeable</td>
<td>1</td>
</tr>
<tr>
<td>Total number of isolates tested</td>
<td>255</td>
</tr>
</tbody>
</table>

3.2.5. PFGE

Pulsotype numbers (P numbers) were assigned to PFGE pulsotypes based on their relatedness (Section 2.10.4). The 255 isolates were assigned to eighty-six different pulsotypes. Several pulsotypes occurred in multiple food sectors, but only three pulsotypes, P44, P46 and P59 occurred in all industry sectors (Fig. 3.2).
Figure 3.2. Distribution of the different *L. monocytogenes* pulsotypes obtained from different food sectors in the processing environments and food obtained from 54 food businesses in Ireland. The asterix indicate that these pulsotypes were persistent (isolated more than once at least 6 months apart in a single facility).

Overall, there was great diversity in the isolates obtained, as seen in the minimum spanning tree (Fig. 3.3). The majority of pulsotypes (69/86) were not seen to persist at a given facility and are likely to represent an incidence of sporadic contamination rather than persistent contamination. Except for 2 cases, all strains within a single pulsotype belonged to the same serogroup.

The distribution of the pulsotypes around the country can be seen in Figure 3.4. From these data it is clear that certain pulsotypes are prevalent in Ireland and persistent isolates are found in each of the four food sectors included in the study.
Figure 3.3. Minimum spanning tree of *L. monocytogenes* pulsotypes obtained from processing environments and food from multiple food sectors from 54 food businesses in Ireland. This was created in Bionumerics (version 7.5) using default settings except maximum distance between nodes in the same position of 12. Pulsotypes containing 10 or more strains are identified.
Figure 3.4. Summary of the *L. monocytogenes* pulsotypes detected in each country throughout Ireland. Unique pulsotypes are in black, persistent pulsotypes are in red, persistent pulsotypes found at multiple locations are in red and underlined, pulsotypes found in multiple sites but are not persistent are in blue. D – dairy; M – meat; S – seafood; V – Vegetable; U – untypeable strain.
3.2.6. Persistence

PFGE analysis also allowed for the identification of persistent strains, defined as indistinguishable strains (by PFGE analysis) isolated at least six months apart from the same processing facility (Fig. 3.5). Sixteen processing facilities had at least one persistent *L. monocytogenes* strain over the three-year period. Seventeen different pulsotypes were observed as being persistent. Five pulsotypes were observed to persist in multiple facilities; P59 in two facilities, P6 in two facilities, P10 in three facilities, P32 in three facilities and P44 in three facilities. Cases of persistence decreased in several facilities over the three-year sampling period. In six facilities, (D16, M1, M3, M7, M8 and M10) persistence was observed in 2013 and/or 2014 but no persistence was observed in 2015 (Appendix 1).

![Figure 3.5. Examples of *L. monocytogenes* pulsotypes, persistent for at least 6 months within a single facility, isolated from processing environments and food obtained from 54 food businesses in Ireland. FC - food contact area, NFC – non-food contact area.](image)

3.2.7. Comparison with pulsotypes of clinical isolates

From 2010 to 2015, there were 25 *L. monocytogenes* clinical isolates obtained at the National Salmonella, Shigella and Listeria Reference Lab (NSSLRL). The PFGE profiles of the isolates from the current study were compared with these 25 clinical isolates using Bionumerics. Eleven of the industry/food pulsotypes showed close similarity (>85% similarity as calculated by Bionumerics) with the clinical pulsotypes (Fig. 3.6). These 11 pulsotypes were identified in 26 facilities and were found in both processing environment and food samples from all sectors. Seven of these 11 pulsotypes were identified as persistent...
in one (P2, P31, P45 and P48) or several (P6, P32, P44) facilities, and one of them (P44) occurred in all food sectors.

Figure 3.6. Dendrogram showing pulsotypes of clinical isolates in common with strains isolated from food and food processing facilities.

3.2.8. Comparison with pulsotypes identified internationally

The 86 pulsotypes obtained were compared with pulsotypes obtained in 5 other countries, including United States of America, Australia, United Kingdom, France and Romania. Of the 86 pulsotypes obtained in Ireland, 32 were seen internationally, with 11 of these being persistent in this study. P44, P46 and P59, which were obtained in all food sectors in this study, were also seen in at least one other country.

3.2.9. Evidence of cross-contamination from the processing environment to food

There was evidence of cross-contamination between the processing environment and food (indistinguishable pulsotypes found in processing environment samples and food samples) at 12 facilities, representing all food sectors (Appendix 1). In two cases, transfer of more than one pulsotype occurred. The cross-contamination included seven different persistent
pulsotypes (P6, P31, P32, P33, P44, P45 and P59) and five sporadic pulsotypes (P10, P17, P21, P61 and P67).

3.3. Discussion

Many of the studies available in the literature on *L. monocytogenes* occurrence/surveillance were performed at a single facility over time or at a single time-point in many facilities. Such surveys provide an important perspective on the problem of *L. monocytogenes* occurrence. However, the lack of long term continuity and the use of sampling and analytical methods which vary from one study to the next limit their impact. Structured continuous surveillance with some degree of standardisation of methods, as undertaken in this study, is necessary to establish valid conclusions on occurrence and persistence over time.

During the course of this study, the overall occurrence of *L. monocytogenes* in the processing environment of the 54 facilities decreased from 4.7% in 2013 to 3.4% in 2015, while in the foods tested, the decrease was from 5.1% to 2.7%. All companies included in the study submitted samples in all three sampling years, and while there was variability in occurrence among individual facilities (29 showed a reduction in occurrence from 2013 to 2015, 12 showed no change between both years and 13 showed an increase between 2013 and 2015), a trend towards a reduction in occurrence over the sampling period was observed. Over the course of the study, the results of analyses were given to the food business owners every two months, explaining the significance of the results, and a series of workshops were undertaken with the aim of upskilling and further informing the FBOs about *L. monocytogenes*. Additionally, the food business owners or their quality staff were responsible for taking the samples. It is considered that this also helped build awareness and understanding. A trend towards a decrease in *L. monocytogenes* persistence over the 3-year period observed in the majority of facilities (e.g. facilities D16, M1, M3, M7, M8 and M10) indicated that the approach of surveillance combined with heightened awareness can have an impact on good management practices and can contribute to reducing *L. monocytogenes* occurrence (Hoffman et al., 2003; Lappi et al., 2004). The decrease in *L. monocytogenes* occurrence and persistence in food processing environments observed over the 3-year period in the current study contributed to a reduced risk of cross-contamination to food, which was reflected in the reduced occurrence in food observed in 2015. The fact that no attempt was made to “police” the sampling regime, may have biased the positive rate for some facilities making it difficult to establish with certainty if increasing the sampling regime produced a positive effect on the incidence of *L. monocytogenes* in the food industry.
Previous studies have shown the occurrence of *L. monocytogenes* in various food sectors. In smoked fish, a previous study showed that 25 out of 90 food samples were positive for *L. monocytogenes*, of which four exceeded the level of 100 CFU/g (Uyttendaele et al., 2009). In another study of raw and smoked fish and processing environments (over 1,000 samples tested), *L. monocytogenes* was isolated from 3.8% of the raw fish samples (0 to 10%, depending on the plant), and 1.3% of the finished product samples (Thimothe et al., 2004). Different fish type/species may have contributed differently to the occurrence of *L. monocytogenes* in the processing environment, as well as the time of year and the turnover in the processing plant at a given time (Fonnesbech Vogel et al., 2001). In this study 1.8% of 272 food samples from seafood processors were positive for *L. monocytogenes*. The occurrence is considerably lower than that reported in the recent EU baseline survey, where the average EU occurrence was about 10% (EFSA, 2013).

It has been documented previously that dairy farms can be a source of *L. monocytogenes*, either in animal faeces or the wider farm environment, at a prevalence of about 20% (Nightingale et al., 2004) or in bulk tank milk at a prevalence of 1-12% (Oliver et al., 2005). These bacteria can be subsequently disseminated to the milk processing environment, where contamination of milk and dairy products can occur. Post-pasteurization contamination of dairy products with *L. monocytogenes* occurs during the processing, packaging and storage of food. Studies have reported varying levels of contamination of dairy products, ranging from 0.47 to 7% (Fox et al., 2009; EFSA, 2013). The occurrence of 3.7% of *L. monocytogenes* in those dairy samples analysed (totalling 1,920 samples, recovered from food and the processing environment) in this study is in line with similar values in the dairy sector, as reported by others.

Extensive testing of RTE meat in the US over a 10 year period (1900-1999) revealed that contamination by *L. monocytogenes* varied according to meat product type (Levine et al., 2001), while the prevalence in production environments can vary from 0% to over 14% (Rivera-Betancourt et al., 2004). Thus, the reported prevalence of 3.5% in the Irish meat industry (1332 samples tested) is relatively low compared to other studies, although the average occurrence of 7.5% (349 samples tested) in food samples is surprising. The high occurrence is due, in part, to three meat processing facilities that did not produce RTE meat, but were included in the surveillance programme because they were considered relevant due to the risk of cross contamination. The occurrence at these facilities was 8.1%, 10.8% and 20.0%.
The largest disease outbreak related to *L. monocytogenes* occurred in the U.S.A. in 2011 and was associated with consumption of contaminated cantaloupe (Laksanalamai *et al.*, 2012), highlighting the risks associated with fruit and vegetables. Several publications on *L. monocytogenes* on fruit and vegetables report contamination of around 2% or lower (Kovacevic *et al.*, 2013; Sant’Ana *et al.*, 2014), although some reports indicate higher contamination (Cordano and Jacquet, 2009). In the present study, the vegetable processing environment was the most highly contaminated, and had the greatest diversity of pulsotypes. This may reflect the ubiquitous nature of *L. monocytogenes* and its association with soil (Weis and Seeliger, 1975). Cross-contamination of *L. monocytogenes* from the vegetable processing environment to the produce was seen, and, at 5.8% occurrence, vegetable contamination was the second highest identified.

A large variability existed in *L. monocytogenes* occurrence among the food businesses. Thus, whilst *L. monocytogenes* occurrence was observed at above 10% for 5 facilities, 10 facilities showed a 0% occurrence over the 3-year period. It is tempting to speculate that appropriate management and hygiene practices implemented in each of these latter facilities may have contributed positively to the *L. monocytogenes*-negative status. This finding warrants further study to explore the nature of the microbiome that may have existed and which potentially could have contributed to this observation (Hoelzer *et al.*, 2012; Fox *et al.*, 2014). It is well recognised that changes in the occurrence of *L. monocytogenes* in food production facilities are often associated with certain types of intervention. For example, in one facility there was an increase in occurrence from 5 to 23% between the years 2013 to 2014, while in another a decrease from 14 to 0% was recorded. The former coincided with the installation of new equipment, while the latter was attributed to the introduction of a 0.5% peracetic acid rinse in the cleaning protocol, suggesting that management practices can influence occurrence of *L. monocytogenes* in processing facilities.

All the isolates obtained during this surveillance were characterised by molecular methods, which allowed for the identification of patterns of contamination, which were reported to the food producer when they were available. The fact that 80.2% of the 86 distinct pulsotypes isolated along the food chain were classified as non-persistent indicates the diversity of strains that exists in the food processing environment. Continuous sporadic contamination identified in a processing facility could be regarded as an indication of a breakdown in hygiene protocols and therefore, addressing such an issue, from a food safety standpoint, may reduce the risk of the food products being contaminated.
To address persistent contamination requires a different approach than that required to address sporadic contamination. The identification of persistent strains may be a symptom of process control failures or resistance of the strains to the cleaning methods used, and therefore the strains continue to exist in the manufacturing facilities. Based on the PFGE patterns and the definition used in this study, 16 facilities showed persistent contamination, while 28 facilities showed sporadic contamination but no persistent strains. All facilities which showed persistent contamination were also seen to have sporadic contamination. This would indicate a general need for updating both the cleaning procedures, with the aim of eliminating persistent strains, and the hygiene barrier systems, with the aim of preventing initial and sporadic contamination events.

Seventeen out of the eighty-six distinct pulsotypes identified along the food chain in the current study were considered persistent, according to the definition of persistence previously given. The failure to find other persistent pulsotypes does not necessarily indicate their inability to persist in the environment but could also reflect their lower relative abundance in the environment or even the existence of limitations/inconsistencies in the sampling regimes used. More extensive sampling could have resulted in the repeated isolation of strains that were isolated infrequently following the current sampling approach.

The occurrence of apparently persistent strains could also be due to re-contamination of environments from the exterior of the processing facility. However, if that was the case, persistence would have occurred in the external source. Persistence in food facilities is thought to primarily arise because of the availability of suitable sites (so-called harbourage sites) within the facility that can sustain a population, in combination with the genetic properties of particular strains that allow them to colonise those sites, e.g. sanitizer resistance, ability to use different carbon sources, ability to form biofilms, etc. (Carpentier and Cerf, 2011). In the current study, several pulsotypes were considered as persistent in more than one facility, which suggests that strains belonging to those pulsotypes may have some superior survival or colonization abilities in comparison to strains from other pulsotypes. Whole genome sequencing analysis and further phenotypic characterisation of these strains may help confirm whether this is the case. On the other hand, the identification of persistent pulsotypes common to several facilities might be due to the higher relative abundance of those pulsotypes in the environment.

Cross contamination from the processing environment to food has been previously reported and indeed has been shown to be the cause of disease outbreaks (Pérez-Rodríguez et al., 2008; McCollum et al., 2013). Evidence of cross-contamination was seen in 12 of the 54
facilities in this study where indistinguishable pulsotypes were seen in both food and processing environment samples. This could be cross-contamination from the processing environment to the food or vice versa. Further research would be required to distinguish between these two scenarios. Furthermore, as this was a general study on occurrence, rather than one focused on contamination events, the number of food samples (about 36 from each company over 3 years) may not have been high enough to draw conclusions on sources of contamination.

From the 255 isolates included in this study, 43.1% of isolates belonged to lineage I and 56.1% belonged to lineage II. This is in general agreement with other studies where lineage I and II isolates are found frequently and lineages III and IV isolates are rarely found (Orsi et al., 2011; Chenal-Francisque et al., 2013). Serotypes identified in this study are in line with the general prevalence of serotypes found in the processing environment. Namely, the highest prevalence of 1/2a strains, followed by 4b, 1/2b and 1/2c (Todd and Notermans, 2011). Because of the ease of analysis, serogrouping by PCR is more frequently undertaken than serotyping through the use on antisera, yet there is little information correlating serogroup with serotype. In this study, 100% of isolates in serogroups 1/2a-3a, 1/2b-3b-7, 1/2b-3b and 4b-4d-4e belonged to serotypes 1/2a, 1/2b, 1/2c and 4b-4e, respectively. Similar results were obtained previously, indicating validity in serogroup rather than serotype analysis as a single serotype is significantly overrepresented in each serogroup (Murugesan et al., 2015). The fact that, in two cases, strains within a single pulsotype belonged to different serogroups highlights the need to use more than one method of typing to help counteract errors which would arise from using only a singular typing method.

*L. monocytogenes* strains have the ability to cause foodborne disease and indeed some strains show a variable ability to cause disease. Several reports have described apparently avirulent *L. monocytogenes* strains with polymorphisms in the *inlA* gene leading to a truncated non-functional protein (Chen et al., 2011b). The comparison of the 255 isolates with the 25 clinical isolates from Ireland showed that 11 pulsotypes from the food/processing environment surveillance matched 11 of the pulsotypes of clinical isolates. Seven of these 11 pulsotypes were persistent, and eight of them were found in several facilities, among these was pulsotype P44, which was found in all four food categories and showed a PFGE pattern indistinguishable from that of a blood isolate obtained from a patient in 2012. This strain was repeatedly found in the food chain over the entire three-year period of the study (2013-2015). This indicates that some strains frequently present in food processing environments, are capable of persisting and contaminating food products and are closely related to strains capable of causing disease. Further investigation of these...
pulsotypes through whole genome sequencing analyses and phenotypic characterisation may reveal further information on their virulence traits.

Global clones of *L. monocytogenes* are known to exist (Chenal-Francisque *et al.*, 2013). The results of this study support the existence of global clones as 32 of the 86 pulsotypes seen were also identified in other countries, 11 of which were seen to persist in the food processing environment. The significance of such global clones in terms of clinical cases is not clear, although 10 of the pulsotypes identified internationally also appeared as clinical isolates. The movement of strains into and out of Ireland is likely facilitated by an open economy where there is a continuous large scale movement of goods and people.

In conclusion, this 3-year study has shown the prevalence of *L. monocytogenes* in 54 food processing facilities in Ireland and highlighted the diversity of *L. monocytogenes* strains that exist in the food sector, particularly in the vegetable sector. The overall rates of occurrence in food and food processing environments are broadly in line with reports from other countries. The finding that many of these strains have similar PFGE profiles to clinical isolates highlights the public health risk that this pathogen presents. The awareness and vigilance created by an extensive 3-year surveillance programme can contribute to a reduction of *L. monocytogenes* in food and food processing environments, leading to a decreased risk to public health.
CHAPTER 4

Phenotypic characterisation of food isolates from seafood facilities in Ireland
4.1. Introduction

*L. monocytogenes* is a robust foodborne pathogen capable of surviving over long periods of time in food processing environments (Dauphin *et al.*, 2001; Autio *et al.*, 2003; Lundén *et al.*, 2003; Leong *et al.*, 2017). By subtyping strains isolated from food processing facilities, it is possible to identify persistent isolates from non-persistent isolates. Strains shown to be the same molecular type through subtyping methods such as PFGE are assumed to be the same clone and this can help determine if a strain is persisting in the processing environment or if cross contamination between the industry environment and food product has occurred. Persistence of this bacterium has been loosely defined as re-isolation of a particular clone over a long period of time (Carpentier and Cerf, 2011; Ferreira *et al.*, 2014; Larsen *et al.*, 2014). The amount of time defining persistence varies between studies with some defining it as the re-isolation of the same isolate multiple times in a two month period, whilst others have defined it as the re-isolation over years (Dauphin *et al.*, 2001; Autio *et al.*, 2003; Lundén *et al.*, 2003; Carpentier and Cerf, 2011; Ferreira *et al.*, 2014; Larsen *et al.*, 2014; Magalhães *et al.*, 2016). In this study, persistence was defined as a sub-type (characterised by PFGE) that was re-isolated with an interval of at least twice at least six months apart between isolation events (See Section 3.1). It has been disputed as to whether strains of *L. monocytogenes* ‘persist’ within niches or harbourages within the processing environment, or whether they are simply reintroduced back into the environment by personnel, or through product or equipment (Hansen *et al.*, 2006; Lomonaco *et al.*, 2009; Chen *et al.*, 2010b; Carpentier and Cerf, 2011). It is also possible that a non-persistent isolate may become persistent in a different environment under different conditions (Kastbjerg and Gram, 2009; Carpentier and Cerf, 2011). It can be difficult to identify persistent isolates by sampling processing sites as only a sub-sample of the population in the environment is captured. This means it possible that sub-types of *L. monocytogenes* may be persisting in the processing environment and may only be isolated sporadically from the site through the sampling process (Ferreira *et al.*, 2014; Larsen *et al.*, 2014).

Different studies have tried to identify characteristics that facilitate the survival of *L. monocytogenes* in harsh conditions encountered in food processing facilities (Lundén *et al.*, 2003; Lundén *et al.*, 2008; Lourenço *et al.*, 2009; van der Veen and Abee, 2010; Magalhães *et al.*, 2016). However, it is disputed as to whether a genotype exists that contributes to persistence of this microorganism (Carpentier and Cerf, 2011). $\sigma^B$ has been shown to help *L. monocytogenes* overcome some stresses it may encounter within food processing environments (O'Byrne and Karatzas, 2008; van der Veen and Abee, 2010; NicAogáin and
O’Byrne, 2016). These conditions are often tested when examining differences between persistent and non-persistent isolates. Lundén et al. (2008) investigated whether there were differences between persistent and non-persistent strains in response to heat shock and acidic conditions. They concluded that it was likely that no one factor contributed to persistence, but instead a range of factors allowed the bacterial cells to persist within a food processing environment (Lundén et al., 2008). Growth of persistent and non-persistent isolates exposed to different temperatures, salt concentrations and pH were also tested by Magalhães et al. (2016). They observed some differences between the lag time and growth rate of the persistent and non-persistent cells in response to different NaCl concentrations and weak acidic conditions. They also looked at the response of the strains to the commonly used sanitizers, benzalkonium chloride and hydrogen peroxide, but as reported in other studies they found no significant differences between the two groups of strains (Magalhães et al., 2016).

Strains isolated from the work detailed in Chapter 3 of this thesis were selected for phenotypic characterisation. This chapter describes characterisation of both environmental and food isolates of interest under a range of different conditions to try identify if there is a difference between the survival of some strains over others under harsh conditions. We also compare the isolates from the surveillance study to the lab strain 10403S to investigate if strains adapted to the food environment can grow or survive better under conditions associated with this environment. Strains characterised as being genetically related through PFGE were compared to examine whether they behave the same when under stress. The activity of σ^B was also investigated and compared between isolates to determine if this contributed to survival in processing facilities.

4.2. Results

4.2.1. Isolates chosen for characterisation

From the 15 strains isolated in chapter 3 at NUIG, six isolates of interest were chosen for further characterisation. These isolates are shown in Table 4.1 and will be referred to as food isolates throughout this chapter. Different assays were used to attempt to characterise the isolates and investigate if the strains displayed any distinguishing phenotypes that enabled them to overcome different stresses encountered in the food processing environment. Of these strains, some had the same pulsotype when tested by PFGE (Section 2.10.4) and the same serogroup determined by multiplex PCR (Table 4.1; Section 2.8.1.2; Table 2.8).
13 and 1_02-15 were characterised as a persistent clone isolated from FBO 1 at the beginning and end of the project whilst 2_10-13 and 2_02-14 were isolated 4 months apart at FBO 2 and so were characterised as a non-persistent clone (based on definition of persistence given in Section 4.1). This isolate from FBO 2 was chosen as an interesting strain as it demonstrated an example of cross contamination from the processing environment to food. 2_10-13 was isolated from an environmental swab sample taken from a drain in October 2013. In December of the same year, this company experienced problems with contamination of food products that led to a product recall for the company. Following this, in February 2014, 2_02-14 was isolated, through this study, from a smoked salmon product from the same producer. PFGE was carried out to confirm that it was the same clone. The results were compared with samples taken by an external lab during the course of the recall and it was confirmed that the isolate had the same PFGE profile. This suggests that cross contamination from the food processing environment can lead to contamination of the food product produced by that facility and therefore presented an interesting clone for characterisation.

