Characterization of *Listeria monocytogenes* responses to food-related stress and population dynamics in soft cheese

By

Thulani Sibanda

Submitted in partial fulfillment of the requirements for the degree

PhD (Food Science)

In the

Faculty of Natural and Agricultural Sciences

University of Pretoria

July 2018

DECLARATION

I, Thulani Sibanda, declare that the thesis, which I hereby submit for the degree PhD (Food Science) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:…………………………………………..

Date:…………………………………………………..

ACKNOWLEDGEMENTS

ABSTRACT

Characterization of *Listeria monocytogenes* responses to food-related stress and population dynamics in soft cheese

Supervisor: **Prof Elna M. Buys**

Degree :   **PhD (Food Science)**

The success of *Listeria monocytogenes* as a pathogen depends on its ability to survive the many environmental stresses that it encounters along the food chain. However, response heterogeneity among strains and individual cells within populations affects the physiological states of the cells which subsequently influences the behavior and fate of the pathogen in contaminated foods. This study sought to investigate the influence of stress on the physiological states of *L. monocytogenes* strains and the potential influence on the persistence and survival of the pathogen in a ready-to-eat (RTE) food. Furthermore, the study also sought to elucidate the bimolecular changes related to the acid stress response of the pathogen.

Flow cytometry coupled with cell membrane integrity indicators showed that the degree of cell injury in *L. monocytogenes* strains (69, 159/10, 243 and ATCC19115) subjected to acid, osmotic and heat stress treatments was influenced by individual strain susceptibilities and the extent to which the stress exposure affects cell membrane integrity. Regardless of strain susceptibilities, acid stress induced the highest level of cell damage with osmotic stress causing the least. Following sorting of injured cells, the lag phase duration was the main difference in the resuscitation behavior of the stress-injured *L. monocytogenes* strains an indication that repair of cell injury was influenced by strain heterogeneity and extent of cell membrane damage. Importantly, once the injury was repaired, the resuscitated cells possessed a growth potential similar to non-injured cells regardless of strain or stress treatment

differences. Despite having a lower level of cell membrane injury, heat-injured cells were incapable of resuscitation in the majority of strains, an indication that the cellular targets of heat-induced injury are not necessarily limited to the cell membrane. Thus on its own, membrane integrity may not be a sufficient indicator of cell injury.

Stress pre-exposure and individual strain susceptibilities also influenced the survival responses and population dynamics of the pathogen in a lactic soft cheese. Kinetic model analysis revealed that while acid and osmotic stress pre-exposures resulted in sensitization of the susceptible strain (69), the same exposures resulted in induction of tolerance responses that protected tolerant strains against the acidity of the cheese. Although the osmotolerance response conferred cross-protection to food stress, it was not as high as the acid tolerance response. Genetic diversity analysis of surviving populations from mixed strain inoculations of the soft cheese revealed that after 15 days of storage, one persistent strain (159/10) remained as the dominant survivor.

As revealed by Fourier Transform Infrared (FT-IR) spectroscopy analysis, the effects of acid stress on both stress-susceptible (strain 69) and tolerant (159/10) strains involved disruptions in protein secondary structure, conformational changes in nucleic acids, and disruptions in cellular lipids and polysaccharides. However, changes in cell membrane lipid acyl chains related to membrane fluidity appeared to be an important factor in the acid stress response of the susceptible strain. Scanning electron microscopy showed that biomolecular changes were accompanied by a physical damage to the cell surface structures.

When subjected to lethal acid stress, a highly tolerant and persistent cell subpopulation that survived, owed its persistence to a phenotypic differentiation into a metabolically inactive state characteristic of persister cells. When the persister survivors were re-grown under

mildly acidic and cold conditions, the stress response gene expression profiles of the re-grown cell populations were not different from control cells indicating a general lack of heritable stress resistance. However, there was an exception with respect to the reduced expression levels of the phosphotransferase system (PTS) coding gene *lmo1038*. A down-regulation of PTS systems potentially infers a suppressed role for sugar uptake systems in persister survivors with a subsequent carry-over of such expression patterns in re-grown cells through epigenetic means.