2_08-13 and 5_04-15 were also included for characterisation as although they had only been isolated on a single occasion during testing at NUIG (August 2013 and April 2015 respectively), when the data was examined from the wider study, it was found that both of these strains had persisted within another food processing facility and therefore were included as other persistent isolates. Interestingly, 5_04-15 represented the persistent pulsotype, pulsotype 44 (P44), which was isolated from all food sectors included in the surveillance study (See Fig. 3.2). All experiments conducted in this study included 10403S and 10403S ΔsigB as lab comparisons as they were characterised as being more closely related to the food isolates than EGD-e through both serogrouping and PFGE (Chapter 3).

<table>
<thead>
<tr>
<th>Date of isolation</th>
<th>Company</th>
<th>Source</th>
<th>Food Sector</th>
<th>Name</th>
<th>Pulsotype</th>
<th>Serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>06/2013</td>
<td>FBO 1</td>
<td>Drain Swab</td>
<td>Seafood</td>
<td>1_06-13</td>
<td>P64</td>
<td>1/2a or 3a</td>
</tr>
<tr>
<td>02/2015</td>
<td>FBO 1</td>
<td>Floor Swab</td>
<td>Seafood</td>
<td>1_02-15</td>
<td>P64</td>
<td>1/2a or 3a</td>
</tr>
<tr>
<td>10/2013</td>
<td>FBO 2</td>
<td>Drain Swab</td>
<td>Seafood; Vegetables</td>
<td>2_10-13</td>
<td>P61</td>
<td>1/2a or 3a</td>
</tr>
<tr>
<td>02/2014</td>
<td>FBO 2</td>
<td>Smoked Salmon</td>
<td>Seafood; Vegetables</td>
<td>2_02-14</td>
<td>P61</td>
<td>1/2a or 3a</td>
</tr>
<tr>
<td>08/2013</td>
<td>FBO 2</td>
<td>Drain</td>
<td>Seafood</td>
<td>2_08-13</td>
<td>P45</td>
<td>1/2a or 3a</td>
</tr>
<tr>
<td>04/2015</td>
<td>FBO 5</td>
<td>Floor</td>
<td>Dairy; Meat; Seafood; Vegetables</td>
<td>5_04-15</td>
<td>P44</td>
<td>1/2a or 3a</td>
</tr>
</tbody>
</table>
4.2.2. **Inhibitory effects of salt or light stress on food isolates**

Salt tolerance assays using 1 M NaCl (Fig. 4.1 a) were carried out to test the growth of the isolates under osmotic conditions (Section 2.5.1.3). Growth was detected at the $10^3$ dilution for 10403S under the condition tested. 2-08_13, 2-10-13 and 2-02_14 also grew at this dilution whilst $\Delta$sigB and the other strains all grew at the $10^4$ dilution. Even though the increase in growth for the $\Delta$sigB mutant was surprising, it is not unprecedented. As discussed later in Chapter 5, $\Delta$sigB has the ability to grow better under certain stresses compared to its isogenic parent strain (Section 5.2.3). The isolates characterised as the same clone by PFGE behaved similar to each other in this assay, confirming that the differences in salt tolerance are inherent genetic traits of these strains. For example, 1_06-13 and 1_02-15 both grew to $10^4$ whilst 2_10-13 and 2_02-14 showed growth only to the $10^3$ dilution. Of the other two strains tested, 5_04-15 grew better than 10403S whereas, 2_08-13 had an intermediate phenotype between 10403S and $\Delta$sigB. These data demonstrate that salt tolerance is most probably a variable property of wild isolates of *L. monocytogenes* whose mechanism is probably quite complex.

A second assay (Fig 4.1 b) was set up to subject the persistent isolates to blue light (470nm; 1.5-2 mW cm$^{-2}$; Section 2.5.1.1). As with salt tolerance, $\Delta$sigB grew better than 10403S when exposed to blue light. This effect has also been demonstrated for EGD-e and its isogenic $\Delta$sigB mutant (Section 5.2.3). Again growth was detected for $\Delta$sigB at $10^4$ and at $10^3$ for 10403S. All food isolates demonstrated an intermediate phenotype with 2_08-13 appearing to have blue light tolerance closest to $\Delta$sigB. These results suggest that the food isolates tested exhibit a small increase in tolerance to blue light compared to the lab strain 10403S.
4.2.3. Increased acid tolerance by food isolates

The tolerance of the food isolates to pH 2.5 over a 5 h period was investigated (Fig. 4.2). Weak and strong acids can be encountered in both the food processing environment and within food so it is hypothesised that some strains can overcome these conditions better than others. Overnight cultures were incubated at 37°C for 16 h. One microliter of each culture was spun down and resuspended in BHI acidified to pH 2.5 with HCl (Section 2.5.6). Samples were taken every 30 min for 2.5 h initially. However, it was observed that all the strains showed increased tolerance in comparison to 10403S and ΔsigB at this pH and no difference could be deciphered between the strains. The duration of the experiment was subsequently increased to 5 h with samples taken every hour to potentially identify differences between the strains. Overall, the lab strain, 10403S ΔsigB, was more sensitive compared to the food isolates and the parental strain, 10403S, at all time points, as expected from other studies (Ferreira et al., 2003; Abram et al., 2008). After 4 h, the data showed two distinct groups. 5_04-15 was the most sensitive of all the food isolates and by the fourth hour displayed similar CFU ml⁻¹ to the lab strains, with an approximate 4 log reduction observed over the course of the experiment. The remaining samples mostly grouped...
together. In this second group, 2_02-14 was slightly more sensitive than the others with around a 1 log reduction observed for this isolate after 4 h. The rest showed no loss of viability after 4 h. This trend repeated consistently between independent experiments.

For the 5 h time point, more variation was observed between the strains. At this time point there were no viable cells detected for the 10403S or ΔsigB. 2_08-13 survived the best out of all of the food isolates, whilst the other strains grouped into two groups based on their PFGE profile. Strains that were genetically related tended to group together. 1_06-13 and 1_02-15 displayed a 2 log reduction and 2_10-13 and 2_02-14 showed around a 5 log reduction. 5_04-15 remained the most sensitive of all the isolates after a 5 h period. Overall, this experiment demonstrated that all the food isolates had an increased tolerance to pH 2.5 compared to the lab strain 10403S but strain dependent variation occurred between the food isolates with genetically related strains behaving similar to each other.

![Figure 4.2. Survival of isolates at pH 2.5 over 5 h.](image)

One millilitre of overnight cultures was resuspended in BHI acidified to pH 2.5 with HCL. Samples were taken every hour for 5 hours and plated onto BHI agar. (▲) represent genetically related strains isolated from FBO 1 with the open (∆) and (▲) closed symbols distinguishing between different sample dates. (♦) represents related strains from FBO 2. Again the open (◊) and closed (♦) symbols represent different isolation dates. Data shown is a representative data set taken from three independent replicates.
4.2.4. Biofilm formation of the food isolates at 37°C, 30°C and 16°C

Bacteria create biofilms in order to become established in an environment. L. monocytogenes can adhere to surfaces and establish biofilms within food processing environments. Biofilm forming capacity of the food isolates at different temperatures was investigated. Biofilm assays were performed in DM supplemented with 0.4% (v/v) glucose and stained using crystal violet (Section 2.5.7). They were incubated at 37°C, 30°C and 16°C. Plates at 37°C (Fig. 4.3 a & b) and 30°C (Fig. 4.3 c & d) were incubated for 24 h or 48 h, as highlighted in the figure, whereas assays at 16°C (Fig. 4.3 e) were incubated for one week. At all temperatures with the exception of 30°C for 48 h (Fig. 4.3 d), ΔsigB produces more biofilm than 10403S. However, this result was only shown to be significant for biofilm grown at 37°C for 24 h (P ≤ 0.05; Fig. 4.3 a). This could suggest that the presence of σB somehow contributes to repressing biofilm formation in L. monocytogenes.

At 37°C, all the food isolates produced less biofilm when compared to the lab strains (Fig. 4.3 b). 2_10-13 produced the most biofilm compared to the other isolates, with 2_02-14, its genetically related counterpart producing slightly less at this temperature at 24 h and 48 h. The differences observed between 2_10-13 and 2_02-14 was not significantly different but the differences shown between 2_10-13 and the other strains were significantly different (P ≤ 0.05). 2_08-13 produced significantly less biofilm compared to the other isolates at both time points at 37°C (P ≤ 0.05). At both the other temperatures, significantly more biofilm was produced by the food isolates compared to the lab strains, with the highest biofilm production observed at 30°C (Fig. 4.3 c & d). Interestingly as the temperature decreases, the lab strains shifted from producing more biofilm than the food isolates at 37°C to producing less at 30°C and 16°C. This could indicate that food isolates have adapted to produce more biofilm at temperatures more commonly associated with the food processing environment.
Figure 4.3. Biofilm production of food isolates in DM at 37°C, 30°C and 16°C. Overnight cultures were spun down and 7 µl of each sample was added to 7 ml DM supplemented with 0.4% glucose. 200 µl of each sample was added to a 96 well plate with 6 replicate wells for each sample. Plates were wrapped in aluminium foil and grown at 37°C for (a) 24 h or (b) 48 h, 30°C for (c) 24 h or (d) 48 h or (e) 16°C for 1 week. Samples with the same bar fill patterns are genetically related isolates. Error bars represent the standard deviation between three separate independent replicates.
4.2.5. Predation by *Tetrahymena pyriformis* does not differ between strains

As bacteria can encounter protozoa within the environment and protozoa are often considered as a reservoir of pathogens, an assay investigating predation of the food isolates by *Tetrahymena pyriformis* was designed. This assay was conducted by adding 100 protozoal cells to 10⁷ bacterial cells and predation was determined by measuring the bacterial survival as a function of time (Section 2.5.8.). Whilst predation of each of the strains did occur, it was found that there was no difference between the strains. Overall, there was approximately a 2 log reduction of cells for all strains tested over 48 h in the presence of protozoa (Fig. 4.4). When the strains were incubated in PAS (Section 2.3.2) with no protozoa there was no reduction observed (Data not shown).

![Figure 4.4. Predation of *L. monocytogenes* by *Tetrahymena pyriformis*. Co-culture experiments were set up by adding 100 protozoa with 10⁷ *L. monocytogenes* in PAS. The co-culture was incubated at 28°C and samples were taken at preselected time points for viability counts. Error bars represent the standard deviation of 2 independent replicates, which each contained 3 technical repeats. Through fluorescence microscopy, using a strain of *L. monocytogenes* EGD-e tagged with GFP, it was possible to follow the predation of the bacterial cells by *T. pyriformis*. Fluorescence was observed within the food vacuoles of the protozoa and the level of fluorescence increased over time as observed in Figure 4.5. Over the time course, it was noted that fluorescence increased over the first 6 h (Fig. 4.5 A, B and C), but at 17 h (Appendix 3) and 24 h time points (Fig. 4.5 D), fluorescence decreased. These findings
suggest that most predation occurs at the earlier stages of co-culture and plateaus around 17 - 24 h, suggesting that the bacteria were digested within the food vacuole. However, this data is not consistent with the viability results presented in Figure 4.4. These results suggest that by 24 h only half of the predation had occurred and by 48 h cell viability had decreased by another 1 log. The discrepancies between these two sets of data could be that the cell density of the culture tested may not be high enough to image fluorescent cells as cultures have decreased by 1 log. If the cell density was too low to image, it may suggest that bacterial cells are continuing to be digested by the protozoa but it is no longer possible to identify the cells under the microscope as there are fewer clusters of bacterial cells at this cell density.

The biggest loss of viability was shown between 6 h and 22 h with losses of approximately 0.9 - 1.2 log observed. The second 16 h gap from 30 h to 46 h only showed losses of approximately 0.15-0.6 log. Therefore the protozoa do not appear to be consuming as many bacterial cells as before which also may make it more difficult to see under the microscope and this could help to explain why predation seems to be plateauing in the microscopic images.
Figure 4.5. Fluorescent microscopy showing predation of *L. monocytogenes* by *T. pyriformis* over a 24 h period. Samples taken at (A) 0 h, (B) 2 h, (C) 6 h and (D) 24 h. The strain used for these experiments was EGD-e PNFB::GFP. Bright field images are shown on the left and fluorescent images using a GFP filter are shown on the right. The images are taken of different protozoal cells at different time-points.
4.2.6. Sequence alignment comparison of the sigB operon

The sigB operon of 30 L. monocytogenes isolates, L. innocua, L. marthii, L. welshimeri, L. seeligeri, L. ivanovii and Bacillus subtilis were compared by sequence alignment using Geneious 10.0.5 (Section 2.12). Although Bacillus subtilis is the model organism for which much of the research on L. monocytogenes is based, the phylogenetic tree in Figure 4.6 shows the divergence between the two species. Out of the Listeria species tested, L. marthii and L. innocua are the most closely related with L. grayi being the most divergent compared to L. monocytogenes. For L. monocytogenes, two distinct branches have formed. Strains, CFSAN010068, N2306, R2-50, Scott and FSL_R8-5487 have all grouped together. These strains are all food isolates (Burall et al., 2014; Stasiewicz et al., 2015; Tasara et al., 2015; Chen et al., 2017a) but they are more divergent than the food isolates from this present study. All the food isolates from this study grouped into the second branch where they diverged further into two groups. 10403S was also found in this second branch suggesting that the food isolates from our study were more closely related to this lab strain. 2_08-13, 2_10-13 and 2_02_14 grouped together while 5_04-15, 1_06-13 and 1_02 grouped together. Genetically related strains also grouped together providing further evidence that these are clones of each other.

![Figure 4.6. Listeria Inter- and Intra-species relatedness. This phylogenetic tree is based on the comparison of the nucleotide sequence of the sigB operon. Text in red shows the positions of the food isolates compared during this study. The tree was generated using Geneious 10.0.5 software (Section 2.12).](image-url)
The amino acid sequence of the *sigB* operon was further compared by sequence alignment comparing the food isolates and 10403S. Genomic sequences were obtained for each of the food isolates and 10403S (Section 2.8.6). The *sigB* operon was highly conserved between the food isolates compared to 10403S with only two strains showing any changes within the operon. 5_04-13 showed a single amino acid change at position 196 within RsbU, where a glutamic acid was changed to a glycine residue (E196G; Fig. 4.7) and 2_08-13 showed two changes in RsbX. One was found at position 146 where an Aspartic acid changed to a Glycine (D146G) and another was found at position 174 with an Isoleucine changing to Threonine (I174T). Other than these changes the *sigB* operon was 100% conserved in the food isolates compared to 10403S.

As RsbU is a phosphatase involved in the activation of σ^B^ (Dufour and Haldenwang, 1994; Yang *et al*., 1996; Section 1.3), it is possible that mutation E196G may affect the interaction of RsbU with its target, the phosphorylated form of RsbV, which could in turn affect the interaction of σ^B^ with RNA polymerase. RsbX is also a phosphatase that dephosphorylates RsbS-P and RsbR-P to return the σ^B^ activation cascade to its original state (Yang *et al*., 1996; Chen *et al*., 2004; Section 1.3). Therefore, it may be possible that mutations within this protein could render σ^B^ in a constant active state.

All three mutations were compared with the sequences from the other 23 *L. monocytogenes* sequences used to generate the phylogenetic tree (Fig. 4.6). The mutation in RsbU, E196G was only identified in 5_04-15 (Fig. 4.7) making it an uncommon mutation among the strains tested. D146G in RsbX was identified in two other strains used in the larger comparison, one of which was EGD-e. I174T in RsbX was also found in four other isolates including EGD-e. Since the numbers of isolates with these mutations was low it suggests that they are not common mutations within the *sigB* operon.
Figure 4.7. Amino acid sequence alignment of RsbU. This comparison of the RsbU protein compares the amino acid sequence of the food isolates with 10403s. The mutation E196G is identified in white in 5_04-15.
4.2.7. Genomic analysis of stress related genes of the food isolates compared to 10403S

Sequence alignments using Genious 10.0.5, were carried out to compare the amino acid sequences of different proteins involved in stress tolerance in *L. monocytogenes* (Fig. 4.8). Proteins encoded for by genes under σ^B^ control were chosen along with proteins involved in resistance to stresses such as low pH or osmotic shock (See Section 1.7). The amino acid sequences of proteins in the sigB operon and proteins thought to be components of the stressosome complex involved in sensing different stresses were also included (Section 1.3). Finally, some virulence genes known to be under partial σ^B^ control were added for this analysis (Section 1.8.2). When the sequences of the sensory proteins were examined, there was variation between the sequences when compared to 10403S. RsbR and the light sensing protein Lmo0799 were 100% conserved between all strains. Lmo0161 and Lmo1842 showed very small differences between the food isolates and 10403S (Fig. 4.8). All strains had 99.6% identity for Lmo0161 compared to 10403S. This corresponded to one amino acid change at position 254 (E245D). 2_10-13 and 2_02-14 were the only two isolates to show any difference for Lmo1842 (99.6%) compared to 10403S. Out of all the sensory proteins Lmo1642 appeared to show the biggest differences between the strains when compared to the lab strain. 2_08-13 and 5_04-15 were 98.5% similar to 10403S, 2_10-13 and 2_02-14 had 96.6% identity but 1_06-13 and 1_02-15 showed the most difference with only 90% identity with Lmo1642 from 10403S. To show that 90% similarity is quite low, percentage identity was compared between the protein sequences encoded for by three house keeping genes, RpoB, RplD and Gap. All of these proteins were 100% conserved between all the food isolates and 10403S (Fig. 4.8). Of the other proteins, variation was seen for them all with the exception of Lmo2230 and PrfA. PrfA is the master regulator of virulence in *L. monocytogenes* (Section 1.8.2). The biggest differences observed for any of the genes was for gadD1, gadT1 and inlA. The operon containing gadD1T1 appeared to be missing for strains 2_10-13, 2_02-14 and 5_04-15. This operon is involved in survival of *L. monocytogenes* during exposure to weak acids and it is not unusual for this operon to be missing in some strains (Cotter et al., 2005). Other proteins involved in acid tolerance as part of the ADI system (Section 1.6.3.2) including ArcA (>99.5%), ArcB (100%), ArcC (>99.6%), ArcD (100%) and ArgR (100%) were also examined and these were shown to very similar between all strains. InlA is involved in attachment of *L. monocytogenes* to host epithelial cells within the gut (Section 1.4). When the sequence of this gene was examined, a premature stop codon was found in strains 2_10-13 and 2_02-14. This means that the inlA protein is present but probably in a truncated form, which is predicted to lead to
lower invasion of this strain in human host cells expressing E-cadherin, the InlA receptor. Sequence alignments (Section 2.12) revealed that strains isolated from the same company had identical sequences for the sub-set of proteins examined in this chapter. In addition to the serogroup and PFGE analysis described previously, sequence alignments indicate that they are indeed the same strain re-isolated over a period of time.
Figure 4.8. Heat map showing percentage identity between protein sequences of the food isolates (strain names across the top) compared to 10403S. Sequences were compared using Geneious. GadD1 and GadT2 are in bold to indicate that they are missing from some strains. InIA is underlined as this sequence contained a mutation leading to a premature stop codon in two of the isolates as discussed in text.
After examining the amino acid sequences of the \textit{sigB} operon for each of the strains compared to 10403S, it was decided that $\sigma^B$ activation should be investigated for the food isolates to see if the mutations in 2\_08-13 or 5\_04-15 had any effect on $\sigma^B$ activation (Fig. 4.9). Each of the strains was transformed with pKSV7-\textit{P}_{2230}::egfp and grown to stationary phase before being examined by fluorescence microscopy (Section 2.6.4 & 2.11.1). Cells were equalised to OD$_{600\text{nm}}$=0.6 and approximately the same number of cells were examined per frame. As expected, $\Delta\textit{sigB}$ showed a low number of fluorescence cells compared to 10403S. The fluorescence of the cells was quantified using Image J (Section 2.11.1). There was a significant difference between 10403S and all of the food isolates ($P \leq 0.001$). It was shown that 2\_08-13 and 5\_04-15 had the lowest number of fluorescent cells under the conditions tested, however, the fluorescence of 2\_08-13 did not significantly differ from $\Delta\textit{sigB}$ or any of the food isolates and 5\_04-15 was only significantly different to 2\_02-14. These strains were the only two strains of the food isolates to exhibit any changes within the \textit{sigB} operon. 2\_08-13 had two mutations in RsbX, whilst 5\_04-15 had a single amino acid change within RsbU (Section 4.2.6). It is possible that these mutations could negatively affect $\sigma^B$ activity and potentially explain the lower level of fluorescence observed for these strains compared to the others but these results should be interpreted carefully with a need for further work to be carried out to confirm the effect of these mutations.
Figure 4.9. Number of cells expressing \textit{sigB}. Each isolate was transformed with pKSV7-\textit{P}_{2230}:egfp. Cells were grown to stationary phase at 37°C and equalised to OD_{600nm} = 0.6 before being fixed in a 1:1 Ethanol: Methanol mix (Section 2.11.1). Cultures were examined by fluorescence microscopy. Each bar represents 15 frames taken from 3 independent experiments. Error bars represent the standard deviation between the 3 replicates.

4.3. Discussion

The definition of \textit{L. monocytogenes} persistence in food processing environments varies within the literature (Autio \textit{et al.}, 2003; Lundén \textit{et al.}, 2003; Chen \textit{et al.}, 2010a; Carpentier and Cerf, 2011; Magalhães \textit{et al.}, 2016). It is even disputed as to whether there are any specific properties that make \textit{L. monocytogenes} persistent or if all strains can potentially attach and avoid eradication by surviving within niches in a factory (Carpentier and Cerf, 2011). Strains that may be persistent in a processing environment may only be identified sporadically through the sampling process and therefore there may be an underestimation of the number of persistent subtypes (Ferreira \textit{et al.}, 2014; Larsen \textit{et al.}, 2014). Through the surveillance study conducted in Chapter 3, multiple isolates of interest were identified through PFGE (Table 4.1). When the isolates were first selected for phenotypic characterisation, PFGE data was only available for those strains isolated at NUIG. 2.08-13 (P45) and 5.04-15 (P44) were selected as controls as presumptive non-persistent isolates, because they had only been isolated once over a period of 3 years by the NUIG seafood study. However, when all the data was gathered for the entire project, it was observed that
some of the sporadic strains isolated in NUIG were actually persistent isolates found in other processing facilities. P45 (2_08-13) was identified in two other seafood facilities whilst P44 (5_04-15) was a persistent isolate detected in all food sectors tested (Section 3.2). This could suggest that a non-persistent strain may be capable of becoming persistent within another suitable environment as suggested by Kastbjerg and Gram (2009) and Carpentier and Cerf (2011) or alternatively that the strain was only isolated sporadically in this facility because of limitations in the sampling regime used (Ferreira et al., 2014). If this is the case, then this makes it difficult to choose an appropriate control for these studies. If non-persistent controls are chosen as a comparison when investigating if persistence increases resistance of strains to different stresses, how can one be sure that a non-persistent control strain is truly sporadic and hasn’t just been missed due to the sampling process or is not persisting in another food environment? It may be better to use a strain that has been isolated from a natural environment such as soil. If an isolate has never come into contact with a food processing environment, this may be a better control to use as it should not have encountered particular stresses associated with the food environment.

Different assays were used in this study to attempt to phenotypically characterise the selected food isolates. From the results it was obvious that strain-to-strain variation occurred between the food isolates throughout these assays. None of the experiments showed an increased resistance for all the persistent isolates over non-persistent strains included in this study, although the number of strains tested was somewhat limited making it difficult to make definitive conclusions. In the literature there is conflicting data as to whether persistence of an isolate conveys resistance to a strain over different stresses. Magalhães et al. (2016) found that persistent isolates had a shorter lag time and higher growth rate when grown under high salt concentrations or at pH 5 and Lundén et al. (2008) demonstrated that persistent isolates were more resistant to pH 2.4 but did not convey higher tolerance to heat stress than non-persistent strains. Based on experiments conducted in our study, the data does not provided evidence to suggest that persistence of an isolate conveys resistance of that strain to common food associated stresses. When exposed to 1M NaCl or blue light (1.5-2.0 mW cm$^{-2}$; Fig. 4.1), strains exhibited variation regardless of whether they were persistent or not and they did show significant differences compared to the lab isolates. The strains all formed biofilm and there were some significant difference observed between the food isolates and the lab strains at each temperature (Fig. 4.3). It appeared that the food isolates were capable of forming biofilm at all three temperatures. At 37°C they formed less biofilm than 10403S and ΔsigB but this shifted at lower temperature with 10403S and ΔsigB forming less biofilm than the food isolates at lower temperatures. 10403S is a streptomycin resistant
variant of 10403, which was originally isolated from a human skin lesion (Edman et al., 1968; Bishop and Hinrichs, 1987). This could help to explain why the lab strains are more able to form biofilm at 37°C compared to the food isolates and why biofilm production decreases at lower temperatures for 10403S. Food isolates may be more adapted to a lower temperature closer to that of a food processing environment and this could explain why they are more capable of forming biofilm at 30°C and 16°C compared to the lab strains.

Some research has been done on how foodborne pathogens, such as L. monocytogenes, survive the harsh conditions of the fresh food produce they encounter to gain entry into the food processing environment. One suggestion is that foodborne bacteria can survive and multiply within protozoa and exit from the food vesicles (Gourabathini et al., 2008; Pushkareva and Ermolaeva, 2010). Therefore, this could have significant implications for food safety. In this study, predation of L. monocytogenes by T. pyriformis was investigated (Fig. 4.4). It was observed that predation did occur but there was no detectable differences between the food isolates and the lab strains. A previous study has shown that L. monocytogenes can escape the food vacuole of T. pyriformis and replicate in the cytoplasm of the protozoal cell using LLO to escape the vesicle (Pushkareva and Ermolaeva, 2010). For this to occur, the virulence gene regulator PrfA would need to be present and intact for expression of the hly gene, which encodes LLO. PrfA was shown to be present and 100% conserved in all the food isolates tested (Fig. 4.8). However it was not evident in our study that L. monocytogenes was capable of escaping from the food vacuole. When fluorescence microscopy was carried out on co-cultures (Fig. 4.5), it was observed that predation of the cells occurred over the first 6 h but then plateaued at approximately 17 h. The protozoal cells appeared to be intact at all time points tested. This observation is in agreement with results compiled by Gourabathini et al. (2008), where in comparison to other foodborne pathogens such as Salmonella enterica; few vesicles were produced from the protozoa containing L. monocytogenes suggesting that the bacterial cells had been digested within the vacuole.

Significant variation was observed between the food isolates and the lab strains at pH 2.5 (Fig. 4.2). It was shown that the strains separated into three groups after 5 h at pH 2.5. 5_04-15, 2_10-13 and 2_02-14 all appeared to be more sensitive to pH 2.5 than the other food isolates, but were more resistant than the laboratory strains (10403S and the ΔsigB derivative). This was interesting as when the gene sequences were examined for these strains, the GadD1T1 operon was missing for all of these isolates (Fig. 4.8). Whilst it is not unusual for strains to lack this operon, it has been suggested that this operon may play a role in the growth of L. monocytogenes at mildly acidic pH but not in survival at extreme low pH (Cotter et al., 2005). Although, in another study it was proposed that different strains can be
more reliant on different components of the Gad system (Feehily et al., 2014). Therefore, it is possible that the missing operon affects survival at low pH. It is also possible that other acid tolerance systems such as the ADI system may have a role to play in higher tolerance (Section 1.7.3.3). When the sequences of proteins associated with the ADI system were aligned, it was seen that ArcB and ArgR were 100% conserved. In the strains lacking GadD1T1, 2_10-13 and 2_02-14 had two mutations in ArcA (L203V & N248S) compared to 10403S. L203V was common to all the food isolates but N248S was unique to 2_10-13 and 2_02-14. 5_04-15 had one other mutation besides L230V in ArcA. This mutation was A29V in ArcD and was the only strain to have this mutation. It is possible that these unique mutations could also be contributing to the decrease in acid resistance observed for these isolates. Besides the mutations observed in the proteins involved in the ADI system, 5_04-15 also had a mutation (E196G) in RsbU in the sigB operon (Fig. 4.7). If this mutation affects the activity of σB, it is possible that it also could be contributing to the decrease in acid tolerance of this strain compared to the other food isolates. ArcA and ArgR have been shown to be σB dependent (Ryan et al., 2009) and GadD2T2 and GadD3 are at least partially under the control of σB (Kazmierczak et al., 2003; Wemekamp-Kamphuis et al., 2004). Therefore it is possible that the mutation in RsbU could be affecting resistance to extreme acidic conditions. To further investigate acid resistance of the food isolates, additional strains should be examined and experiments could be carried out to investigate the survival of strains lacking GadD1T1 at different growth phases. Experiments investigating the transcript levels of the arc genes and the gad genes could be conducted for 5_04-15 to investigate if the RsbU mutation has an effect in this strain. It is also possible that the individual mutations identified here could be constructed in another background to test their effects individually.

The genomic sequences of all the strains tested were acquired through WGS (Section 2.8.6). Since many of the phenotypes tested were associated with σB activation, the sigB operon was compared between 30 L. monocytogenes strains including the food isolates and 10403S used in this study, along with six other Listeria sp. This showed that the sigB operon was highly conserved amongst all L. monocytogenes strains. Five food isolates whose sequences were obtained from the NCBI database appeared to be the most divergent of all L. monocytogenes isolates (Fig. 4.6). The isolates used in this study were the more conserved with them all grouping similarly to the majority of L monocytogenes strains analysed. It also showed that the sigB operon was conserved among Listeria sp. with L. grayii being the most divergent and L. marthii the most similar (Fig. 4.6). Ferreira et al. (2004) compared the sigB operon between different bacteria, including Bacillus subtilis and Staphylococcus aureus. They
found that whilst all the genes of the operon were not present in all Gram positive bacteria, in cases where they were, the amino acid sequence was highly conserved. Although the operon was highly conserved for the food isolates tested here, there was a slight variation seen for SigB activity levels (Fig. 4.8). The lowest levels were seen for the two strains that had mutations within the sigB operon suggesting that these mutations may have an effect on the number of fluorescent cells in this experiment. Further experiments could be conducted to investigate transcript levels or expression levels of σB dependent genes. It would also be possible to investigate if either of the mutations in RsbU or RsbX is affecting the phosphatase activity of either of these proteins. In Bacillus subtilis, the N-terminal domain of RsbU has been shown to interact with RsbT (Delumeau et al., 2004). The mutation identified in 5.04-15 in this study was identified at position 196 meaning that this falls within the C-terminal domain. This terminus is the catalytic domain of this protein (Delumeau et al., 2004). Therefore a mutation in this domain could affect dephosphorylation of RsbV leaving σB bound to RsbW. Hardwick et al. (2007) identified 4 residues in RsbU that affected both interaction of RsbU with RsbT and the phosphatase activity of RsbU. These residues were present at positions 24, 28, 74 and 78 (Hardwick et al., 2007). By creating a mutant in a clean background with the mutation E196G it may be possible to see if this residue has any role to play the dephosphorylation of RsbV through phosphatase activity assays.

Comparing other proteins of the food isolates (Fig. 4.8), variance was found in many of the amino acid sequences (as little as 90% similarities for some). The biggest differences were as mentioned before, GadD1 and GadT1, were missing from three of the isolates. It was also noted that in two of the strains, there was a mutation causing a truncated form of InlA. This has recently been seen to occur in clinical and food isolates and has been associated with reduced invasion in Caco-2 cells and low virulence in a host (Olier et al., 2003; Gelbičová et al., 2015; Ferreira da Silva et al., 2017). InlA is required for binding to host cells through interactions with E-cadherin (Mengaud et al., 1996; Section 1.4). A truncated InlA may no longer be able to bind with this receptor and this could be why invasion and virulence of strains with truncations are affected, however, it is not known why these mutations occur. Throughout alignment of all the sequences, it was observed that all mutations were consistent between strains that were genetically related. It was also shown that in the phenotypic experiments conducted, these clones also tended to group together. Taking this data along with the sequencing data, PFGE profiles and serogrouping, it suggests that these strains are indeed the same strains isolated at different time points but further analysis of the genomic sequences would be required to confirm this.
In conclusion, it is clear that strain variation occurs between food isolates from different sources. It may be possible that the bacterium does not possess a particular phenotype but requires multiple factors to be able to persist within a food processing environment. It is possible that the cells do not become persistent but instead can survive in niches within the facility evading cleaning strategies leading to re-isolation over a long period of time. As the number of strains used in this study is very limited a more comprehensive study of all the strains isolated in Chapter 3 would be needed to make definitive conclusions regarding the persistence of *L. monocytogenes*.
CHAPTER 5

Blue-light inhibition of *Listeria monocytogenes* growth is mediated by reactive oxygen species and is influenced by $\sigma^B$ and the blue-light sensor Lmo0799

Note. The majority of work presented in this chapter has recently been published in the joint author paper ‘Blue-light inhibition of *Listeria monocytogenes* growth is mediated by reactive oxygen species and is influenced by $\sigma^B$ and the blue-light sensor Lmo0799’ (O’Donoghue et al., 2016, Applied and Environmental Microbiology). Figures 5.4 -5.9 were contributed by Beth O’Donoghue and Figures 5.10-5.13 is relevant data not included in the publication.
5.1. Introduction

*Listeria monocytogenes* is a Gram positive bacterium commonly found in the environment. It is a foodborne pathogen capable of causing a severe systemic infection in humans and is associated with mortality rates of up to 30% (Farber and Peterkin, 1991; Mead *et al*., 1999; EFSA, 2015). Although incidence levels remain far lower than those for outbreaks involving pathogens, such as norovirus and *Salmonella*, cases of listeriosis rose annually in Europe between 2009 and 2013 and are linked primarily with the consumption of ready-to-eat foods (EFSA, 2015). *L. monocytogenes* displays a number of stress adaptations that aid its survival in a wide range of habitats including foods, food-processing environments and the mammalian gastrointestinal tract. In particular, it displays a notable tolerance to osmotic stress, low temperature and bile (Ferreira *et al*., 2001; Hardy *et al*., 2004; Moorhead and Dykes, 2004; Sue *et al*., 2004), and has a potent adaptive tolerance response to acid (Davis *et al*., 1996).

Many of the stress-resistant properties of *L. monocytogenes* are under the control of the stress-inducible sigma factor, SigB (σ^B^), which drives the transcription of the general stress regulon (Kazmierczak *et al*., 2003; Hecker *et al*., 2007; O’Byrne and Karatzas, 2008; Toledo-Arana *et al*., 2009). The regulation of σ^B^ is not fully understood in *L. monocytogenes* but it is believed to involve a signal transduction cascade that ultimately modulates the availability of σ^B^ to associate with RNA polymerase. Environmental signals are thought to be sensed and integrated into the regulatory pathway by a high molecular weight multisubunit complex called a stressosome (O’Byrne and Karatzas, 2008; Heavin and O’Byrne, 2012). The structure of this complex has recently been determined in *L. monocytogenes* (Impens *et al*., 2017) and it is thought to be similar to the stressosome from *B. subtilis* (Marles-Wright *et al*., 2008), since all of the genes involved are conserved between these species (Ferreira *et al*., 2004). In *B. subtilis* the current model proposes that stress signals are sensed by protrusions on the surface of the stressosome that are formed by the N-terminal domains of RsbRA and its paralogues, RsbRB, RsbRC and RsbRD (Marles-Wright *et al*., 2008; Marles-Wright and Lewis, 2008). These sensory signals are then transduced to the core of the stressosome resulting in phosphorylation events that lead to the release of RsbT from the stressosome. RsbT then interacts with RsbU to bring about the activation of σ^B^ (Section 1.3). It is thought that RsbRA and its paralogues (RsbR, Lmo0799, Lmo0161, Lmo1642 and Lmo1842 in *L. monocytogenes*) can integrate different environmental stress signals, allowing σ^B^ activation under a variety of stress conditions, but thus far, only blue light has been shown to be sensed by the stressosome (Ávila-Pérez *et al*., 2006; Möglich and Moffat, 2007; Ondrusch and Kreft, 2011; Tiensuu *et al*., 2013).
Blue light sensing in *L. monocytogenes* requires Lmo0799, a widely-conserved protein predicted to be associated with the stressosome that has a light-oxygen-voltage (LOV) domain at its N terminus and a sulphate transporter, anti-sigma factor (STAS) domain at its C-terminal (Ondrusch and Kreft, 2011). Mutants lacking *lmo0799* have increased motility in the presence of light compared to the wild-type, fail to show enhanced invasion into mammalian cells in response to light (Ondrusch and Kreft, 2011), and are unable to form rings on semisolid agar in response to repeated cycles of light and dark (Tiensuu *et al.*, 2013). The mechanism of light sensing by Lmo0799 has not yet been fully elucidated, although it is clear that it exhibits photochemical activity similar to the related light sensor from *B. subtilis*, YtvA, and the formation of a flavin-cysteinyi adduct in response to light was postulated (Chan *et al.*, 2013). In YtvA this adduct forms between C-4 of the flavin mononucleotide (FMN) ring and the cysteine residue at position 62 (Möglich and Moffat, 2007), which is conserved in the *L. monocytogenes* protein but located at position 56. In the present study, we sought to elucidate the mechanism of sensing by mutating Cys56 to determine the impact on light sensing and to clarify the contribution of Lmo0799 to the growth and survival of *L. monocytogenes*.

Although blue light is known to activate σB in *L. monocytogenes* the impact of visible light on growth and survival in this pathogen has’t been studied in detail. Visible light has been explored as an antimicrobial treatment in numerous studies, with effectiveness demonstrated against a diverse range of medically important bacteria and fungi (for reviews see Maisch *et al.*, 2004; St Denis *et al.*, 2011; Maclean *et al.*, 2014). In most cases, high intensity violet-blue (405 nm) light has been used (Endarko *et al.*, 2012; Murdoch *et al.*, 2012; Maclean *et al.*, 2013). High intensity light at 405nm was found to be effective at inactivating *L. monocytogenes* in liquid suspensions (Endarko *et al.*, 2012) and on surfaces, including acrylic, glass and agar-based growth medium (Murdoch *et al.*, 2012; McKenzie *et al.*, 2013). The presence of photosensitising compounds such as sodium chlorophyllin (Luksiene *et al.*, 2010) or 5-aminolevulinic acid (Buchovec *et al.*, 2010) enhance the killing effect of visible light on surfaces. The mechanism of killing by visible light hasn’t been addressed in *L. monocytogenes* to date, although studies on other bacteria suggest that excitation of endogenous porphyrins by light under aerobic conditions can lead to the production of damaging reactive oxygen species (Nitzan *et al.*, 2004). Indeed the photosensitising effect of 5-aminolevulinic acid is thought to be due to its role as a precursor in the biosynthesis of porphyrins (Buchovec *et al.*, 2010).

In this study, we investigate the influence of visible light on the physiology of *L. monocytogenes*. We used blue light with a wavelength of 460 to 470 nm since this is the
wavelength that has been shown to activate the general stress response via the light sensor Lmo0799 and σB. We show that quite low doses of light at this wavelength inhibit the growth of this pathogen in both liquid and solid growth media. We show that the inhibitory mode of action is dependent on the production of reactive oxygen species. The role of σB and Lmo0799 in the response of L. monocytogenes to blue light is further clarified and the conserved cysteine residue in Lmo0799 is shown to be essential for light sensing. We further demonstrate that L. monocytogenes is able to survive in seawater for a prolonged period of time. We investigate the effect of ambient light on survival in seawater and whether the presence σB or Lmo0799 play a role in the survival of L. monocytogenes in seawater when exposed to UV-A or visible light.

5.2. Results

5.2.1. Blue light inhibits L. monocytogenes growth

While previous studies have shown that L. monocytogenes responds to blue light, the effects on growth have not been investigated. We devised an apparatus to deliver a uniform dose of light at 460 to 470 nm to agar plates and liquid cultures in 96-well microtiter plates (Section 2.4.1 & 2.4.2). When overnight cultures of L. monocytogenes EGD-e were spotted onto BHI agar plates (using a dilution containing approximately $10^4$ cells ml$^{-1}$) and incubated at 30°C for 24 h under a blue light irradiance of 1.5-2.0 mW cm$^{-2}$ (Section 2.5.1.1), no growth was detected, whilst normal growth was observed on the dark control (Fig. 5.1 a). Blue-light inhibitory effects on growth were also recorded for L. monocytogenes 10403S and several food environment isolates (Section 4.2.2). The cells were not killed by this exposure to blue light, since a subsequent incubation of the plates inoculated with L. monocytogenes EGD-e in the dark allowed the colonies to form after a further 48 h at 30°C (data not shown). In liquid BHI medium (Section 2.5.2), the same light irradiance significantly inhibited both the culture yield (final optical density at 24 h) and the lag time (taken as time to reach OD$_{600nm}$ of 0.1), although growth was not completely inhibited (Fig. 5.1 b).
Figure 5.1. Growth inhibition of EGD-e by blue light. EGD-e was illuminated with blue light (460 to 470nm, 1.5-2.0 mW cm⁻²) either on BHI agar (a) or in a BHI liquid culture (b). (b) White bars represent growth following continuous illumination, and the black bars represent a dark control. The graphs show final OD₆₀₀nm after 24 h (left) and the difference in lag times (right) between the two conditions. Overnight cultures were standardised to OD₆₀₀nm of 1.0 and diluted to 10⁻⁵ (approximately 10⁷ cells ml⁻¹). Cells were incubated at 30°C for 24 h. The values represent the means of the results from three independent replicates. The error bars represent the standard deviations between replicates. Student’s t test was carried out to determine the statistical difference (P ≤ 0.05, indicated with an asterisk) between cultures grown in light and dark.