The findings of this study indicated that stress-injured, and stress hardened tolerant cells can be a food safety risk if conditions in contaminated foods allow for their growth or survival. The stress-induced formation of persister cells provides a potential explanation for the challenge of *L. monocytogenes* persistence in food processing environments.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1   CHAPTER 1: PROBLEM STATEMENT

Listeria monocytogenes is a Gram-positive, facultatively anaerobic, psychrotrophic non-spore forming rod that is the aetiological agent of the human disease listeriosis, a rare but highly fatal food-borne infection (Donovan, 2015). The organism survives largely as an environmental saprophyte with major sources being water, soil, and vegetation (Linke et al., 2014). Human infections of *L. monocytogenes* arise from the ingestion of contaminated foods which in most healthy individuals, are largely without any consequences or at worst can manifest as self-limiting gastroenteritis (Warriner and Namvar, 2009). However, in the major risk groups such as immunocompromised persons, pregnant women, the elderly and infants, *L. monocytogenes* infections result in a highly invasive form of the disease characterized by septicemia, meningitis, encephalitis, perinatal infections, and abortions (Drevets and Bronze, 2008). While the frequency of occurrence of invasive listeriosis is relatively lower compared to infections caused by other food-borne pathogens such as *Salmonella* and *Campylobacter*, the high fatality rate of 20-30% in outbreaks makes it the deadliest of all food-borne infections (Warriner and Namvar, 2009).

The main vehicle foods associated with listeriosis outbreaks are contaminated ready-to-eat (RTE) foods, such as processed meats, smoked fish, soft cheeses, and salads (Angelo et al., 2017; Currie et al., 2015; Gillesberg Lassen et al., 2016; Heiman et al., 2016; Stephan et al., 2015). The absence of any thermal processing of these foods prior to consumption means that in the event of any post-processing contamination, control of the pathogen depends largely on the physicochemical characteristics of the foods such as pH, and water activity (a_w) (Shabala et al., 2008). In these RTE foods, the risk of listeriosis is related to the ability of the food product to support *L. monocytogenes* growth during the product shelf-life. Owing to its

psychrotrophic nature, the organism can readily multiply to dangerous levels in low-temperature stored RTE foods especially if the physicochemical hurdles are insufficient to prevent its growth (Wemekamp-Kamphuis et al., 2002). Based on Codex Alimentarius Commission (CAC) categorization, RTE foods with a pH > 4.4, a_w > 0.92, or a combination of pH > 5.0 and a_w > 0.94 are considered capable of supporting *L. monocytogenes* growth and are therefore high risk (CAC, 2009). Notwithstanding this risk-based profiling, a number of food products historically considered low risk have since been associated with outbreaks in recent years (Centre for Disease Control and Prevention (CDC), 2015a, 2015b, 2015c; Fretz et al., 2010; McCollum et al., 2013). These atypical vehicle foods historically not expected to support *L. monocytogenes* growth, include whole fruits (e.g. cantaloupe, apple, stone fruit), frozen foods (e.g ice-cream) and acid soft cheeses (CDC, 2015a, 2015c; Fretz et al., 2010). These realizations have raised questions about the current understanding of the ecology of *L. monocytogenes* growth and survival in foods (Buchanan et al., 2017).

Like all pathogens, the success of *L. monocytogenes* as a pathogen depends on its ability to survive and overcome the many environmental stresses that it encounters along the food chain as well as in the host. In the food processing environments, the pathogen may be faced with stresses ranging from fluctuations in temperature, pH, relative humidity, a_w as well as nutrient stress (Dikici and Calicioglu, 2013; Sadeghi-Mehr et al., 2016). Studies have shown that responses to these stresses vary among *L. monocytogenes* strains, serotypes and even among individual cells within a single population (Aryani et al., 2015a; Aspridou and Koutsoumanis, 2015; Lianou et al., 2006; Shen et al., 2014). Remarkably, these variations in stress responses affect the physiological states of the cells which subsequently influence the behavior and fate of the pathogen in contaminated foods, yet a good understanding of this behavior is central to the development of accurate models for prediction, risk quantification

2

as well as effective control measures. A lot of work has already been done to resolve variations in individual strains, with findings, for instance, showing that nearly all post-process contamination of foods is associated with the so-called persistent strains that colonize and establish permanent residence in food processing facilities (Wang et al., 2015; Wulff et al., 2006). However, the extent to which the physiological states of the cells within populations of the same strain affect *L. monocytogenes* responses in foods is a subject still to be elucidated. What has been apparent from researches involving single cell analysis techniques such as flow cytometry, cell sorting, and vibrational spectroscopy is that the net effect of the variations in response among individual cells is the formation of survivors in different physiological states (Kennedy et al., 2011; Krämer et al., 2016). These survival states consist of sub-lethally injured cells with a reversible damage to their cell components as well as non-injured stress tolerant cells.