The inhibitory effects of blue light (470 nm) were found to be dependent on the cell density, since only more-dilute cultures (those containing <10⁷ CFU ml⁻¹) were found to be inhibited on agar plates (Fig. 5.2 a). This was also the case in liquid BHI medium, in which the effects of blue light on the 24 h culture OD₆₀₀nm and the lag time were more pronounced as the cell concentration decreased (Fig. 5.2 b). At high cell densities (>10⁷ CFU ml⁻¹), essentially no inhibition of growth was observed at this dose of light (Fig. 5.2 a & b).
Figure 5.2. Cell density influences the extent of growth inhibition of EGD-e by blue light. Dilutions of EGD-e were illuminated with blue light (460 to 470nm, 1.5–2.0 mW cm⁻²) either on BHI agar (a) or in BHI liquid culture (b). For (a) overnight cultures were standardised to OD₆₀₀nm of 1.0 and diluted to 10⁻⁸. Four microliters of each dilution was spotted in triplicate onto BHI agar and grown at 30°C for 24 h. In (b), white bars represent growth in the presence of light and the black bars represent the dark control. The graphs show final OD₆₀₀nm measurements (left) and lag times (right). The number over the lag time indicates the time taken to reach OD₆₀₀nm of 0.1. Starting cells were equalised to OD₆₀₀nm of 0.05 and diluted to 10⁻⁶. Cultures were grown in 96 well plates at 30°C for 24 h. Values represent the mean of three individual replicates. Error bars represent the standard deviations between replicates. Student’s t-test was carried out to determine the statistical difference (P ≤ 0.05, indicated with an asterisk) between cultures grown in light and dark.
5.2.2. Inhibitory effects of blue light are dependent on the generation of reactive oxygen species

One possible interpretation of these data was that oxygen levels in the medium were influenced by the population cell density, and that this variable might influence the sensitivity to blue light, since other studies have reported that light can lead to the generation of reactive oxygen species (ROS) (Ávila-Pérez et al., 2009; Endarko et al., 2012). To investigate this hypothesis, we measured the inhibitory effect of blue light on cells grown in BHI medium containing the ROS scavenger dimethylthiourea (DMTU). When included at a concentration of 20 mM (Section 2.5.1.2 & 2.5.2), DMTU had no significant effect on dark-incubated cultures, but it conferred a significant protective effect against blue light both on solid medium and in liquid medium (Fig. 5.3 a & b). Strikingly, in liquid BHI medium, DMTU completely reversed the potent inhibitory effect of blue light in low cell density cultures; both the lag phase and the final OD_{600nm} at 24 h were restored to the same levels as the dark control when DMTU was present (Fig 5.3 b).
Figure 5.3. **ROS scavenger DMTU mitigates the inhibitory effect of blue light.** EGD-e cells were illuminated with blue light (460 to 470nm, 1.5-2mW cm\(^{-2}\)) either on BHI agar (a) or in liquid culture (b) with or without 20 mM DMTU. (a) Overnight cultures were standardised to OD\(_{600nm}\) of 1.0 and diluted to 10\(^{-8}\). Four microliters of each dilution was spotted in triplicate onto BHI agar (-) or BHI agar supplemented with 20 mM DMTU (+) and grown at 30°C for 24 h. (b) Final OD measurements (left) and difference in lag time (right). Starting cells were equalised to OD\(_{600nm}\) of 0.05 and diluted to 10\(^{-6}\). Cultures were grown in 96 well plates at 30°C for 24 h in BHI broth with or without 20 mM DMTU. Values represent the mean of three individual replicates. Error bars represent the standard deviation between samples. Student’s t-test was carried out to determine the statistical difference between cultures grown with and without DMTU. In (b) the asterisks indicate significant differences (\(P \leq 0.05\)).
Very similar results were observed when catalase (Section 2.5.1.2 & 2.5.2) was included in either the agar-based (Fig. 5.4 a) or liquid BHI medium (Fig. 5.4 b); the presence of catalase reversed the growth inhibition and reduced the lag phase caused by blue light. Together these results suggest that blue light causes the production of ROS in the medium, including hydrogen peroxide, and this is likely to be the principal reason for growth inhibition.

**Figure 5.4. Catalase alleviates growth inhibition of *L. monocytogenes* by blue light.** (a) Wild-type overnight cultures were standardised, diluted serially and spotted on BHI agar spread with catalase (+) or on agar plates without catalase(-). Plates were incubated in the presence of light for 24 h. (b) Wild-type overnight cultures were diluted to an OD$_{600nm}$ of 0.05 and diluted 10-fold to a $10^6$ dilution. Dilutions were grown for 24 h in the presence or absence of light, with or without catalase at a concentration of 125 U ml$^{-1}$. The 24 h endpoint and the time taken to reach OD$_{600nm}$ of 0.1 were calculated for each strain condition at each dilution. Error bars represent standard deviations between samples. Unpaired $t$ tests were used to determine the significance of differences ($P \leq 0.05$, indicated by asterisks) between endpoint and lag-phase values between cultures with and without catalase.
5.2.3. A mutant lacking $\sigma^B$ has decreased sensitivity to blue light

As earlier studies have shown that $\sigma^B$ is activated in response to blue light, we investigated the effect of a $\text{sig}B$ deletion mutation on the sensitivity of *L. monocytogenes* to blue light. On solid BHI medium the $\Delta\text{sig}B$ mutant was found reproducibly to grow better than the parental control when exposed to blue light for 24 h (1.5-2 mW cm$^{-2}$). Growth was detected from the $10^{-3}$ dilution for the $\Delta\text{sig}B$ mutant, whilst this dilution failed to grow for the wild-type under the same conditions (Fig. 5.5 a). The *L. monocytogenes* 10403S $\Delta\text{sig}B$ mutant also displayed a growth advantage under these conditions (Section 4.2.2). In liquid medium, the final OD$_{600\text{nm}}$ of the EGD-e $\Delta\text{sig}B$ mutant was significantly higher than the wild-type, especially for cultures inoculated with low starting cell numbers ($10^5$ and $10^6$ dilutions; Fig. 5.5 b). The $\Delta\text{sig}B$ mutant also exhibited a shorter lag time than the wild-type during illumination with blue light (almost 3 h shorter for the lowest cell density inoculum; Fig. 5.5 b).
Cells lacking SigB have decreased sensitivity to blue light. Shown is the influence of blue light (470nm, 1.5-2 mW cm$^{-2}$) on the growth of *L. monocytogenes ΔsigB* compared to the wild-type EGD-e on BHI agar (a) and in BHI liquid culture (b). Overnight cultures were standardised to OD$_{600nm}$ of 1.0 and diluted to $10^{-8}$. Four microliters of each dilution was spotted in triplicate onto BHI agar and grown at 30°C for 24 h. (b) shows final OD measurements (left) and difference in lag time (right). Starting cells were equalised to OD$_{600nm}$ of 0.05 and diluted to $10^{6}$. Cultures were grown in 96 well plates at 30°C for 24 h. Values represent the mean of three individual replicates. Error bars represent the standard deviation between samples. A Student $t$-test was carried out to determine the statistical difference between EGD-e and ΔsigB. The asterisks in (b) indicate $P \leq 0.05$.

This somewhat surprising result might suggest that at sublethal doses of light, the cost of deploying a σB-controlled stress response is associated with a negative impact on growth rate. As the stress becomes more severe, a survival advantage might be expected. To investigate this, we exposed wild-type and ΔsigB mutant cells to a lethal dose of blue light (8 mW cm$^{-2}$). Under these conditions, the ΔsigB mutant was found to be significantly more sensitive to light than the wild-type, with a 10,000-fold reduction in survivors detected in the mutant populations after 12 h exposure to light (Fig. 5.6). This effect was also observed at 16°C when a lower power density was tested; after 24 h at 6.5 mW cm$^{-2}$, the sigB mutant had
4-fold fewer survivors than the wild-type (data not shown). These data suggest that $\sigma^B$ plays an important role in protecting cells against lethal doses of blue light.

![Graph showing survival of L. monocytogenes ΔsigB and Δlmo0799 in blue light](image)

**Figure 5.6.** *L. monocytogenes* ΔsigB displays a survival defect in higher-intensity blue light. Overnight cultures were washed and resuspended in PBS and exposed to 8 mW cm$^{-2}$ blue light. Viable cell counts were performed at 0 h, 6 h and 12 h time points. The values represent the means of the results from six individual replicates. The error bars represent the standard deviations between samples.

### 5.2.4. Role for the blue-light sensor Lmo0799

Since $\sigma^B$ activation in the presence of blue light occurs via the blue-light sensor protein Lmo0799, we investigated the impact of loss of this sensor on light sensitivity. A deletion mutant, Δlmo0799, and a missense mutant with an alanine replacing the conserved cysteine at position 56 of the Lmo0799 protein (C56A) were constructed by Beth O’Donoghue (O’Donoghue, 2016). The *lmo0799* C56A mutant was constructed to genetically test the idea that this residue is essential for the light-sensing function of the protein, as proposed by others (Möglicn and Moffat, 2007; Ávila-Pérez *et al.*, 2009; Chan *et al.*, 2013). Two phenotypes known to be associated with the loss of Lmo0799 function were tested (Section 2.5.3); derepressed motility in the presence of light and loss of ring formation in response to light-dark cycles (Ondrusch and Kreft, 2011; Tiensuu *et al.*, 2013). In response to blue light, motility is repressed in the wild-type but this repression is lost in a ΔsigB mutant (Fig. 5.7). This repressed motility was also found to be lost in the Δlmo0799 and *lmo0799* C56A mutants (Fig. 5.7). Ring formation in response to 12 h cycles of ambient light and dark is
observed in the wild-type, and this is abolished in a mutant lacking σB (Fig. 5.8). This phenotype is also lost in the Δlmo0799 and lmo0799 C56A mutants. These data show that light sensing via Lmo0799 is essential for the repression of motility by ambient light and for ring formation. Furthermore, they provide strong genetic evidence that the cysteine residue at position 56 is essential for the light sensing function of Lmo0799.

**Figure 5.7.** The *lmo0799* C56A 'blind' mutant displays derepressed motility in light. Strains were inoculated on 0.3% agar and colony diameter was measured 60 h after exposure to ambient white light/ incubation in dark at 30°C. The values represent the means of the results from four biological replicates. The error bars represent the standard deviations between samples. The asterisk indicates $P \leq 0.05$. 

![Figure 5.7](image.png)
In the presence of an inhibitory dose of blue light, the Δlmo0799 mutant was found to display a similar decreased sensitivity phenotype to the ΔsigB mutant. This effect was observed on both BHI agar plates and in liquid medium (Fig. 5.9 a & b). When tested on agar plates, the reintroduction of lmo0799 on a plasmid pMK4 removed this growth advantage, and it was observed that the presence of additional Lmo0799 (via pMK4 lmo0799) negatively affected growth of the wild-type strain (Appendix 4). The lmo0799 C56A mutant consistently displayed a less-pronounced phenotype, showing only a slight decrease in sensitivity to blue light on agar plates when compared to the wild-type (Fig. 5.9 a) and no significant difference in liquid medium (Fig. 5.9 b). These data may indicate that the C56A mutant version of Lmo0799 is still able to transmit light-related signals that lead to σB activation (and a corresponding decrease in growth). At lethal doses of light (8 mW cm⁻²), neither the Δlmo0799 mutant nor the lmo0799 C56A mutant displayed any increase in sensitivity to blue light compared to the wild-type (Fig. 5.6). This result was unexpected but may reflect the fact that at higher doses of light additional stress signals are generated (e.g. ROS) that still lead to σB activation even in the absence of the capacity to sense blue light itself.
Figure 5.9. Removal of SigB or Lmo0799 decreases the inhibitory effect of light on cell growth.
(a) Cultures were standardised to an \( \text{OD}_{600\text{nm}} \) of 1 and diluted, first 1:10, followed by 1:5 dilutions in PBS. Dilutions were spotted on BHI agar and incubated in the presence or absence of light. (b) Overnight cultures were diluted to an \( \text{OD}_{600\text{nm}} \) of 0.05 and diluted 10-fold to a \( 10^{-6} \) dilution. Dilutions were grown for 24 h in the presence or absence of light. The 24 h endpoint and the time taken to reach \( \text{OD}_{600\text{nm}} \) of 0.1 were calculated for each strain at each dilution. Student’s \( t \)-tests were used to identify endpoint and lag-phase values that differed significantly from that of the wild-type strain. The values represent the means of the results from three individual replicates. The error bars represent the standard deviations between samples. The asterisks indicate \( P \leq 0.0167 \), adjusted for Bonferroni correction.
5.2.5. *L. monocytogenes* can survive for long periods in seawater

Next the impact of light on the survival of *L. monocytogenes* in a natural environment was investigated. First the survival of *L. monocytogenes* in seawater without light was measured. Cells of overnight cultures were first washed twice in PBS and then were used to inoculate duplicate flasks containing 100 ml of non-sterile unfiltered seawater with approximately 1 x 10^8 CFU ml⁻¹.(Section 2.5.4) Flasks containing the cultures were wrapped in aluminium foil to prevent light exposure. One set of flasks were placed outside to allow the seawater temperature to fluctuate with the local ambient temperature ranging from 4°C to 14°C, whilst the second set of flasks were placed at 16°C to keep the temperature stable. Samples were removed for counting at suitable intervals over a 25 day (600 h) period. Over the first 180 h *L. monocytogenes* remained stable and in excess of 1 x 10^7 CFU ml⁻¹ (Fig. 5.10 a). After that a steady decline in survivors was recorded over the following 420 h; however survivors were still detected after 25 days (600 h; Fig. 5.10). A very similar pattern of survival was observed at 16°C, with the numbers remaining stable for the first 180 h and then gradually declining to approximately 1 x 10^4 CFU ml⁻¹ after 600 h (Fig. 5.10). These data show that *L. monocytogenes* is capable of persisting for a number of weeks in marine environments.

![Graph showing survival of L. monocytogenes in seawater](image)

**Figure 5.10.** *L. monocytogenes* survives for up to 600 h in live seawater. The survival of EGD-e in live seawater was investigated at ambient temperature and 16°C. Bacterial cultures were incubated in the dark under both conditions. Error bars represent the standard deviation of three replicates.
5.2.6. **Daylight significantly reduces* L. monocytogenes survival in seawater**

Since the initial experiments were performed with flasks wrapped in aluminium foil to exclude light, it was investigated whether ambient light could influence the survival of *L. monocytogenes* in seawater. Flasks were inoculated with 1 x 10^8 CFU ml\(^{-1}\) and incubated outdoors as before, either with or without aluminium foil cover. Daily irradiance values were recorded and samples were removed at suitable intervals for counting. Strikingly *L. monocytogenes* lost viability much faster in the light-exposed flasks than in the dark controls (Fig. 5.11). Survivors were only detected in the light-exposed flasks for up to 80 h, while the dark control showed no significant loss in viability over this period. The biggest losses in viability appeared to coincide with the midday irradiance peaks at 32 h and 56 h (Fig. 5.11). Together these observations indicate that the survival of *L. monocytogenes* in seawater is strongly influenced by ambient sunlight.

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![Figure 5.11](image-url)  
**Figure 5.11. Ambient light affects survival of L. monocytogenes in seawater.** The survival of *L. monocytogenes* EGD-e was tested for 80 h in live seawater. Samples were incubated in ambient light at ambient temperature outdoors over 80 h (open symbols). Dark control samples were also included in the experiment (closed symbols). The dashed line represents the average irradiance values (W m\(^{-2}\)) over the sample period. Error bars indicate the standard deviation of 6 replicates.
5.2.7. Visible and UV light affect the survival of *L. monocytogenes* in seawater

To gain insights into the region of the light spectrum that was detrimental to *L. monocytogenes* in seawater, the survival of EGD-e either in the presence of blue light (470 nm) or in the presence of UV-A light (385 nm) at 16°C was analysed. The power from both lights sources was 6.7 mW cm$^{-2}$, which is slightly less than that of a typical sunny day (10 mW cm$^{-2}$). The UV-A light treatment produced the fastest killing with no wild-type survivors detectable after 10 h (Fig. 5.12 b). However, blue light also had a significant impact on survival of the wild-type in seawater; a steady decline in viability was recorded over 50 h compared to the dark control, which showed no loss of viability over this period (Fig. 5.12 a).
Figure 5.12. Inhibition of *L. monocytogenes* in seawater occurs at a quicker rate in the presence of UV-A light compared to blue light. Inactivation of EGD-e (○), ΔsigB (□) and Δlmo0799 (Δ) in seawater in the presence of (A) 470 nm blue light (6.7 mW/cm²) and (B) 385 nm UV-A light (6.7 mW/cm²) (open symbols). Cells were incubated at 16°C for a period of 50 h for blue light and 24 h for UV-A light in a 96 well microtitre plate. A dark control plate was also set up (closed symbols). Values represent the mean of three replicates and error bars indicate the standard deviation.
5.2.8. Role for the general stress response in seawater survival in the presence of Visible or UV-A light

The role of $\sigma^B$ and the light sensor, Lmo0799, in surviving both UV-A light and blue light was investigated in live seawater (Fig. 5.12 a & b). Interestingly, in the presence of blue light $\sigma^B$ was found to aid survival since cells lacking this sigma factor lost viability much faster than either the wild-type or the mutant lacking Lmo0799 (Fig. 5.12 a). Under UV-A illumination there was no obvious difference in the killing rates between the strains (Fig. 5.12 b). These results indicate that whilst $\sigma^B$ contributes to protection against light-associated stress in seawater the blue light sensor Lmo0799 does not appear to play a role. These data further suggest that $\sigma^B$ is primarily required to protect against the damaging effects of visible light, in the blue region of the spectrum, rather than UV-A light.

5.2.9. Components of live seawater (unfiltered) may influence the survival of *L. monocytogenes* in the presence of ambient light

To investigate whether components of live seawater have a role in the reduction of viability of *L. monocytogenes* caused by ambient light, seawater samples were filter sterilised to attempt to remove contents of the seawater. Samples were filtered as autoclaving could influence the composition of the seawater. Samples were treated as before and unfiltered samples were run in parallel as a control. It was observed that sterilising the seawater had an effect on the survival of EGD-e with cells in filtered water surviving for up to 24 h longer than those in unfiltered seawater (Fig 5.13). The dark controls showed no loss of viability in either sample over the time period tested (Fig 5.13). This result suggests that there may be components of seawater that influence the survival of the bacterium in the presence of ambient light.
Figure 5.13. Filtering seawater may influence the inhibition of *L. monocytogenes* by ambient light. Filtered seawater samples were filtered prior to addition of bacteria. Samples were incubated in the presence of ambient light (open symbols) for 50 h. A dark control was also set up (closed symbols). Error bars represent the standard deviation of three independent replicates, each plated in triplicate.

5.3. Discussion

In this study, we have shown that the growth of *L. monocytogenes* is negatively affected by blue light (460 to 470nm) at comparatively low irradiance levels (< 10 mW cm\(^{-2}\)) on both agar-based medium (Fig. 5.2 a) and in liquid culture (Fig. 5.2 b). We have also demonstrated that ambient light effects the survival of *L. monocytogenes* in a natural environment. The data show that blue light extends the lag time of cultures in liquid broth and that this effect can be reversed by addition of the scavengers of reactive oxygen species (ROS) dimethylthiourea (DMTU; Fig. 5.3 b) and catalase (Fig. 5.4 b). These results suggest that blue light triggers the formation of ROS and that these species have an inhibitory effect on growth, presumably because they cause oxidative damage of macromolecules in the cell (Imlay, 2013). Previous studies have suggested the link between killing of bacteria by high intensity visible light and ROS production (Maclean *et al.*, 2008; Endarko *et al.*, 2012). The mechanisms underlying ROS production remain to be elucidated, but endogenous porphyrins have been implicated in light-inactivation studies with other bacteria (Gabor *et al.*, 2001; Nitzan and Ashkenazi, 2001; Hamblin and Hasan, 2004). As the genes for heme biosynthesis are present in *L. monocytogenes* (Panek and O'Brian, 2002), it seems likely that
photosensitising porphyrin intermediates such as the uroporphyrinogen III, could contribute to the light sensitivity observed. Indeed, the addition of the porphyrin precursor 5-aminolevulinic acid (5-ALA) to the growth medium increases the sensitivity of *L. monocytogenes* cells to violet light (Buchovec et al., 2010). The explanation for the protective effect of high cell densities on the extent of growth inhibition (Fig. 5.2 a & b) remains unknown, but two possible explanations are suggested: either the total amount of catalase present in the population helps to mitigate the effect of ROS or the oxygen level is depleted to a greater extent in dense cultures by respiratory activity and this limits ROS production. Further studies will be required to differentiate between these possibilities.

The protective response to oxidative stress is known to be partially under σB control (Ferreira et al., 2001) and so it was somewhat unexpected to discover that the ΔsigB (and Δlmo0799) mutant displayed an enhanced capacity to withstand the inhibitory effects of light at 1.5-2 mW cm⁻². However enhanced growth of sigB mutants has been reported by others both in *L. monocytogenes* and *B. subtilis*. Abram et al. (2008) observed that a 10403S ΔsigB mutant had a faster growth rate than the wild-type parent in a chemically defined medium with 0.5 M added NaCl. Mutations in *rsbT* and *rsbV* that are predicted to negatively affect σB activity also produce a fast-growth phenotype under some conditions (Chaturongakul and Boor, 2004). A ΔsigB mutant of *B. subtilis* is known to have a significant growth advantage compared to the wild-type when grown in a glucose-limited medium (Schweder et al., 1999). Although these results have not been explained to date, it has been suggested that these effects might arise from sigma factor competition for core RNA polymerase (O’Byrne and Karatzas, 2008). In this model the increase in growth rate of the mutant lacking sigB arises because the housekeeping sigma factor, σA, has greater access to the core polymerase and can therefore more efficiently transcribe genes with growth-related functions. A similar model has been proposed to account for the accumulation of *rpoS* (encoding σS, the general stress response sigma factor) mutations in *E. coli* when grown under limiting conditions (Nyström, 2004; King et al., 2006). The energetic cost of deploying the general stress response (with associated homeostatic and repair energy requirements) may also contribute to the negative effect on growth rate. As both the ΔsigB and Δlmo0799 mutants behaved in a similar manner at inhibitory low doses of blue light (Fig. 5.9 a) it suggests that the activation of the general stress response by σB at this dose of light, which occurs following light sensing and signal transduction by Lmo0799 (Ondrusch and Kreft, 2011), produces a negative effect on cell growth. Indeed, we have recently observed that a nonsense mutation in *rsbU*, which is predicted to negatively affect SigB, also produces reduced light sensitivity to low intensity light. However, once the dose of blue light reaches a lethal level (8 mW cm⁻²
it is clear that $\sigma^B$ contributes positively to survival (Fig. 5.6), highlighting the importance of the general stress response for surviving damage caused by visible light. Surprisingly Lmo0799 did not appear to be required for survival at lethal doses of blue light. It is possible that indirect stress signals (e.g., oxidative damage caused by ROS) are generated at this higher dose of light, and these signals can be sensed independently of Lmo0799, perhaps via RsbR or one of its paralogues (the putative sensory subunits of the stressosome), thereby ensuring an effective stress response independently of light sensing. We are currently pursuing this line of investigation.

The genetic evidence presented here provides strong support for the model proposing that the conserved cysteine residue at position 56 of Lmo0799 plays a crucial role allowing this protein to sense blue light (Fig. 5.7 & 5.8). Previous modelling studies have found the Lmo0799 protein structure to be nearly identical to that of YtvA in Bacillus amyloliquefaciens (Ogata et al., 2009; Ondrusch and Kreft, 2011) with both having the conserved cysteine in the FMN-binding pocket. Gaidenko et al. (2006), have shown that the YtvA conserved residue C62 is critical for light-induced SigB activation of the stress-response in B. subtilis. The photocycle of the Lmo0799 protein has been elucidated by Chan et al. (2013), and the authors predicted an important role for C56. Replacement of this residue with an alanine produced phenotypes (loss of ring formation during light-dark cycles and increased motility in the presence of blue light) similar to those observed for the removal of the full Lmo0799 protein and suggests that the predicted light-induced cysteinyl-flavin mononucleotide adduct is crucial for light sensing (Ondrusch and Kreft, 2011; Tiensuu et al., 2013). This single amino acid change likely results in the uncoupling of the Lmo0799 LOV domain from its FMN chromophore, resulting in the loss of blue-light sensing capacity. The enhanced growth phenotype of the lmo0799 C56A mutant was not as pronounced as the Δlmo0799 or ΔsigB mutants on BHI agar plates in the presence of a sublethal blue-light dose (Fig. 5.9 a), suggesting that $\sigma^B$ might be partially activated by light in this strain, but this difference was not found to be significant in liquid BHI medium (Fig. 5.9 b). It is worth noting that the impact of Lmo0799 on the overall structure and assembly of the stressosome has not been investigated in L. monocytogenes, and it is possible that the deletion mutation might have indirect effects on the sensing capacity of the stressosome independent of the loss of the Lmo0799 protein itself. This might have contributed to the subtle differences in the behaviour of the lmo0799 C56A and Δlmo0799 mutants on BHI agar plates in the presence of light. Alternatively, the Lmo0799 protein might have a secondary sensing function, as has been postulated by Chan et al. (2013), which remains unaffected in the lmo0799 C56A mutant.
In a natural environment such as seawater, solar irradiation is a major factor affecting the survival of *L. monocytogenes* (Fig. 5.11). When light is eliminated the organism can survive for long periods in seawater, raising the possibility that contamination of shellfish and other aquaculture produce might occur prior to harvesting. Although surface seawater is subjected to solar irradiation on a daily basis, local factors can influence the extent of this irradiation. Examples include weather conditions, concentrations of suspended solids, marine algae or biomass content, all of which can vary significantly in the coastal waters where most aquaculture is located. Indeed it is known that light transmittance through the water column is typically much lower in coastal waters than in the open ocean (Tedetti and Sempéré, 2006). When this is considered together with the likelihood of *L. monocytogenes* being washed into the sea periodically (Colburn *et al.*, 1990; Rodas-Suárez *et al.*, 2006), particularly during extended periods of rainfall in coastal areas, this study highlights an important potential source of contamination for the fish farming industry.

σB appears to be required for surviving irradiation with blue light in live seawater, since irradiation at 470 nm with 6.7 mw cm⁻² produced a greater killing effect on the *sigB* mutant than on the wild-type (5.12 a). The blue light sensor Lmo0799 again appeared to be dispensable for this effect (Fig. 5.12 a). Although the effect is quite small, it was shown that removal of components of seawater through filtration increased survival of EGD-e in the presence of ambient light (Fig 5.13) compared to live seawater suggesting that contents of the seawater could contribute to the killing effect observed. This could be due to compounds produced by other living organisms which may be removed by filtering. For example, organisms present in the water may produce chlorophyll for photosynthesis. Chlorophyllin, a derivative of chlorophyll, has previously been shown as a photosensitizer which can be used in combination with light to decrease viability of *L. monocytogenes* (Luksiene *et al.*, 2010; Luksiene and Paskeviciute, 2011). This could mean that if extracellular components similar to this photosensitizer are removed by filtration, the survival levels could increase as seen in Figure 5.13. This experiment could be repeated with more time-points included to see if the difference between filtered and live seawater is more obvious at earlier time-points or whether the effect is very small throughout.

In this study we have demonstrated that ambient light and blue light (460 - 470nm) exhibit inhibitory effects on the growth and survival of *L. monocytogenes* in culture media and in seawater and show that these effects are caused by the production of reactive oxygen species (ROS). Whilst a number of other studies have also shown the inhibitory effects of visible light on a variety of bacterial species (Bialka and Demirci, 2008; Maclean *et al.*, 2008; Maclean *et al.*, 2009; Kairyte *et al.*, 2012; Murdoch *et al.*, 2012), most have been conducted
with high intensity violet-blue light (400-405 nm) rather than blue light (470 nm). Thus this is the first demonstration that *L. monocytogenes* growth and survival can be affected by exposure to blue light. Overall the study raises the interesting possibility that blue light-emitting diode lights, which are comparatively cheap, energy efficient and widely available, might be used to control the growth of this pathogen in food processing environments or even in certain amenable food groups.
CHAPTER 6

Characterisation of a putative cation transporter and investigation of its role in $\sigma^B$ activation
6.1. Introduction

As discussed before, (Section 1.10; Section 5.1) different genes such as \textit{lmo0799} have been identified as being involved in $\sigma^B$ activation in \textit{L. monocytogenes}. $\sigma^B$ is an alternative sigma factor that controls the GSR in \textit{L. monocytogenes} (van Schaik and Abee, 2005; Chaturongakul \textit{et al.}, 2008; O’Byrne and Karatzas, 2008; NicAogáin and O’Byrne, 2016; Section 1.3) This bacterium can overcome and survive a wide range of stressful conditions, partially due to the activity of $\sigma^B$. Whilst analysing motility plates, Tiensuu \textit{et al.} (2013) discovered that \textit{L. monocytogenes} was capable of forming rings when exposed to oscillating cycles of light and dark on semi-solid agar plates. Interestingly, the isogenic EGD-e $\Delta$sigB mutant was unable to form rings under these conditions. A mutant lacking \textit{lmo0799} was also incapable of producing rings in response to cycles of light and dark but when complemented with a plasmid containing \textit{lmo0799}, the phenotype was restored. This suggested that the ring formation occurred in an Lmo0799 / $\sigma^B$ dependent manner (Section 1.10).

To identify other genes that contribute to ring formation, Tiensuu \textit{et al.} (2013) developed a transposon insertion library. They identified 48 mutants that could no longer form rings in response to cycles of light and dark. This could indicate that $\sigma^B$ regulation is affected by disruption of the target gene by the transposon. The transposon mutants were categorised into three groups (Section 1.10); genes encoding proteins involved in $\sigma^B$ regulation, transport or maintenance of the cell wall and regulatory proteins. Following this, they further analysed genes with functions in transport or cell wall, maintenance and regulation to assess if the activity of $\sigma^B$ was affected. It was found that H$_2$O$_2$-induced activity was affected in most of these mutants. Interestingly of the 48 transposon mutants with loss of ring formation, a transposon insertion was identified in the gene \textit{lmo0818}. Further study of \textit{lmo0818::Tn} showed that this strain had other phenotypes consistent with a sigB mutant, such as an acid sensitive phenotype (Dr C. O’Byrne, Personal communication). The protein encoded by this gene has been characterised by Hein \textit{et al.} (2012) as a P-type ATPase cation transporter (Section 1.10.1) and is homologous to PacL in \textit{Synechococcus} sp. (Berkelman \textit{et al.}, 1994) and YloB in \textit{B. subtilis} (Raeymaekers \textit{et al.}, 2002). PacL has been confirmed as a P-type ATPase (Berkelman \textit{et al.}, 1994) calcium transporter, while YloB appears to have a role to play in sporulation (Raeymaekers \textit{et al.}, 2002). This is not the first P-type ATPase described in \textit{L. monocytogenes}. Recently Faxén \textit{et al.} (2011) characterised LMCA1, a Ca$^{2+}$-dependent ATPase. This calcium transporter demonstrates a low Ca$^{2+}$ affinity, and activity is induced by a high pH. P-type ATPases within prokaryotes have been shown to have functions associated with virulence and survival of the cells at high extracellular cation concentrations found in the host (Francis and Thomas, 1997; Rosch \textit{et al.}, 2008). Cytosolic calcium has
been proposed to play a role in different cell functions, for example, chemotaxis, stress response, gene transcription, cell division, and maintaining cell wall structure (Dominguez et al., 2015). Whilst structural and sequence analysis suggest that Lmo0818 could be a cation transporting ATPase, specifically involved in transporting Ca\(^{2+}\), no biological data exists to date to confirm this (Hein et al., 2012; Section 1.10.1).

\textit{lmo0818} is located in an operon with three other genes, \textit{lmo0819}, \textit{lmo0820} and \textit{lmo0821} (Toledo-Arana et al., 2009) (Fig. 6.1). To date \textit{lmo0819} has not been characterised but BLAST searches against the non-redundant NCBI database revealed that this gene has no homologues outside of the \textit{Listeria} genus, whilst \textit{lmo0820} appears to encode an acetyltransferase homologue (Listilist \url{http://genolist.pasteur.fr/ListiList/}; Moszer et al., 1995). No function has yet been assigned to \textit{lmo0821}. There is a promoter sequence located upstream of \textit{lmo0818} and another one located just before \textit{lmo0820}. Two putative terminator sites are also located within the operon, one following \textit{lmo0819} and one behind \textit{lmo0820}.

![Figure 6.1. The \textit{lmo0818} operon. \textit{lmo0818} is transcribed in an operon along with \textit{lmo0819}, \textit{lmo0820} and \textit{lmo0821}. Transcriptional start sites (blue arrow), terminators (red arrowhead) and a SigB dependent promoter (green arrow) are all marked. Information was taken from Listilist (http://genolist.pasteur.fr/ListiList/; Moszer et al., 1995) and The Weizmann Institute of Science’s Listeria Browser (http://www.weizmann.ac.il/molgen/Sorek/listeria_browser/; Wurtzel et al., 2012). Interestingly, Wurtzel et al. (2012) undertook a genome wide comparative transcriptomic study to globally map the transcription start sites (TSS) of both \textit{L. monocytogenes} and \textit{L. innocua}. They compared both of these organisms when grown under different conditions including 30 and 37°C in different growth phases, and also included \textit{Δ}sigB and \textit{Δ}prfA mutants. Using this browser (http://www.weizmann.ac.il/molgen/Sorek/listeria_browser/; Wurtzel et al., 2012), it appears that there is a σ\(^B\) dependent promoter sequence located about two thirds of the way through \textit{lmo0818}. This can be determined as the TSS is present under all conditions except in the \textit{Δ}sigB mutant suggesting that SigB may play a role in the transcription of the end of this gene or possibly \textit{lmo0819} may be partially under the control of SigB. To investigate the possible role of Lmo0818 in σ\(^B\) regulation and to elucidate the biological role of Lmo0818, three mutants were constructed; two deletion mutants (EGD-
Δlmo0818 and EGD-e Δlmo0819) and a mutant with the internal σ^B promoter sequence mutated. This mutant had silent mutations within the -10 and -35 consensus sequences of the σ^B promoter internal to the lmo0818 ORF, to try to disrupt any potential binding of RNA polymerase at this site.

6.2. Results

6.2.1. Construction of mutation cassettes for each of the three mutants

The sequences of genes required for the construction of EGD-e mutants in this study were obtained from the ListiList database (http://genolist.pasteur.fr/ListiList/). Deletion cassettes for Δlmo0818 and Δlmo0819 had the entire gene deleted with the exception of 6 base pairs at the 5’ and 3’ ends of the gene. Cassettes were designed to include 294 base pairs (bp) upstream and downstream of the genes as well as 6 bp of the beginning and end of the gene making both cassettes 600 bp each (Fig. 6.2). At the 5’ and 3’ ends of the cassette, a digestion site for EcoRI was added to ensure easy removal of the cassette from the cloning vector. The lmo0818ΔPsigB cassette was designed slightly differently. A SigB dependent promoter site had been identified about two thirds of the way through lmo0818 (Fig. 6.2). Identification of this promoter sequence was achieved through sequence analysis using the known consensus σ^B promoter sequence in conjunction with the Weizmann Institute of Science’s Listeria Browser (Wurtzel et al., 2012). Upstream of the transcriptional start site, five bp changes were created with two falling within the -35 consensus sequence and 1 within the -10 consensus sequence. The other two fell in the sequence between these two regions to help ensure that RNA polymerase would not bind to this promoter sequence (Fig. 6.2). The changes were silent mutations and therefore would not affect the amino acid sequence of the Lmo0818 protein. This cassette consisted of a region of 39 bp which fell included the -35 consensus sequence up to the start codon thereby including the 5 bp mutations. 129 bp upstream and 132 bp downstream of this region were also included, giving this cassette a final size of 300 bp. This cassette also had two EcoRI digestion sites added, one at the 5’ and one at the 3’ end. The mutation cassettes were all commercially synthesised (MWG Eurofins; Section 2.9.1).
**Figure 6.2. Construction of the mutant cassettes.** (a) Cassettes for ∆lmo0818 and ∆lmo0819 were created by removing the middle of the target gene leaving only 12 bp segment of the gene along with 294bp upstream and downstream to create a cassette 600 bp long. IN represents the whole mutation cassette (b) The cassette for lmo0818∆PsigB was created by making 5 bp changes within the sequence of the SigB dependent promoter located two thirds of the way through lmo0818. The mutations were silent and had no effect on the amino acid sequence. The cassette had 129 bp upstream and 132 bp downstream of the 39 bp that contained the mutated region. This created a cassette 300 bp long. The -35 (underlined in blue) and -10 (underlined in red) consensus sequences are marked. (c) Confirmation by PCR using M13 primers of successful transformation of the cassettes carried on pKSV7 into L. monocytogenes (See Section 2.9.1 & Table 2.7). The size of the cassettes is demonstrated here. (1) ∆lmo0818 – 600 bp (2) ∆lmo0819 – 600 bp (3) lmo0818∆PsigB – 300bp. (d) The pKSV7 used as a vector for the new constructs. The multi-cloning site is located within the lacZ gene on the plasmid and the M13 primers are located either side of this site.

### 6.2.2. Purification and cloning of each cassette into pKSV7

The three mutant cassettes synthesised by Eurofins MWG were supplied already cloned into the plasmid pEX-A (Table 2.2). pEX-A was digested with EcoRI and the band corresponding to the cassettes were identified based on size, and purified (Section 2.8.7). The purified product was phosphatase treated (Section 2.8.8) before it was ligated to purified
pKSV7 (Table 2.2) in a 3:1 molar ratio (vector:insert; Section 2.8.9). The ligation was confirmed by confirming the size of the plasmid combined with the insert on an agarose gel (pKSV7::Δlmo0818 – 7.5 kb; pKSV7::Δlmo0819 – 7.5 kb; pKSV7::lmo0818ΔPsigB – 7.2 kb). The ligation mix containing the insert was transformed into electrocompetent *E. coli* Top 10 cells (Sections 2.6.1 & 2.8.10). Transformants were screened using M13 primers specific to the pKSV7 plasmid (Table 2.7; Fig 6.2 d). Successful clones were identified for each of the constructs and were named pKSV7::Δlmo0818, pKSV7::Δlmo0819 and pKSV7::lmo0818ΔPsigB, respectively.

### 6.2.3. Chromosomal integration of mutation cassettes

Following confirmation of the constructs in *E. coli*, plasmids containing the inserts were re-isolated and transformed into electrocompetent *L. monocytogenes* EGD-e cells. These were plated on BHI agar containing 10 μg ml⁻¹ chloramphenicol as a selective marker. Positive clones were identified by screening transformants using M13 primers to identify the presence of the plasmid construct. Transformants carrying each of the three plasmids were identified (Fig. 6.2 c).

Chromosomal integration involves integration of the plasmid containing the deletion cassette into the host genome at the region homologous to the cassette. pKSV7 is an ideal shuttle vector as it contains a thermosensitive origin of replication that inhibits independent replication at a non-permissive temperature. Colonies for each of the constructs were selected and grown at 42°C in BHI broth containing 10 μg ml⁻¹ chloramphenicol to select for colonies where the plasmid had integrated into the host genome (Section 2.9.1.2). Once consistent growth started to appear after re-culturing the cells in BHI broth, this was taken to indicate integration of the plasmid into the chromosome. To screen for possible integration, two different sets of primers were used in different combinations firstly to check for integration but secondly to check which type of integration had occurred. One set of primers were designed to fall just upstream and downstream of the region on the construct for Δlmo0818 and Δlmo0819, therefore existing on the wild-type chromosome (Fig. 6.3). However, for lmo0818ΔPsigB, the forward primer was designed to be specific for the mutated region of the sequence (Table 2.7). These primers were used in combination with M13 primers which are specific to pKSV7 and therefore outside the region of the construct. Depending on the size of fragments observed from a PCR, the integration of the plasmid could be determined (Fig. 6.3). For Δlmo0818, integration occurred downstream of the target
gene (Fig. 6.3 a), while the other two recombinations occurred upstream of the target loci on the chromosome (Fig. 6.3 b & c).

Figure 6.3. Confirmation of integration of the three cassettes into the host chromosome.
(a) Downstream integration of pKSV7::Δlmo0818 confirmed with primers M13F / 872 producing a product of 3293 bp (1) and M13R / 873 producing a product of 656 bp (2). (b) Integration of pKSV7::Δlmo0819 upstream of the wild-type gene detected with primers M13F / 875 producing a product of 759 bp (3) and M13R / 876 with a product of 1622 bp (4). (c) Upstream integration of pKSV7::lmo0818ΔPsigB detected with primers M13F / 878 (5) giving a product of 523 and
M13R/877 (6) giving a product of 150 bp. The diagrams below each of the gel images show the orientation of the plasmid when integrated into the genome for each individual mutant. The angle arrows on the diagram indicate the location of the primers used and IN represents the mutation cassettes for each of the mutants. The primer sequences are available in Table 2.7.

6.2.4. Plasmid excision and confirmation of mutant alleles

For plasmid excision, the strain is passaged at 30°C without antibiotic selection (Section 2.9.1.3). This is a second homologous recombination step in which pKSV7 is excised from the genome of the bacterial cell. During excision the plasmid exits the genome taking with it the target wild-type gene or the mutant cassette (Fig 6.4) and the loss of the plasmid is characterised by sensitivity of the strain to chloramphenicol. Approximately 100 colonies were screened for sensitivity to chloramphenicol for each mutant between passages 3-8. Each passage consisted of approximately 18 generations. Sensitive colonies were detected by the 4th passage for all strains. Mutants were confirmed using primers designed for the region outside of the mutation and a PCR was conducted to ensure the removal of pKSV7 using M13 primers. Positive colonies were selected for further study (Fig 6.4). These were named EGD-e Δlmo0818, EGD-e Δlmo0819 and EGD-e lmo0818ΔPsigB. The region containing the silent mutations in EGD-e lmo0818ΔPsigB was amplified by PCR and sequenced on both strands to confirm the expected mutations were present. These mutant strains were then available for further characterisation and phenotypic analyses.
6.4. PCR confirmation of mutants. (a) Confirmation of EGD-e Δlmo0818 using primers 872/873. Lane 1 to 2 shows the size shift from the Wild-type gene (3349 bp) to the deletion (769 bp). (b) Confirmation of EGD-e Δlmo0819 using primers 875/876. Lanes 3 and 4 show the Wild-type gene (1781 bp) and the mutated gene (981 bp) respectively. (c) Confirmation of EGD-e lmo0818ΔPsigB using primers 877/873. Using these primers 877 is designed to only bind the mutated region. Lanes 5 and 6 demonstrate that this primer doesn’t bind in the wild-type (5; red cross) but show the band for the mutant (6) (1337 bp). The diagrams under each gel indicate the location of the primers used and the size of the band expected in the Wild-type and the new mutants.

6.2.5. Confirmation of ring phenotype in mutants

Tiensuu et al. (2013) described the development of a transposon mutant library that was screened for mutants unable to produce rings in response to cycles of light and dark (Section 6.1) One such mutant harbour ed a transposon insertion in the lmo0818 gene. To confirm lack of ring formation in our newly constructed mutants, the ring phenotype assay was conducted. Strains were grown on 0.3% BHI agar and under oscillating cycles of light and dark (Section 2.5.3). In response to these conditions, the results of Tiensuu et al. (2013)
repeated. EGD-e formed rings but ΔsigB did not in response to light/dark cycles. It was also confirmed that the transposon mutant, lmo0818::Tn did not produce rings (Fig. 6.5).

To characterise the newly constructed mutants and determine how they responded to light, the mutants were exposed to five 12 h cycles of light and dark. Since the transposon had disrupted lmo0818, it was hypothesised that deletion of this gene would result in loss of ring formation as was demonstrated for lmo0818::Tn. However, all three mutants formed rings in response to these conditions (Fig. 6.5). This suggested that neither lmo0818 or lmo0819 contribute to ring formation by L. monocytogenes. Ring formation has been shown to occur in a σB-dependent manner with the sigB mutant unable to form rings. It has also been demonstrated that a mutant lacking lmo0799, which activates σB in response to blue light, does not form rings (Tiensuu et al., 2013; Section 6.1). The ability of lmo0818 to produce rings, suggests that σB activation is intact in this strain. This means that there must be another reason why the transposon mutant is not forming rings in response to light/dark cycles.