The possibility of contamination with either of the two cell states presents different challenges to the safety of RTE foods. For injured cells, the ability to predict their fate and behavior in foods is difficult as such cells require conditions that permit the repair of cellular damage and resuscitation which also makes their detection in foods difficult (Wesche et al., 2009). On the other hand, non-injured survivors may develop adaptive stress tolerance responses that can enhance survival and persistence with the consequence that such survivors are protected against normally effective food preservation hurdles (Yousef and Courtney, 2003). The role of stress tolerance responses in the enhanced survival and persistence of *L. monocytogenes* is particularly important in food safety given the now established link that *L. monocytogenes* genotypes isolated from contaminated foods are nearly always related to the so-called persistent strains (Castro et al., 2018; Ferreira et al., 2014; Li et al., 2017). These are processing plant resident strains that are associated with repeated contamination of

products from the same plant over a long period of time (months and even years) (Nowak et al., 2017; Wang et al., 2015; Wulff et al., 2006). In the most recent listeriosis outbreak in South Africa, the outbreak strain (Sequence Type 6, ST6) was traced to an RTE meat processing plant environment, from where persistent contamination of the product occurred for more than a year (NICD, 2018). While stress resistance and ability to form biofilms have often been used to explain *L. monocytogenes* persistence in food plants, available literature does not support the conclusion that persistent strains are more stress resistant or better biofilm formers (Carpentier and Cerf, 2011). Thus there is a need for an improved understanding of the underlying basis of *L. monocytogenes* persistence.

2   CHAPTER 2: LITERATURE REVIEW

2.1   Introduction

Although the incidence of human diseases caused by *L. monocytogenes* is comparably lower than other food-borne pathogens such as *Salmonella* serotypes and *Campylobacter*, human listeriosis is the most fatal of all food-borne diseases. The most recent outbreak in South Africa, resulted in a record of 1060 cases and 216 deaths (as of 26 July 2018 statistics) (DoH, 2018) making it the deadliest of all food-borne diseases and the biggest outbreak in history (WHO, 2018). Notwithstanding current interventions by food industries to eliminate the risk of listeriosis in processed foods, available evidence shows that the pathogen is still a major food safety challenge. This review provides an analysis of the available literature on *L. monocytogenes* stress responses and how these responses influence the survival of the pathogen in foods and food processing environments. The review focuses on the history and taxonomy of this species, its ecology, as well as its transition from life as an environmental saprophyte to life as an intracellular pathogen. The organism's responses to specific stress factors such as acid, heat, osmotic and cold stress are also discussed in detail. The implications of stress responses on the safety of foods in the context of evolving food processing technologies driven by changing consumer demands for fresh and ready-to-eat (RTE) foods are also explored. Studies involving the relationship between *L. monocytogenes* stress response and its persistence in food processing environments are also reviewed.

2.2   History and taxonomy of the genus *Listeria*

The genus *Listeria* consists of Gram-positive, facultatively anaerobic non-spore forming rods. *L. monocytogenes*, the prototype species of this genus was the first to be described. The organism was first described by Murray in the United Kingdom in 1926 as a cause of