Figure 6.5. Ring formation occurred in response to oscillations of light and dark in the newly constructed mutants. Overnight cultures were equalised to OD₆₀₀nm = 2.0 and 2 μl was spotted onto BHI agar (0.3% w/v). Plates were incubated in the dark for 60 h or exposed to five consecutive 12 h periods of ambient light and dark. (A) EGD-e, (B) ΔsigB, (C) lmo0818:: Tn, (D) Δlmo0818, (E) Δlmo0819, (F) lmo0818ΔPsigB

6.2.6. Identification of a secondary mutation within lmo0818::Tn

Genomic DNA was prepared from both lmo0818::Tn cells and the parent strain EGD-e and sent for whole genome sequencing (WGS) by MicrobesNG to investigate the possibility of secondary mutations within the transposon mutant that could be responsible for the loss of ring formation. The genome sequence of lmo0818::Tn confirmed the presence of the transposon insertion within lmo0818. As σB activity appeared to be intact in lmo0818, it was
hypothesised that a mutation in the sigB operon could have led to the loss of ring formation in lmo0818::Tn. Examination of the sigB operon in both strains led to the identification of a base pair deletion at position 403 within rsbU in lmo0818::Tn that was not present in EGD-e or the ∆lmo0818 mutant (Fig. 6.6). This mutation caused a frameshift that created a premature stop codon at position 166 of RsbU, potentially inactivating the protein by truncating the protein by 50%. rsbU encodes for a phosphatase protein involved in the regulation of SigB via the dephosphorylation of RsBV (see section 1.3). A stop codon in this gene would likely result in an inability to activate σB, which in turn prevents RNA polymerase from transcribing stress related genes. Since the formation of rings has been shown to be σB-dependent, this would explain why lmo0818::Tn no longer produced rings in response to cycles of light and dark if it was unable to activate σB. It also explains the presence of a normal ring-forming phenotype in the ∆lmo0818 mutant, which had a wildtype rsbU allele. Thus the original phenotype detected in the lmo0818::Tn strain (Tiensuu et al., 2013) is most likely not attributable to the transposon insertion but is due to the presence of a secondary mutation in the rsbU gene.
Figure 6.6. Nucleotide and amino acid sequence alignment of \textit{rsbU} EGD-e compared to \textit{lmo0818::Tn}. A deletion at nucleotide position 403 (a) created a nonsense mutation leading to the formation of a premature stop codon at position 166 (b) within RsbU of \textit{lmo0818::Tn}. Sequence alignments were performed using Geneious 10.0.
6.2.7. Phenotypic characterisation of Δlmo0818, Δlmo0819 and lmo0818ΔPsigB

Following indication that Lmo0818 may not contribute to SigB activation, a series of phenotypic assays were carried out to attempt to characterise each of the newly constructed mutants. Salt tolerance assays were carried out using 1 M NaCl (Section 2.5.1.3). No reproducible differences in salt tolerance were observed between the strains when compared to the parental strain (Fig. 6.7 a). The ΔsigB mutant grew better than the parent EGD-e strain and the three mutants, as observed previously (Section 4.2.2 and Section 5.2.3). Blue light growth assays (1.5-2.0 mW cm\(^{-2}\)) were also undertaken with the mutants and similar results to the salt assay were observed (Fig. 6.7 b).

![Figure 6.7. Growth of mutants was similar to EGD-e in the presence of 1M NaCl or blue light.](image)

Overnight cultures were standardised to OD\(_{600nm}\) = 1.0 and diluted to 10\(^{-8}\) in PBS. Four microliters was spotted onto BHI agar with 1M salt and wrapped in aluminium foil (a) or exposed to blue light (b; 1.5 -2.0 mW cm\(^{-2}\); 470 nm) or BHI agar only incubated in the dark (c). All plates were incubated at 30°C for 24 h. Each plate represents a minimum of three independent replicates.

The acid tolerance of the mutants was measured at pH 2.5 over 2.5 h (Fig. 6.8). Whilst ΔsigB exhibited expected sensitivity to acid, no differences were observed between the three mutants and the parent strain in this assay. Therefore no phenotype was discernible within the range of assays tested here.
Figure 6.8. No difference in survival between mutants and EGD-e at pH 2.5. One millilitre of
overnight culture was resuspended in BHI acidified to pH 2.5 with HCl (Section 2.5.6). Samples were
taken every half hour for 2.5 h and plated onto BHI agar. Error bars represent the standard deviation
between three replicates.

6.2.8. Addition of EDTA leads to a growth defect in EGD-e

As discussed in the introduction of this chapter (Section 6.1), the structure of Lmo0818 has
been crystallised and is homologous to PacL in Synechococcus sp. (Hein et al., 2012). In
Synechococcus sp. this protein has been characterised as a Ca\textsuperscript{2+} P-type ATPase (Berkelman
et al., 1994). As a homologue, Lmo0818 may also be involved in cation transport,
specifically calcium transport into or out of the cell however, no functional assays have been
conducted with the protein to date. To determine which type of transport Lmo0818 may be
involved in, high and low levels of different cations were tested. These experiments could
also give information about the cationic substrate of the putative Lmo0818 transporter. If
the protein is involved in uptake of cations into the cell, limiting the concentrations of
cations in the medium may help to determine this as the mutant may grow poorly when the
cation is limited in the medium. Alternatively, increasing levels of cations in the medium
could determine if Lmo0818 is involved in exporting excess cations out of the cell. In this
case, a mutant lacking the transporter might be expected to show a cation-sensitive
phenotype.

To investigate the effect of low levels of cations on the cell, EDTA was added to DM
supplemented with 0.4 % glucose. EDTA chelates cations present in the medium. The total
concentration of divalent cations present in this medium was calculated as 1.67 mM. Therefore 1.67 mM of EDTA was added to give a 1:1 ratio of cations: EDTA. This was added to the medium prior to filtration (Section 2.2.8) and cells were equalised to OD_{600nm} of 0.05 before grown in a 96 well microtitre plate for 24 h at 37°C. Molecular biology-grade water was used to prepare the media in an attempt to limit the amount of additional cations that may be present in trace amounts in non-pure water. Addition of 1.67 mM EDTA appeared to inhibit the growth rate of cells when compared to that of the control cells grown in DM without EDTA (Fig. 6.9 a). The growth rate of cells grown with EDTA was 0.0136 h\(^{-1}\) compared to 0.0936 h\(^{-1}\) for cells in DM minus EDTA. The final OD_{600nm} of cells without EDTA was approximately 0.5, whereas when cells were grown in the presence of EDTA the final OD_{600nm} reached only 0.21 (P ≤ 0.001) after 24 h. These results suggest that the chelation of cations within the medium leads to a reduced growth rate of \textit{L. monocytogenes} (Fig. 6.9 a).

Addition of the normal amount (the amount required for normal growth in DM) of calcium (37 µM), magnesium (1.62 mM) or zinc (12.4µM) or cobalt (3.46µM), separately to the medium containing EDTA restored growth to some extent (Fig 6.10 b). The restoration of growth rate was higher for calcium (0.0617 h\(^{-1}\)) and magnesium (0.0892 h\(^{-1}\)) compared to the other cations (zinc 0.014 h\(^{-1}\) and cobalt 0.0109 h\(^{-1}\)). It is possible that this effect was caused by a simple titration of the EDTA from the medium by the additional divalent cations added. Supplementing 1.67 mM of the combined cations back into the medium also restored the growth rate (0.0566 h\(^{-1}\)) of the cells to some extent (Fig 6.9 a). This suggested that the repression of growth seen with the addition of a 1:1 ratio of cations: EDTA was due to the chelation of the cations and not due to other unexpected toxic effects of EDTA on the cells.
Figure 6.9. Growth of EGD-e in the presence of EDTA. Overnight cultures were grown in DM supplemented with 0.4% glucose for 22 h. They were diluted to a starting OD\textsubscript{600nm} = 0.05 in fresh DM which had (a) 1:1 EDTA : cations (1.67 mM EDTA) or 1:1 EDTA: cations with additional 1.67 mM cations. (b) 1:1 EDTA : cations with additional cations added individually at the concentration normally required for growth in DM. Graphs are representative of the trend seen in two replicate experiments.

6.2.9. Varying concentrations of cations had little effect on the growth of *L. monocytogenes* EGD-e

Using DM supplemented with 0.4% glucose, it is possible to vary the concentrations of cations present in the medium. This can be achieved by making up an elements solution without the cations and adding the desired concentrations of cations separately from pre-prepared stocks (Section 2.2.8.10). Examining the effect of different concentrations of
cations on the growth of *L. monocytogenes* was carried out to determine any role of Lmo0818 in cation transport. Firstly different concentrations of Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Co$^{2+}$ and Mn$^{2+}$ were tested on the wild-type to determine a concentration that produced a measureable growth effect before trying those conditions in an experiment with the ∆lmo0818, ∆lmo0819 and lmo0818ΔPsigB mutants. Concentrations varied from 1000X less than the normal concentration to 100X more. The highest and lowest levels tested for each cation are shown here (Fig. 6.10 a & b). The medium included all the cations at normal required concentrations apart from the one being tested. Magnesium was the only cation that at low levels led to a decrease in growth of *L. monocytogenes* under these conditions. The final OD$_{600nm}$ of cells grown in low magnesium only reached approximately 0.26, whilst cells grown under the other conditions all reached 0.55-0.6 (Fig. 6.10 a). This result suggests that the cells entered into exponential phase similar to the other cultures but entered stationary phase quicker than the other cultures. To further confirm this, there was no significant difference between the growth rates of any of the strains tested. Alternatively, addition of high concentrations of cations had very little effect on the growth of EGD-e, with only zinc and cobalt showing significant inhibitory effects (Fig. 6.10 b). Interestingly, calcium appears to have no effect on the growth of EGD-e at high amounts or when no calcium is added to the medium. For further experiments the same concentrations of cations were used as shown in Figure 6.10 a and b.
Overnight cultures were grown in DM supplemented with 0.4% glucose for 22 h. They were diluted to OD\text{600nm} = 0.05 in fresh DM which had (a) low levels (100-fold reduced with the exception of calcium for which none was added) of each cation or (b) high levels (100-fold excess) of each cation present in the medium. Graphs are representative of the trend seen in two replicate experiments.

6.2.10. Growth of mutants was inhibited by EDTA but was not affected by varying concentrations of cations

A similar experiment was conducted as before to determine the effect of EDTA on the growth of ∆lmo0818, ∆lmo0819 and lmo0818∆PsigB compared to ∆sigB and EGD-e. Again the same effect was observed as seen for EGD-e where the growth was inhibited in the presence of 1:1 cations: EDTA (Fig. 6.11 a & b). This effect was also observed for all of the...
mutants under these conditions (Fig. 6.10 b). No significant difference was recorded between any of the strains ($P > 0.05$).

![Graph](image)

**Figure 6.11.** Growth of EGD-e, ΔsigB Δlmo0818, Δlmo0819 and lmo0818ΔPsigB in the presence of EDTA. Overnight cultures were grown in DM supplemented with 0.4% glucose for 22 h. They were diluted to a starting OD$_{600\text{nm}}$ = 0.05 in either (a) fresh DM or with (b). 1:1 EDTA : cations (1.67 mM cations). Graphs are representative of the trend seen in two replicate experiments.

All of the cations in section 6.2.9 were tested with the three constructed mutants along with ΔsigB and EGD-e at high and low concentrations. No significant differences were observed between any of the strains at high or low calcium, zinc or cobalt concentrations. As a representative of this data Figure 6.12 b and c show the results for the strains when grown in DM with no calcium and DM with 3.7 mM Ca$^{2+}$ (100X excess) respectively. No differences were noted for the endpoint OD$_{600\text{nm}}$ values ($P > 0.05$) for any of the strains under either of
these conditions. The growth rate of the strains was approximately 0.1-0.12 h⁻¹ with the exception of EGD-e, which was 0.064 h⁻¹ in DM with no calcium (Fig. 6.12 b). However, a similar growth rate was also observed for EGD-e in DM control during these experiments (Fig. 6.12 a). Therefore, there could have been some variability in the batch of media causing this result. There were no differences observed for the growth rate in excess calcium (Fig. 6.12 c). Since no obvious cation based phenotype was observed for the mutant lacking Lmo0818, this suggests that this protein may not be involved in the transport of Ca²⁺. Alternatively, it is possible that another system may be compensating for the loss of Lmo0818 and which may explain the lack of a phenotype.
Figure 6.12. Growth of EGD-e, ΔsigB Δlmo0818, Δlmo0819 and lmo0818ΔPsigB in the presence of no calcium and high concentrations of calcium. Overnight cultures were grown in DM supplemented with 0.4% glucose for 22 h. They were diluted to an OD$_{600nm}$ = 0.05 in either (a) fresh DM or DM that had (b) no calcium or (c) high amounts of calcium (3.37 mM). Graphs are representative of the trend seen in two replicate experiments.

Following on from the results in Figure 6.10a where a low concentration of magnesium had an effect on growth of EGD-e, a similar experiment was conducted using the mutant strains (Fig. 6.13). This experiment showed similar results where all of the cultures seemed to enter into stationary phase at an earlier stage than those grown in DM with the required level of magnesium. Cells grown in DM appeared to enter stationary phase at approximately 14 h (Fig. 6.13 a) but when the concentration of magnesium was limited in the medium, the cells entered stationary phase around 10 h (Fig. 6.13 b). Again there was only a very small difference in growth rate observed between the strains.
Figure 6.13. Growth of EGD-e, ΔsigB Δlmo0818, Δlmo0819 and lmo0818ΔPsigB in the presence of low magnesium. Overnight cultures were grown in DM supplemented with 0.4% glucose for 22 h. They were diluted to an OD_{600nm} = 0.05 in either (a) fresh DM or DM that had (b) a low concentration of magnesium (16.2 µM). Graphs are representative of the trend seen in two replicate experiments.

6.2.11. Expression of Lmo0818 is increased in the presence of EDTA

Expression levels of Lmo0818 and Lmo0819 were investigated by Western blotting using polyclonal antibodies against both proteins. The efficiency of the antibodies was first tested using cells grown to stationary phase over 16 h at 37°C in BHI broth (Fig. 6.14). For this experiment the Wild-type and ΔsigB were included along with the three newly constructed mutants. Lmo0818 was detected in all strains except for Δlmo0818 mutant. Expression levels were higher in EGD-e compared to ΔsigB. A similar pattern was observed for
Lmo0819 expression. Expression of both proteins was reduced in lmo0818ΔPsigB compared to EGD-e and ΔsigB, whilst deletion of lmo0819 also decreased expression of Lmo0818 compared to the wild-type. Deletion of lmo0818 led to a decrease in expression of Lmo0819. However, for Lmo0819, there appeared to be a faint band present in Δlmo0819. To confirm that this was due to nonspecific binding, the deletion in this strain was reconfirmed by PCR and Lmo0819 was blasted against the L. monocytogenes genome to check that there was no other possible binding site for the antibody. These were both confirmed suggesting the presence of some nonspecific binding to a protein with similar mass to Lmo0819 by the antibody.

![Image](image.png)

**Figure 6.14. Determining the efficiency of anti-Lmo0818 (105 kDa) and anti-Lmo0819 (34 kDa) antibodies in BHI broth.** Protein extractions were carried out on stationary phase cells grown for 16 h. Protein concentrations were equalised to 3.5 mg ml⁻¹. Fifteen microliters of protein was loaded to each lane with 0.05 mg of total protein per lane.

As Lmo0818 is thought to be involved in cation transport, Western blots were conducted to investigate the effect of the cation chelator EDTA on the expression levels of Lmo0818 and Lmo0819. For Western blots, the experiments had to be scaled up to harvest enough protein. Cultures were grown in 25 ml of DM in 50 ml Starstedt tubes to limit addition of cations that could be present in glassware. However, under these conditions, the growth of the strains was very slow and after 24 h of growth, the cultures had not reached the required OD to harvest the cells. For this reason, the overnight cultures were first grown in DM for 22 h at 37°C. They were subsequently re-inoculated into 25 ml of fresh DM supplemented with 0.4% glucose and grown for another 22 h. Cells were re-inoculated into fresh DM once more and grown to the required cell density (OD₆₀₀nm = 0.8-0.9) before the protein extractions were performed. For samples treated with EDTA, once they had reached this OD, 1.67 mM EDTA was added for 1.5 h prior to performing protein extractions. Western blots were carried out on the samples comparing treated and untreated cells (See section 2.7.3). In the untreated samples, expression of Lmo0818 was increased in ΔsigB compared to EGD-e (Fig. 6.14).
This is the opposite of the result observed in Figure 6.14 which means that the expression of Lmo0818 in ΔsigB is increased in DM but is reduced in BHI. This could suggest that SigB is acting as a repressor of Lmo0818 in the wild-type strain grown in DM. Expression Lmo0818 in Δlmo0819 and lmo0818ΔPsigB was also increased in the untreated samples compared to EGD-e. When EDTA was added to the cultures, expression of Lmo0818 increased in EGD-e, ΔsigB and Δlmo0819 compared to the untreated samples (Fig. 6.15). Expression of Lmo0818 decreased in lmo0818ΔPsigB in response to EDTA. Compared to expression levels in EGD-e with EDTA, Lmo0818 expression was increased in ΔsigB and Δlmo0819 but similar expression was observed between the wild-type and lmo0818ΔPsigB. As EDTA works by chelating cations in the medium, the increased expression of Lmo0818 in EGD-e treated with EDTA could suggest that this protein may be involved in the uptake of cations into the cell when levels of cations in the environment are low. Again it appears that this expression could be repressed by σB in the wild-type as levels were increased in the sigB mutant.

Lmo0819 expression levels were harder to make many conclusions from (Fig. 6.15). Compared to the band seen for Lmo0819 in Δlmo0819 in Figure 6.14, a much stronger band appeared for Δlmo0819 in DM despite the fact that a higher concentration of acrylamide bisacrylamide was used when preparing the SDS gel to try to separate out the bands further. Thus, without further purification of these anti-Lmo0819 antibodies it is difficult to arrive at any conclusions regarding factors influencing its expression.

![Figure 6.15. Addition of EDTA increases expression of Lmo0818 in EGD-e and ΔsigB strains.](image)

Bacterial cultures were first grown overnight for 22 h in DM supplemented with 0.4% glucose at 37°C. The overnight culture was diluted into 25 ml fresh DM to an OD600nm = 0.05 and grown to exponential phase (OD600nm = 0.8-0.9). Cells were re-inoculated into fresh media and grown to OD600nm = 0.8-0.9 before protein extractions were carried out. Extraction were equalised to 0.25 mg ml⁻¹ for each sample and 2.5 µg of total protein was loaded per lane (Section 2.7.2). Samples treated with EDTA (+) had 1:1 EDTA: cations (1.67 mM) added to the media and these samples were grown for a further 1.5 h.
6.2.12. Addition of excess cations to the medium leads to increased expression of Lmo0818 in ΔsigB, Δlmo0819 and lmo0818△PsigB

After observing that treatment of the cells with EDTA led to increased expression of Lmo0818 in EGD-e, further Western blots were carried out to test the effect additional cations had on expression levels. Experiments were conducted as before (Section 6.4.4) but this time 100X more calcium (3.37 mM) or 162 mM magnesium (162 mM) was added to the medium (Section 2.2.8.10). Whilst the expression levels of Lmo0818 in EGD-e increased in the presence of EDTA, they did not decrease in the presence of excess cations as might be expected if expression levels had increased to counter the low levels of available cations. Levels of Lmo0818 increased in EGD-e when excess calcium or magnesium (Fig. 6.14) was added to the cells. Again as before, the level of expression was also increased in the sigB mutant compared to the isogenic parent strain. Levels were also increased in the Δlmo0819 and lmo0818△PsigB mutant strains. The increase in expression of Lmo0818 in the sigB mutant compared to the wild-type in the presence of excess calcium or magnesium repeated for two independent experiments. However, this experiment was only carried out once with each of the newly constructed mutants and therefore these would need to be repeated before definite conclusions could be made.

![Figure 6.16. Addition of calcium and magnesium increases expression of Lmo0818.](image)

Bacterial cultures were first grown overnight for 22 h in DM supplemented with 0.4% glucose at 37°C. The overnight culture was diluted into 25 ml fresh DM to an OD_{600nm} = 0.05 and grown to exponential phase (OD_{600nm} = 0.8-0.9). Cells were resubbed twice more, before protein extractions were carried out. Extractions were equalised to 0.2 mg ml^{-1} for each sample. Samples treated with Calcium (+) had 3.37 mM Ca^{2+} or with Magnesium (+) had 167 mM added to the media and these samples were grown for a further 1.5 h. Controls were included where cells were grown in plain DM (-).
6.3. Discussion

In this study we investigated the biological role of a suspected cation transporter in *L. monocytogenes*. Through crystallisation of Lmo0818, it was predicted that this protein could be involved in transportation of calcium (Hein *et al.*, 2012). It was also suggested that *lmo0818* may be involved in the activation of the GSR through regulation of the alternative sigma factor, σ^B (Tiensuu *et al.*, 2013). Through the successful generation of three mutants in an EGD-e background, we have demonstrated that *lmo0818* does not appear to be involved in ring formation by *L. monocytogenes* in response to light and dark. Tiensuu *et al.* (2013) showed that rings are produced in response to oscillating cycles of light and dark in a σ^B/Lmo0799-dependent manner (Section 6.1). In this study, we confirmed that ∆*sigB* and *lmo0818::Tn* have indeed lost the ability to form rings in response to cycles of light and dark. It was also shown that the newly constructed ∆*lmo0818*, along with ∆*lmo0819* and *lmo0818∆PsigB* all produce rings under these conditions (Fig. 6.5). This suggests that Lmo0818 does not have a role to play in ring formation and that σ^B can be activated in all three mutants. To further confirm this, an acid sensitive phenotype characteristic to a *sigB* mutant (Abram *et al.*, 2008), was not observed for any of the mutants suggesting that *sigB* function was not impaired in these mutants (Fig. 6.6).