monocytosis in rodents and Murray named it *Bacterium monocytogenes* (Murray et al., 1926). In the following year, a similar organism was also described by Pirie in South Africa as a cause of death in gerbils. In honor of Joseph Lister, Pirie proposed the name *Listerella* for the new organism (Pirie, 1927). When the two scientists discovered they were dealing with the same organism, they called it *Listerella monocytogenes*. However, the nomenclature of the organism was later changed to *Listeria monocytogenes* in 1940 (Pirie, 1940). Historically, the genus *Listeria* was considered to consist of six species, namely *L. monocytogenes L. grayi*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, and *L. seeligeri* (Rocourt and Buchrieser, 2007; Wagner and McLauchlin, 2008). However, the classification of the genus has evolved with the discovery of new putative species since 2009 (Chiara et al., 2015; den Bakker et al., 2014, 2013; Weller et al., 2015). At present, the genus is deemed to consist of 17 species divided into two distinct groups, *Listeria* Sensu Strictu and *Listeria* Sensu Lato (Orsi and Wiedmann, 2016) (Figure 2.1). The group *Listeria* Sensu Strictu comprises six species that form a monophyletic cluster and share common phenotypic characteristics, namely *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, and *L. innocua* as well as *L. marthii* (Orsi and Wiedmann, 2016). With the exception of *L. marthii* that was only described in 2010 (Graves et al., 2010), the other five species had historically been identified as part of the genus having been described before 1985 (Rocourt and Buchrieser, 2007). On the other hand, the group *Listeria* Sensu Lato comprises of 11 species namely, *L. grayi* (first described in 1966) (Larsen and Seeliger, 1966) as well as the recently described species *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, *L. cornellensis*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, *and L. booriae* (Chiara et al., 2015; den Bakker et al., 2014, 2013; Weller et al., 2015). Although *L. grayi* is phylogenetically close to the *Listeria* Sensu Strictu species, it shares a recently discovered common ancestor with *L. fleischmannii*, *L. floridensis*, *and L. aquatica* (Orsi and Wiedmann, 2016; Weller et al.,

2015). Figure 2.1 illustrates the phylogenetic history of the current genus *Listeria*. Within the *Listeria* Sensu Strictu group, three monophyletic groups can be identified.

Notwithstanding all that is known thus far about this genus, what is emerging is that the taxonomy of this genus is still a long way to be resolved. This has become even more complicated given the continued discovery of new *Listeria*-like organisms such as the recently described *Listeria goaensis* sp. nov. (Doijad et al., 2018) and *Listeria costaricensis* sp. nov. (Núñez-Montero et al., 2018), that are not currently assignable to any existing species. It is against this background that Orsi and Wiedmann (2016) proposed a re-classification of the genus in which the genus name *Listeria* is restricted to *Listeria* Sensu Strictu species while *Listeria* Sensu Lato species are re-classified into three proposed genera (*Murraya*, *Mesolisteria*, and *Paenilisteria*) based on the separate monophyletic groups (Orsi and Wiedmann, 2016).
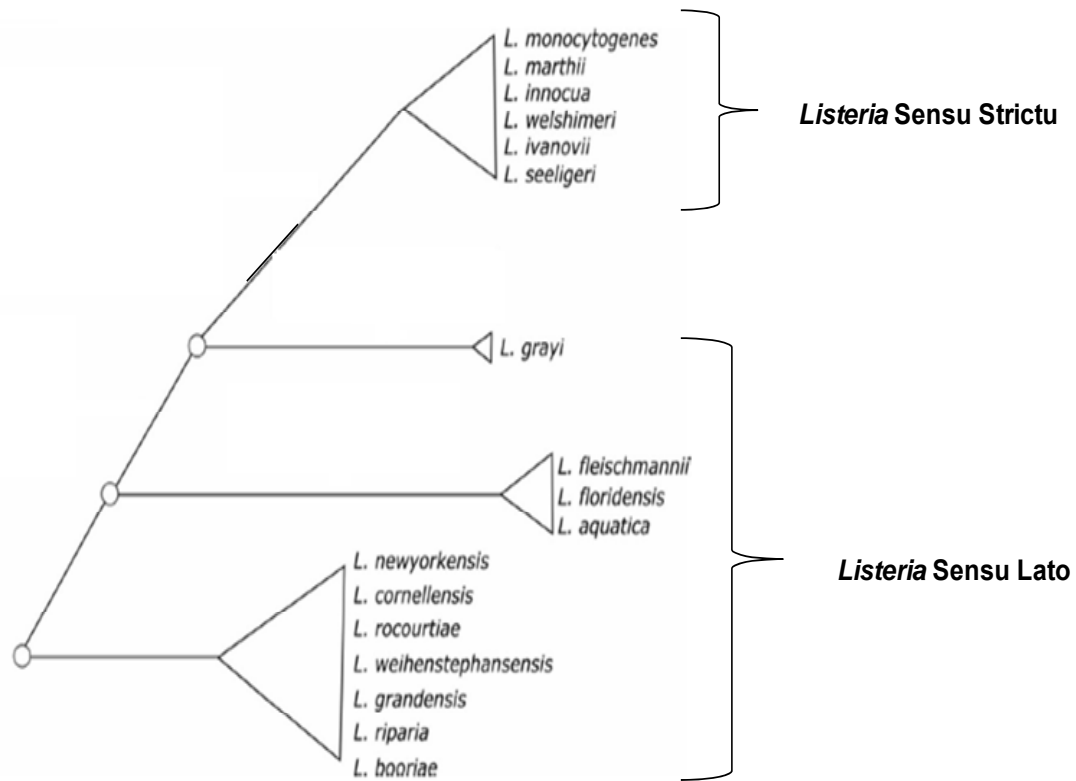
Figure 2.1: Schematic representation of the phylogenetic history of the current genus *Listeria*. Triangles represent the current monophyletic groups proposed. (Adapted from Orsi and Weidman, 2016