Through whole genome sequencing a secondary mutation in the *sigB* operon was identified that is likely to be responsible for the lack of σ^B activation in *lmo0818::Tn* (Fig. 6.7). As this mutation caused a frameshift that led to the formation of a premature stop codon in *rsbU*, the RsbU protein produced in this strain would be truncated by 50%. Since the catalytic domain of RsbU is located in the C-terminus of the protein (Delumeau *et al.*, 2004), RsbU, in *lmo0818::Tn*, is not expected to have phosphatase activity but should still be able to bind RsbT at the N-terminus as essential residues for binding RsbT are still present (Delumeau *et al.*, 2004; Hardwick *et al.*, 2007). As the catalytic domain is not present, RsbV would remain phosphorylated under stress meaning that the anti-sigma factor RsbW remains bound to σ^B. In this state, σ^B would be unable to bind to RNA polymerase and transcribe the GSR regulon (Section 1.3). The *sigB* operon in *L. monocytogenes* has been shown by data presented in Chapter 4 (Section 4.2.5) to be highly conserved between strains of *L. monocytogenes*. Consequently, mutations affecting this region of the genome can be significant regarding the function of SigB. Quereda *et al.* (2013) also found spontaneous mutations that impaired the function of SigB whilst constructing mutants lacking the surface proteins InlG and Vip. In three separate mutants, they identified secondary mutations in *rsbS*, *rsbU* and *rsbV*. All of these mutations resulted in loss of function of σ^B. They also suggest that similar observations were made in un-presented data by Cabanes *et al.* (2005).
Combined with the data presented here, these results suggest that conditions associated with the construction of the primary mutation (the transposon insertion into \textit{lmo0818}), somehow led to the selection of mutations within the \textit{sigB} operon. As discussed previously (See Section 5.2.1 & 4.2.2), deletion of \textit{sigB} can increase growth under certain conditions such as blue light or NaCl exposure. Therefore, spontaneous mutations affecting the \textit{sigB} operon could impart benefits to the cell under some circumstances. Together these findings highlight the need for caution when constructing mutants in \textit{L. monocytogenes} and suggest that whole genome sequencing should be routinely performed following mutant construction to ensure that secondary mutations are not inadvertently introduced.

Since no obvious stress response phenotype could be identified for any of the three mutants, experiments were conducted to investigate the potential role of Lmo0818 in cation transport. As shown in Figures 6.12 and 6.13 no differences could be seen between the mutants and the wild-type under the conditions tested. Magnesium at a low concentration was the only cation which induced any effect on growth in EGD-e (Fig. 6.10 a) but when tested on the mutants this inhibition of growth repeated for all (Fig. 6.13 a). One reason that no significant differences were observed may be due to the concentrations used. For example, it appears that \textit{L. monocytogenes} does not require calcium for growth under the conditions tested (Fig. 6.10 a; 6.12 b) but it was also observed that high levels of calcium had no effect on growth, although it is still possible that the concentration used needs to be higher to observe an effect (Fig. 6.10 b; 6.12 c). It is considered that many bacteria maintain their intracellular calcium concentrations at levels similar to eukaryotes (100 nM – 300 nM; Jones \textit{et al}., 1999, Torrecilla \textit{et al}., 2000, Dominguez, 2004), however it is possible that they are able to survive in higher concentrations For example, \textit{E. coli} maintains its intracellular calcium concentration at approximately 272 nM but when up to 10 mM of calcium was added to the extracellular medium, the intracellular concentration increased for a short period of time before reducing back to its basal level (Jones \textit{et al}., 1999). This suggests that bacteria can deploy mechanisms to ensure survival at high calcium concentrations.

It is possible that \textit{L. monocytogenes} has another transport system that compensates for the loss of Lmo0818. Recently another P-type ATPase calcium transporter has been characterised in \textit{L. monocytogenes}. LMCA1, encoded for by \textit{lmo0841}, is a calcium acquisition pump which exchanges H\textsuperscript{+} ions with Ca\textsuperscript{2+} from the extracellular environment (Faxén \textit{et al}., 2011). When LMCA1 and Lmo0818 are compared with known binding sites of the eukaryotic SERCA1a protein, they both have fully conserved Ca\textsuperscript{2+} site II, but site I is less conserved for both proteins (Hein \textit{et al}., 2012). It is possible that in the absence of Lmo0818, LMCA1 is able to overcome the extra pressure on the cell to keep internal
homeostasis. To determine if this is the case, a double knockout mutant would need to be generated lacking both the lmo0818 and lmo0841 genes. It has been previously observed that a phosphorylated intermediate of LMCA1 by ATP was evident in the presence of Ca$^{2+}$ but when this cation was removed the phosphorylation was abolished (Faxén et al., 2011). A similar approach could be taken with Lmo0818 to determine if the phosphorylation of this protein occurs in response to any particular cation, which might give insights into whether the activity of this transporter is dependent on a specific cation. If this experiment was carried out with a chelator added along with addition of specific cations, it may also help to determine if the formation of a phosphoenzyme intermediate is dependent on any particular cation.

Antibodies against Lmo0818 and Lmo0819 were commercially generated to try to understand the function and expression of these proteins. In BHI the expression of both Lmo0818 and Lmo0819 is lower in the ΔsigB mutant than in the wildtype. Expression was also reproducibly lower in the three mutants than in the wildtype (Fig. 6.14). This suggests that in BHI, σ$^B$ may induce expression of these proteins as removal of σ$^B$ leads to a decrease in expression. When a similar experiment was carried out in DM, the wildtype had the lowest level of expression compared to the other strains. From the Western blots it is very hard to reach a definitive conclusion and there are still many questions remaining unanswered regarding the expression of these proteins. In the Anti-Lmo0819 Western blots there was a nonspecific band present at the same size of the expected protein (Fig. 6.14 & 6.15). This made it extremely difficult to conclude anything from the blots for this protein (Fig. 6.15). Nevertheless, it was still possible to draw some conclusions from the Anti-Lmo0818 blots. It appears that σ$^B$ is involved in regulation of Lmo0818 as levels increased in the ΔsigB strain when grown in DM suggesting that SigB may act as a repressor of this protein in the wild-type strain in DM (Fig. 6.15). On the other hand, levels of Lmo0818 are also increased in Δlmo0819 and lmo0818ΔPsigB strains. This again suggests that lmo0818ΔPsigB is controlled by SigB but there is no evidence to suggest that lmo0819 is under σ$^B$ control. The increase in expression of Lmo0818 in Δlmo0819 could suggest that this gene is somehow involved in the repression of Lmo0818 but further experiments would need to be carried out to confirm this.

Addition of EDTA at the same concentration as cations present in the medium allowed for the chelation of cations. EDTA binds divalent cations meaning that the cations were no longer available for the bacterial cells to use and this resulted in a reduced growth rate (Fig. 6.9 a & 6.11 b). EDTA appeared to increase expression of Lmo0818 in EGD-e and the ΔsigB and Δlmo0819 mutants (Fig. 6.15). Again in DM medium the expression levels in
ΔsigB were higher compared to the wildtype again suggesting that SigB has a role to play in repression of this protein in the parent strain. However, an opposite effect was observed for lmo0818ΔPsigB. Unlike before, the expression level of Lmo0818 in lmo0818ΔPsigB was reduced compared to the wild-type and more interestingly compared to ΔsigB. This result was unexpected because if this is a SigB dependent promoter then it would be expected that both strains should act similarly. It is conceivable that SigB is involved in controlling the expression of Lmo0818 through another mechanism other than the internal σ^B dependent promoter. This could help to explain the differences in expression levels in the presence of EDTA (Fig. 6.15).

Since EDTA caused an increase in Lmo0818 expression in the wild-type compared to expression in DM only, it may suggest that Lmo0818 is involved in acquisition of cations into the cell (Fig 6.15). When the concentration of cations outside the cell is low, then bacteria need to use mechanisms to scavenge cations. However, this is contradicted by the data shown in Figure 6.16. If Lmo0818 was an uptake system, then addition of excess cations into the medium might be expected to lead to a decrease in expression but this was not the case. Instead treatment of the strains with excess calcium and magnesium (Fig. 6.16) led to an increase in expression of Lmo0818 which was the opposite of what was expected. This could suggest that the increase seen under both conditions may not be as a result of EDTA or addition of excess cations per se, but instead may be due to extra stress being put on the cells in response to the environment.

To further investigate whether Lmo0818 is involved in cation transport, to identify its substrate and to understand its regulation, further experiments are required. To investigate further the expression of this protein, studies looking at the transcription levels of the lmo0818 and lmo0819 genes would be required in the presence of different cations. These studies could help to give an insight into the type of pump Lmo0818 is and also which cation it is specific for. A similar experiment was carried out by Wichgers Schreur et al. (2011) whilst trying to characterise a manganese acquisition system in Streptococcus suis. They showed that transcript levels of troA were downregulated in the presence of excess manganese and in combination with other experiments they concluded that TroA was involved in manganese uptake.

Overall in this study, it has been determined that Lmo0818 is unlikely to be involved in activating σ^B in L. monocytogenes. Much more work needs to be carried out to determine the potential role of this protein in the transport of cations into or out of the cell. The expression of the protein does seem to be repressed by the presence of σ^B, but nothing is known about
how this is achieved. It is possible that Lmo0819 also has a role to play in repression of Lmo0818 but further investigation is needed to confirm and understand how this works.
CHAPTER 7

Discussion
7.1. Overview

*L. monocytogenes* is a bacterial pathogen of concern for the food processing industry. Throughout the course of this study, we have investigated the rate of occurrence of this foodborne bacterium within the Irish food industry and whether the alternative sigma factor, $\sigma^\text{B}$, has a role to play in persistence of this organism within food processing facilities. $\sigma^\text{B}$ is involved in the transcription of the general stress response regulon and here we demonstrate that $\sigma^\text{B}$ contributes to survival of the bacterium in the presence of blue light. We also demonstrate that the Lmo0818, a possible Ca$^{2+}$-ATPase, is unlikely to be involved in activation of $\sigma^\text{B}$.

7.2. Isolation and Characterisation of *L. monocytogenes* isolated from food processing facilities

7.2.1. Traditional methods of subtyping may be becoming redundant

During the course of this study, 255 *L. monocytogenes* isolates were obtained over a three year period. PFGE analysis of these samples yielded eighty six different profiles with seventeen being classified as persistent (Chapter 3). PFGE is the most common reference method for surveillance of *L. monocytogenes* and investigating outbreaks of listeriosis (Gerner-Smidt et al., 2006; Martin et al., 2006; Ragon et al., 2008, Moura et al., 2016). This method has been standardised and is recognised internationally through the PulseNet program (Gerner-Smidt et al., 2006). However, although multiple PulseNet networks exist internationally, naming of PFGE profiles is not widely synchronised between these networks. Furthermore, it is difficult to extract information regarding evolutionary relationships between clones through PFGE as many profiles are prevalent. Many PFGE profiles reoccur, and appear to be exceedingly widespread demonstrating a disadvantage of using PFGE to discriminate between strains as WGS has shown that often strains with indistinguishable PFGE profiles are not necessarily the same strain (Stasiewicz et al., 2015). Another subtyping method known as multilocus sequence typing (MLST) is a method of discriminating between strains based on the sequences of seven housekeeping genes, which allows them to be divided into different sequence types (ST; Ragon et al., 2008). This method offers a standardised approach to classifying genotypes of *L. monocytogenes* but still fails to differentiate sufficiently between closely related strains to give information necessary for surveillance of this organism (Moura et al., 2016). The limited information provided by both of these methods means that often strains are classified as persistent within
an environment as the same PFGE profile or ST reoccurs over a long period of time. However if a method with greater resolution was used to further classify these strains, it might identify strains as being unrelated and having different sources. Therefore, developing a method that can provide more information than traditional subtyping methods has become a vital topic of research to help overcome problems of *L. monocytogenes* contamination in the food chain.

### 7.2.2. WGS-based subtyping vs traditional methods

As the cost of whole genome sequencing (WGS) is continuing to decrease, it has been suggested that methods utilising the whole genome sequence are a more useful and beneficial way of scrutinizing differences between strains that could be overlooked by other subtyping methods. Through this approach it is possible to gain information on the evolution of clones in a food processing facility and can help to track the origin and transmission of outbreaks of *L. monocytogenes*. This technique has already been used for surveillance of other foodborne pathogens including *S. typhimurium* and *E. coli* (Joensen et al., 2014; Leekitcharoenphon et al., 2014). Moura et al. (2016) have recently developed a new method of MLST called core genome MLST (cgMLST). This is a genome wide method of strain genotyping that classifies strains based on comparison of 1,748 loci and groups the isolates into cgMLST types (CT). From this method it is possible to gain an insight into the epidemiology of *L. monocytogenes* which other methods of subtyping cannot accomplish. During their analysis, out of 100 *L. monocytogenes* strains tested, 68 individual CTs were identified. This compared to only 36 distinct PFGE profiles identified for the same strains, demonstrating the ability of this technique to discriminate more accurately between strains (Moura et al., 2016). If this technique was applied to the study presented here in Chapter 3 of this thesis, it may be that there were many more subtypes identified and therefore less persistent strains than were classified by PFGE.

Moura et al. (2016) also suggest that cgMLST could be carried out alongside other WGS techniques such as variant calling. Identifying SNPs in the genomic sequence can be used to differentiate more accurately between two strains that may have the same PFGE profile or MLST (Stasiewicz et al., 2015; Fagerlund et al., 2016; Chen et al., 2017b). Stasiewicz et al. (2015) demonstrated by WGS-based subtyping that two isolates with identical PFGE profiles could be differentiated into separate WGS-SNP-based clades and that this specific clade was found in one deli compared to the PFGE type which was found in multiple. Therefore, a strain which may have been perceived as persisting based on PFGE profile may
have actually been two separate isolates. In this study, they also show that PFGE can lead to over differentiation of strains. They found that in some cases when a strain differed by three or less bands, this could actually be due to a single genetic event such as a prophage or plasmid being lost or gained (Stasiewicz et al., 2015). This means that whilst PFGE has differentiated a strain as different from another, it may have originated from a closely related common strain. These relationships can be better resolved through WGS-based subtyping. However, a disadvantage of this method is that it requires the use of reference strains for a comparison. It does not use predefined loci and therefore, it can be difficult to standardise for use as an international form of surveillance of L. monocytogenes. It is also possible that SNPs may arise through culturing of L. monocytogenes and therefore it can be difficult to say if a SNP is a real difference in the isolate encountered in the food facility (Orsi et al., 2008). In the present study one pulsotype, P44, appeared in all four food sectors and appeared to be a persistent isolate (Fig. 3.2). It is possible that this isolate is not persistent within the tested facilities but is in fact being reintroduced into the sites through an external source. This raises the possibility that a strain could be persisting at the site of an external supplier, which could be a common ancestor to clones found within sampled companies in the same sector. WGS-based analysis could help to determine if this was the case. Evidence of reintroduction of L. monocytogenes into food processing facilities has previously been suggested (Lomonaco et al., 2009; Fagerlund et al., 2016).

7.2.3. Genomic sequences can be beneficial for secondary analysis

Having the genomic sequences available for food isolates can also have other benefits apart from epidemiological classification. In studies aimed at identifying features that may contribute to persistence of food isolates it can be difficult to choose tests for phenotypic analysis. Many studies have investigated biofilm formation, salt tolerance, acid survival and resistance to different disinfectants (Lundén et al., 2008; Kastbjerg and Gram, 2009; Lourenço et al., 2009; Magalhães et al., 2016). The present study focused on light and salt exposure, acid tolerance, biofilm production and resistance to predation by protozoa (Section 4.2). All of these physical characteristics may be needed by the bacterium to overcome conditions encountered in the food processing industry. However, as our data shows (Section 4.2), the results for these types of assays are often variable and very little can be concluded definitively about whether the phenotypes contribute to persistence of an isolate. For this reason, having the genomic sequences can prove beneficial for identification of molecular mechanisms that may convey persistence of an isolate. Some have previously
suggested that no single genetic determinant of persistence exists, but instead a variety of factors may contribute to the persistence of this foodborne pathogen in a food processing facility (Carpentier and Cerf, 2011; Ferreira et al., 2014). The virulence potential of strains can also be surmised by analysing virulence associated genes within the genome of the isolates (Fagerlund et al., 2016; Moura et al., 2016). This is information that cannot be gathered from traditional methods of subtyping. Further work using WGS-based techniques could be carried out on the strains isolated in this study to further analyse the epidemiology of the isolates and confirm the information provided from PFGE. Secondary analysis could investigate whether some genes may potentially have a role to play in persistence and this could then be backed up by phenotypic testing rather than blind testing for phenotypes that may not contribute to persistence.

7.2.4. Transmission of *L. monocytogenes*

Studies such as the one conducted here can give beneficial insights into the relationship between food or environmental isolates and clinical isolates. Here, we have shown that when the PFGE profiles of 25 Irish clinical isolates were compared with the strains collected during our study, eleven of the industrial isolates displayed profiles of high similarity with eleven of the clinical isolates (Fig. 3.6). From this it can be speculated that whilst the origin of infection cannot be determined from this information, strains that were isolated throughout the course of this study are capable of causing infections through consumption of contaminated food products. Although it is also possible that these strains may not be closely related and just appear related because of the resolution of the typing method. WGS would be needed to confirm. Previous studies have also identified pulsotypes that are indistinguishable between clinical and food isolates (Fugett et al., 2007; Fox et al., 2012; Chen et al., 2017b). This information can be beneficial if the strain has only been isolated from one food facility or one food sector such as P45. However if as in the case of P44, the isolate has been found in multiple food sectors and is indistinguishable from a clinical isolate, it provides very limited information regarding the likely source of the outbreak. This means that further epidemiological analysis would need to be undertaken to determine the source of the outbreak. In these cases, PFGE could be used as a first source of reference but would need further analysis to confirm the route of transmission. Although, even then confirmation of route of transmission can be difficult because you don’t have information regarding the food the patient actually ate. Stasiewicz et al. (2015), also suggest that similar problems are encountered when using SNP-based techniques. They suggest that even a high
level of WGS similarity may not establish a common link between a particular food and human case especially if the strain is found widely in the environment –like P44- and could therefore get into the found chain in a variety of different ways. It was shown that strains that were nearly identical based on genome sequences could be isolated from different food facilities and therefore it could not be determined which isolate was associated with the human case. This could matter in the case of an outbreak as it would be difficult to determine the source and control the associated outbreak. For this reason, to gain a better understanding of the epidemiology of *L. monocytogenes*, a multi-approach strategy would be recommended including standard subtyping methods combined with WGS-based techniques.

### 7.3. Visible light affects the growth and survival of *L. monocytogenes*

#### 7.3.1. Reactive Oxygen Species mediate the inactivation by blue light under aerobic conditions

Previous studies have shown that bacterial cells can be inactivated by visible light (Maclean *et al.*, 2010; Luksiene and Paskeviciute, 2011; Endarko *et al.*, 2012; Murdoch *et al.*, 2012; Bumah *et al.*, 2015; Hosein *et al.*, 2016; Ramakrishnan *et al.*, 2016). Many report that visible light close to the UV end of the spectrum is capable of efficiently inactivating bacterial pathogens (Maclean *et al.*, 2010; Luksiene and Paskeviciute, 2011; Endarko *et al.*, 2012; Murdoch *et al.*, 2012; Ramakrishnan *et al.*, 2016). In this study, it was demonstrated that blue light in the range of 460 - 470 nm can inhibit the growth of *L. monocytogenes* at low intensities (1.5 – 2 mW cm\(^{-2}\)) and have a killing effect on cells at a higher intensity (8 mW cm\(^{-2}\)) of blue light (Fig. 5.1 & 5.6). Photodynamic inactivation (PDI; Section 1.9.2) as a method of controlling bacterial growth and survival requires oxygen in combination with a light source and a photosensitising agent, which can be added to the medium to increase the killing effect of light (Hamblin and Hasan, 2004; Luksiene *et al.*, 2010; St Denis *et al.*, 2011). Our study revealed that the addition of an exogenous photosensitizer is not required for growth or survival of *L. monocytogenes* to be affected by visible light (Fig 5.1). This result has also been observed in other studies investigating the effects of light on a wide range of bacteria (Maclean *et al.*, 2010; Murdoch *et al.*, 2012; Bumah *et al.*, 2015). Previously, it has been suggested that the photosensitizing agent becomes excited by the absorption of photons, which leads to the transfer of electrons to oxygen resulting in the
creation of reactive oxygen species or singlet oxygen (Sibata et al., 2001; Luksiene, 2003; Luksiene and Zukauskas, 2009; Robertson et al., 2009; Ramakrishnan et al., 2016). Examining our results, addition of reactive oxygen scavengers, catalase and DMTU, provided a protective effect against growth inhibition further confirming that the creation of ROS is essential for PDI of L. monocytogenes (Section 5.2.2.).

It is hypothesised that the inhibitory effect of light would occur at a much lower rate or not at all, in an anaerobic environment due to a lack of ROS production. Due to constraints in experimental design it was not possible for us to test this hypothesis during the study, but it is presumed that under these conditions, levels of growth and survival would be similar to the dark controls included in these experiments. Survival of anaerobes such as Porphyromonas gingivalis, Fusobacterium nucleatum and Protevotella sp. was shown not to differ between illuminated and non-exposed cultures under anaerobic conditions (Henry et al., 1996; Feuerstein et al., 2005). However, one group has more recently contradicted these studies by reporting reduced viability of Prevotella intermedia, Prevotella nigrescens and P. gingivalis following exposure to light in anaerobic chambers, suggesting that oxygen is not essential for inactivation and other components of PDI may contribute (Hope et al., 2013; Hope et al., 2016). They suggest that it is possible that ion radicals might be formed independent of oxygen by means of type I photodynamic reactions. Type I reactions are mediated by the transfer of electrons and/or hydrogen atoms creating excited forms of the photosensitizer (Hamblin and Hasan, 2004; Luksiene and Zukauskas, 2009; Robertson et al., 2009). The excited photosensitizer can interact with cellular components to create free radicals and radical ions, and this type of reaction is considered to be less dependent on oxygen than Type II (Huang et al., 2012). Type II reactions involve electron transfer to molecular oxygen to create singlet oxygen (Sibata et al., 2001; Hamblin and Hasan, 2004; Luksiene and Zukauskas, 2009; Robertson et al., 2009). Both types of reactions occur simultaneously but it has been suggested that under anaerobic conditions, type I reactions may occur independently of oxygen (Ergaieg et al., 2008; Huang et al., 2012; Hope et al., 2013; Hope et al., 2016). The ratio of occurrence of these reactions is dependent on the type of photosensitizer used and also on the microenvironment of the photosensitizer (Huang et al., 2012). This means that under anaerobic conditions, type I reactions may be able to occur independently of type II. Some studies have highlighted that inactivation of bacteria by type I or type II reactions may be species dependent. Gram negative bacteria have been shown to be more susceptible to hydroxyl radicals produced by type I whilst Gram positive bacteria are more susceptible to $^1$O$_2$ produced by type II (Ergaieg et al., 2008; Huang et al., 2012). If this is the case, it suggests that if type I reactions occur under anaerobic conditions,
then Gram negative bacteria may be inactivated at a higher rate than Gram positive microorganisms under these conditions, but this remains to be further investigated.

In contrast to this, effects of the addition of excess oxygen to cultures exposed to visible light have been investigated. Maclean et al. (2008) found that addition of gaseous molecular oxygen decreased viability of light-exposed S. aureus cells by 3 logs more than cultures without excess oxygen. Burns et al. (1996) found that addition of deuterium oxide also lead to reduced viability by Streptococcus mutans in response to light treatment. During the course of our study, we demonstrated that addition of reactive oxygen scavengers, DMTU and catalase, led to a decreased rate of inactivation in cultures exposed to light. This suggests that ROS do play a critical role in the inactivation of L. monocytogenes by blue light (Fig 5.3 & 5.4). Other studies have also shown that the rate of inactivation in the presence of reactive oxygen scavengers is decreased compared to normal oxygen conditions, although it is not completely prevented (Burns et al., 1996; Feuerstein et al., 2005; Maclean et al., 2008; Ramakrishnan et al., 2016). This may be because the scavengers used are often specific for a single type of ROS and therefore may not scavenge all the ROS created. Alternatively, it might be due to the location of ROS production in relation to the cell. Catalase, when added exogenously to the medium, is a H2O2 scavenger (Saito et al., 1998) that cannot penetrate the cell membrane and can only scavenge extracellular H2O2 (Ramakrishnan et al., 2016). H2O2 produced intracellularly can last for up to a few seconds but can also diffuse through the cell membrane. However, if some of the H2O2 does not leave the cell it can cause significant damage to cellular components (Ramakrishnan et al., 2016). DMTU is an intracellular scavenger of hydroxyl radicals (Bruck et al., 2001), but due to extremely short half-life of only \( 10^{-9} \) s of \( \cdot \text{OH} \), it has been suggested that at the concentration of DMTU (100 mM) might not be able to scavenge all the hydroxyl radicals produced (Ramakrishnan et al., 2016).

The requirement of oxygen for PDI could also help to explain the cell density effect observed in our study (Fig. 5.2). Blue light appeared to inhibit growth in cultures with a lower cell density more effectively than cultures with higher cell density. We hypothesise that the oxygen concentration may vary between these cultures, with more cells consuming more oxygen. This means that there would be less oxygen available to create ROS during light exposure leading to less growth inhibition in cultures with a higher cell density. However, to date there is very little evidence suggesting L. monocytogenes carries out aerobic respiration and therefore other explanations should also considered for the cell density effect observed. It could be envisaged that when more cells are present in a culture, there may be a shadowing effect where the mere number of cells present in a high density
culture means that some cells may be protected by others and uniform irradiation of the culture is not achievable as would be in a culture with a lower density of cells. It is also conceivable that more catalase may be produced in a culture with a higher cell density. Production of catalase by L. monocytogenes is encoded for the kat gene (Rea et al., 2005). It is possible that when more cells are present, more catalase would be produced by the cells to deal with the excess H$_2$O$_2$ being produced in response to light. Catalase produced endogenously by cells can penetrate the membrane (Ramakrishnan et al., 2016) and this means that it could diffuse from the cell and combine with catalase produced by other cells to combat H$_2$O$_2$. This means that if more cells are present in a culture, more catalase would be present than in lower density cultures. More research would need to be carried out to confirm this hypothesis.

7.3.2. Endogenous porphyrins act as photosensitizers

PDI involves a photosensitizing agent in addition to oxygen and light. Photosensitizers may be added to products in the form of a preservative or colouring, or may be found as coatings on surfaces. Na-Chlorophyllin, a derivative of chlorophyll is a water soluble food-grade colourant. Some studies have demonstrated that inclusion of this compound in the medium or on the surface of packaging before exposure to light, increased the rate of bacterial inactivation (Luksiene et al., 2010; Luksiene and Paskeviciute, 2011), whilst others have investigated photodynamic effects of the photocatalyst, TiO$_2$ (Yemmireddy et al., 2015; Ananpattarachai et al., 2016). This molecule, typically used as nanoparticles, has been subjected to much investigation in recent times due to its low cost and self-cleaning properties and therefore could be an ideal coating for materials in food processing environments. TiO$_2$ has strong oxidising properties when illuminated by UV-A light, but research is now being conducted to try to enhance this property in response to less damaging visible light (Yemmireddy et al., 2015; Ananpattarachai et al., 2016). In addition to added photosensitizers, it has been discovered that some bacterial cells including L. monocytogenes contain endogenous photosensitizers including molecules such as porphyrins and riboflavin (Panek and O’Brien, 2002; Buchovec et al., 2010; Liang et al., 2013; Matern et al., 2016). Using this as the target photosensitizing agent removes the need for an exogenous photosensitizer to be added for PDI (Maclean et al., 2008; Murdoch et al., 2012; Hope et al., 2013; Hope et al., 2016). In section 5.2 no additional exogenous photosensitizers were added to the experiments but it should be noted that complex media may of course have some. It is hypothesised that endogenous porphyrins became excited by light leading to the transfer of
electrons to create ROS. Porphyrins are known to be produced by *L. monocytogenes* as intermediates in the heme pathway (Panek and O'Brian, 2002). Studies have investigated whether increased levels of endogenous porphyrins can accelerate the inactivation of pathogens and Buchovec et al. (2010) demonstrated that increased quantities of endogenous porphyrins do indeed lead to a higher degree of killing of *L. monocytogenes* by visible light. Nitzan et al. (2004) demonstrated that porphyrin production could also be induced in other bacteria including *S. aureus* and *E. coli* by using a precursor of the heme pathway, 5 – aminolevulinic acid (5-ALA), to stimulate production of porphyrins in the cell and decrease viability of the bacterial pathogens. Collectively these findings suggest that light, under the appropriate conditions, could be used as a mechanism of curtailing bacterial contamination within food processing environments or medical facilities. One study has demonstrated the successful use of High Intensity Narrow Spectrum (HINS) light as an extra method of decontamination in a hospital isolation room (Maclean et al., 2010). They installed two HINS-light units onto the ceiling of the room and they were synchronised with the normal room lighting so they could be used during the day without disrupting the normal work carried out in the room. The lights were designed to irradiate 10 m². This method successfully reduced the bacterial contamination in the room and kept levels low when the lights were switched off without causing any damage to patients or staff. However, bacterial counts were back to similar levels as before two days prior to light exposure. This study also only looked at hard contact points and excluded the floor. Whilst this method may be capable of reducing contamination within a hospital facility, it may be hard to envision it within a food processing facility. On the manufacturing floor of a food processing company, it may be difficult to irradiate all areas with some of the machines potentially containing niches for bacteria to evade illumination. As drains and floors have been shown to harbour *L. monocytogenes* (Section 3.1), illumination of these areas might prove very difficult. Other factors such as sensory effects on food products would also have to be considered.

7.3.3. **Sunlight limits numbers of *L. monocytogenes* cells in seawater**

Sunlight is considered to be a major environmental stressor of bacteria (Rollins and Colwell, 1986; Davies and Evison, 1991; Gourmelon et al., 1994; Besnard et al., 2002; Sinton et al., 2002; Sinton, 2005; Sinton et al., 2007). This study has observed that sunlight has a dramatic effect on the survival of *L. monocytogenes* in seawater (Fig. 5.11). We show that this bacterium can survive over long periods of time in seawater when maintained in dark conditions (Fig. 5.10); however viability decreased rapidly when exposed to sunlight (Fig.
5.11). We have also shown that both visible light (470 nm) and UV-A light contribute to the inactivation of this pathogen in seawater (Section 5.2.7.). These types of light are considered most associated with solar inactivation in seawater. UV-C does not penetrate through the Earth’s atmosphere and it is believed that very little in the UV-B range reaches below the seawater surface (Sinton, 2005).

Other studies have reported on the inactivation of different bacteria in seawater by sunlight (Sinton et al., 2002; Sinton et al., 2007; Sassoubre et al., 2015). For example, Sinton et al. (2007) found that *Campylobacter jejuni*, *Salmonella enterica* and *E. coli* were all inactivated when exposed to sunlight in live seawater, with viability of *C. jejuni* decreasing the fastest. They suggest that there may be a link between salinity and inactivation by sunlight as the viability of these three bacterial species were decreased to a lesser extent in live river water (Sinton et al., 2002; Sinton et al., 2007). Whilst these studies conclude that the bacteria have been inactivated, it has previously been hypothesised that the cells are not inactivated by sunlight but simply transition into a reversible “viable but non-cultur able” (VBNC) state (Gourmelon et al., 1994; Besnard et al., 2002). It has been proposed that this may be a survival strategy employed by the bacterium to overcome the environmental stress encountered. Therefore, after exposure to sunlight the cells may be in a dormant state, but subsequently they may be capable of returning to a culturable state when the environment becomes more favourable. As seawater is a nutrient limiting environment, this could also contribute in combination with sunlight to the transition of bacteria to a VBNC state. This hypothesis has been suggested by different studies as a mechanism employed not only by *L. monocytogenes* but other bacterial species too, including *C. jejuni*, *Salmonella* and *E. coli*. (Rollins and Colwell, 1986; Davies and Evison, 1991; Besnard et al., 2002). Some further work could be carried out to determine whether the cells are dead or whether they are surviving as VBNC cells. Using a Live Dead stain is one method of clarifying this. However one difficulty with this method is that the stain binds to all live cells in the medium, therefore it would be difficult to distinguish between *Listeria* cells and the natural microbiota in the seawater. To overcome background live/dead signal from the natural microbiota in seawater, these experiments were repeated using filtered seawater. Whilst this did remove some of the background staining, it also showed that the survival of *L. monocytogenes* exposed to sunlight in filtered seawater was slightly increased compared to the unfiltered seawater (Fig. 5.13). This result suggests that natural microorganisms in seawater may have a role to play in inactivation of *L. monocytogenes* in live seawater. It could be possible that molecules secreted from the seawater microbiota, may enhance the effect of sunlight on the survival of *L. monocytogenes*. It has also been suggested that the
natural microcosm may include species that prey on bacteria, which could lead to a decrease in survival of the pathogen (Sinton, 2005). However, as the dark control has survived over the course of our light experiments, it is not likely that this plays a role over the time course of the experiment (Fig. 5.11 and 5.12).

7.3.4. $\sigma^B$ is activated in response to light

In the presence of high intensities of blue light ($6.7 - 8.0$ mW cm$^{-2}$), we observed that the absence of $\sigma^B$ had a negative effect on the survival of *L. monocytogenes* (Fig. 5.6) suggesting that $\sigma^B$ is required for survival during exposure to severe light stress. Interestingly, it was also shown that the *L. monocytogenes* blue light sensor, Lmo0799 (Ondrusch and Kreft, 2011), does not appear to contribute to survival of the bacterium at high intensity blue light, as deletion of lmo0799 did not have an effect on the survival under these conditions (Fig 5.6). This suggests that effects generated by lethal doses of light must be sensed independently of Lmo0799 to ensure that the appropriate stress response mechanism is deployed. It could be that another one of the sensors found in the stressosome (Section 1.3) may activate $\sigma^B$ in response to another element other than light directly. As it is known that ROS are produced during light exposure (Hamblin and Hasan, 2004; Ergaieg *et al.*, 2008; Luksiene and Zukauskas, 2009; Huang *et al.*, 2012; Ramakrishnan *et al.*, 2016), $\sigma^B$ could potentially be activated independently of Lmo0799 in response to oxidative stress caused by the production of ROS in response to blue light. Tiensuu *et al.* (2013), identified other genes involved in responding to increased amounts of ROS through the $\sigma^B$-dependent pathway independently of Lmo0799. Boura *et al.* (2016), however, recently showed that in response to 3% H$_2$O$_2$, $\Delta$sigB is notably more resistant than the EGD-e wild-type strain. It is known that ROS, other than hydrogen peroxide, are produced in response to visible light (Hamblin and Hasan, 2004; Ergaieg *et al.*, 2008; Luksiene and Zukauskas, 2009; Huang *et al.*, 2012; Ramakrishnan *et al.*, 2016) and it could be that other ROS lead to the activation of $\sigma^B$ in response to high levels of light.

In the presence of low intensity blue light (1.5-2.0 mW cm$^{-2}$) the sigB mutant demonstrated increased growth compared to EGD-e (Section 5.2.3.). This effect was also noted for the $\Delta$lmo0799 mutant suggesting that at low levels of blue light, the bacterium activates $\sigma^B$ through the blue light sensor. Although no explanation for this result has been confirmed, one possible explanation for increase growth after deletion of sigB could be that activation of the stress response is energetically costly to the cell. The process of deploying the general stress response through signals sensed by Lmo0799 leading to the activation of $\sigma^B$, appear to
have a negative effect on the growth of *L. monocytogenes* under these conditions. A similar effect has also been observed in other studies where a fast growth phenotype was observed for *L. monocytogenes ΔsigB* in response to glucose limiting conditions and loss of function mutations in the SigB operon also led to fast growing phenotypes in response to energy related stress (Chaturongakul and Boor, 2004; Abram et al., 2008). It was also observed in our study that *lmo0818::Tn* exhibited a similar decrease in sensitivity to low intensity blue light, but this strain was shown to have a missense mutation in RsbU (Fig. 6.6), which is thought to affect σB activation. Removal of σB means that there may be less competition between sigma factors for RNA polymerase, suggesting that σA could have greater access to RNA polymerase directing more efficient transcription of growth related genes. A similar effect has been observed when rpoS is deleted in *E. coli*, with deletion of this gene completely inhibiting the transcription of the σS regulon in stationary phase cells, leading to a super-induction of σ70 related genes. This is thought to occur as there is no RpoS present to compete with σ70 for RNA polymerase resulting in transcription of housekeeping genes only (Farewell et al., 1998; Nyström, 2004). Other studies have also observed similar effects in response to mild stress (Schweder et al., 1999; Notley-McRobb et al., 2002).

7.4. **Structural and functional characterisation of Lmo0818**

7.4.1. **Structural studies of P-type ATPases**

Within *L. monocytogenes*, there appears to be at least two possible Ca\(^{2+}\)-ATPases present, LMCA1 and Lmo0818 (Andersen et al., 2011; Faxén et al., 2011; Hein et al., 2012). Through multiple sequence alignment, Faxén et al. (2011) were able to determine that LMCA1 had homology to the eukaryotic SERCA Ca\(^{2+}\)-ATPases. Their study focused mainly on determining the function of LMCA1 without an available crystallised structure. Through mutational analysis, they determined that this protein was involved in exporting Ca\(^{2+}\) from the cell in exchange for one H\(^{+}\) ion (Faxén et al., 2011). Following that study, Andersen et al. (2011) crystallised the structure of LMCA1. They confirmed the presence of Ca\(^{2+}\) binding sites and a structure similar to a SERCA P-type ATPase. However, in the case of Lmo0818, the structure of the protein has been crystallised but no biological function has been determined (Hein et al., 2012). Hein et al. (2012) suggest that since it is homologous and has similar calcium binding sites to other Ca\(^{2+}\)-ATPases, it could be involved in calcium transport but the possibility that it can transport other cations cannot be ruled out. As *L. monocytogenes* has at least two Ca\(^{2+}\)-ATPases, this could make it difficult to determine the
function of Lmo0818. As we have demonstrated here, generation of a mutant of \textit{lmo0818} did not produce any significant phenotype when we attempted to characterise it through phenotypic assays (Fig. 6.6, 6.8 & 6.9). There was also no significant phenotype observed for this mutant when it was exposed to different concentrations of cations including calcium (Fig. 6.11 & 6.12). It may be that in this case LCMA1 could be compensating for the loss of Lmo0818 in the mutant constructed in this study (Section 6.2). To investigate the role of Lmo0818 further, a double mutant of Lmo0818 and LCMA1 should be constructed to try elucidating a function.

Although the role of calcium is well characterised in eukaryotes, its role in prokaryotes still remains elusive. Some hypothetical calcium transporters have been identified in different bacteria but many of these putative transporters have not been well characterised (Dominguez \textit{et al.}, 2015). Many of these proteins are similar to the eukaryotic Ca\textsuperscript{2+}-ATPases, SERCA but structural and functional studies including mutational analyses are required to determine the function of these proteins and confirm a role in calcium transport. Some studies have crystallised the structure of calcium associated proteins but no further functional assays have been performed to confirm the biological role of the protein (Zhao \textit{et al.}, 2010; Hein \textit{et al.}, 2012). Once the structure has been crystallised, it is usually possible to predict the type of transport system and the cation specific to the transporter. However, it can be difficult to elucidate the role of the protein in vivo or in vitro. If a bacterium has multiple transporters for a specific cation, there may be redundancy associated when attempting mutational studies, making it very difficult to identify the role of the transporter in cation transport.

7.4.2. Cation transporters as possible drug targets

Ion homeostasis in prokaryotic cells is of vital importance for growth and survival. Therefore, ion transport systems including P-type ATPases, could potentially be used as drug targets. It has been observed that efflux mechanisms are essential for host pathogenesis and spore germination of many bacteria including \textit{S. pneumoniae}, \textit{B. subtilis}, \textit{C. perfringens} and \textit{M. tuberculosis} (Raeymaekers \textit{et al.}, 2002; Alvarez and Abel-Santos, 2007; Rosch \textit{et al.}, 2008; Novoa-Aponte and Soto Ospina, 2014). If an antimicrobial agent was developed to target essential cation efflux systems, this could potentially create a new viable antimicrobial strategy. CaxP, a Ca\textsuperscript{2+}-ATPase in \textit{S. pneumoniae}, is required for efflux of calcium in the presence of high calcium found in the host (Rosch \textit{et al.}, 2008). Rosch \textit{et al.} (2008) investigated if different inhibitors had an effect on the survival of the bacterium in high
concentrations of calcium. They observed that two of the five inhibitors tested targeted residues in the binding site of CaxP and therefore, inhibited the growth of the cells in high calcium concentrations. As CaxP has conserved homologues in other bacteria, it is possible that inhibitors such as the ones used by Rosch et al. (2008) may also block calcium transport in different bacterial species. However where redundancy exists this approach is unlikely to be successful.

Studies have shown that some *Bacillus* species rely on calcium efflux for spore germination (Raeymaekers et al., 2002; Alvarez and Abel-Santos, 2007). During spore formation, a sporulation-specific Ca\(^{2+}\)-ATPase is responsible for transporting high quantities of calcium into the spores. The calcium interacts with dipicolinic acid to form a complex that makes the spores more resistant to adverse conditions encountered in the environment (Alvarez and Abel-Santos, 2007). YloB has been characterised as a P-type ATPase involved in spore formation. In an *yloB* mutant, spores are formed but are much more sensitive to high temperatures than the wild-type (Raeymaekers et al., 2002). If a compound was designed to block calcium accumulation in the spores, this could potentially reduce the spread of the bacterium through spore formation particularly in hospital settings or food environments.

### 7.5. Concluding Remarks

Whilst *L. monocytogenes* continues to present a very real risk to human health we have a greatly improved understanding of its ecology, genetics and physiology. The ability to rapidly identify the sources of contamination using the latest genetic typing methods (including whole genome sequencing) means that food producers will better know where to target their efforts to reduce occurrence in foods. Through surveillance studies, such as the one presented here (Section 3), it is possible to gain insight into patterns associated with contamination of foods and food processing environments. Further analysis of the strains isolated here, may give more insight into factors affecting survival of *L. monocytogenes* within the food processing industry. Through identification of these factors it may be possible to develop strategies to help food producers to combat *L. monocytogenes* contamination within their facilities. Our understanding of the biology of *L. monocytogenes*, including detailed knowledge of the protective strategies it uses to defend itself against harsh conditions better equips us to design food processing and preservation strategies targeting this pathogen. There are still significant gaps in our knowledge, not least of which concerning the precise mechanisms that *L. monocytogenes* uses to sense its environment and
how it couples its stress response to its pathogenicity, but research in these fields is likely to produce answers to these questions in the near future. The prevalence of this pathogen in the natural environment and its ability to overcome harsh conditions means that eliminating it from the food chain is almost impossible. Development of new antimicrobial strategies should in the long-term help to combat this bacterium in the food processing industry. As we have demonstrated in our study, novel approaches such as inactivation with visible light (Section 5) could be adapted for use in the food processing environment to help reduce numbers of \textit{L. monocytogenes} cells residing in niches within these facilities to reduce the risk of infection to the consumer.
Bibliography

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Ferreira, A., Sue, D., O’Byrne, C.P., and Boor, K.J. (2003). Role of *Listeria monocytogenes* *σB* in survival of lethal acidic conditions and in the acquired acid tolerance response. *Appl Environ Microbiol* 69, 2692-2698.


FSIS (2014). FSIS Compliance guideline: Controlling *Listeria monocytogenes* in post-lethality exposed Ready-to-Eat meat and poultry products


213


216


Appendices
### Appendix 1

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</table>

Appendix.1. Complete results of the occurrence of *L. monocytogenes* in processing environments and food obtained from 54 food businesses in Ireland. Persistent pulsotypes are indicated in bold.
Appendix 2. Summary of sample number and % \textit{L. monocytogenes} positive at different locations and in different food sectors throughout Ireland. For each county, the food sector is shown (D – dairy; M – meat; S – seafood; V – vegetable), followed by the number of processing facilities sampled and the number of samples, followed by the percentage positives at those facilities.
Appendix 3

**Appendix 3. Fluorescent microscopy showing predation of *L. monocytogenes* by *T. pyriformis* after 17 h.** Bright field images are shown on the left and fluorescent images using a GFP filter are shown on the right.

Appendix 4

**Appendix 4. The presence of Lm0799 negatively affects growth in low intensity blue light.** Overnight cultures were standardised to an OD$_{600nm}$ = 1 and diluted, first tenfold followed by 1 in 5 dilutions in PBS. Dilutions were spotted on BHI agar and incubated in the presence or absence of light (470nm, 1.5 - 2.0 mW cm$^{-2}$).