2.2.1 *The species* L. monocytogenes*: Evolutionary lineages*

Within the genus *Listeria*, the species *L. monocytogenes* and *L. ivanovii* are the only ones known to be associated with mammalian infections with *L. monocytogenes* specifically causing human listeriosis (Low and Donachie, 1997). Within the species *L. monocytogenes*, several genetic subtyping methods have since 1989 shown that the population structure of the organism is clonal (Piffaretti et al., 1989). By the year 2008, subsequent studies on the clonal nature of *L. monocytogenes* had identified four evolutionary lineages designated lineages I, II, III and IV (Cheng et al., 2008; Ward et al., 2008). An important aspect of the evolutionary clustering of *L. monocytogenes* is that lineages are linked to ecological niche preferences to the extent that lineages I and II strains are the only ones frequently (> 98%) isolated from

foods and human infected patients (Deng et al., 2010; Ward et al., 2010). Because of their

seeming lack of association with foods and human listeriosis, not much literature is available

about the ecology of lineage III and IV strains except one observation by Jeffers et al. (2001)

that lineage III strains appear to be over-represented in ruminant animals. The ecological

preference of lineage IV strains, the last of the lineages to be described is still poorly

understood (Orsi et al., 2011). Of the two lineages frequently associated with foods and

human infections (lineage I and II), differences also exist as lineage I strains are more

frequently associated with human listeriosis cases, whereas lineage II strains are more

frequent in foods (Lee et al., 2018; Maury et al., 2016).

Because of the contribution of lineage I and II strains in the global burden of food-borne

listeriosis, a lot of attention has been placed in understanding the genetic basis of their

adaptation to the human host and the food environment respectively. In particular, research

has focused on understanding the paradox that although lineage II strains are the most

frequently found in RTE foods, they are ultimately not the major cause of human listeriosis,

instead it is the less frequently encountered lineage I strains (Deng et al., 2010; Ward et al.,

2010). The differentiation between these two lineages is based on several single nucleotide

polymorphisms (SNPs) as well as absence or presence of some genes in their respective

genomes (Deng et al., 2010; Moorhead et al., 2003; Van Stelten et al., 2010). Inter-lineage

specific SNP assays based on multilocus genotyping (MLGT) have been developed using an

array of virulence (such as internalin genes *inlA*, *inlB*; hemolysin gene *hly*; phospholipase C

gene, *plcC*; the ActA protein gene, *actA*) and stress response genes (such as the alternative

sigma factor B gene, *sigB*) (Ward et al., 2010, 2008). Apart from the phylogenetic

importance, SNP genotyping has been useful in unraveling the genetic basis of the ecological

niche preferences between lineage I and II strains. Several studies have shown that lineage II

strains carry numerous mutations in their *inlA* genes that lead to premature stop codons and production of truncated forms of internalin A (da Silva et al., 2017; Nightingale et al., 2008, 2005). This has been supported by observations that mutations in the *inlA* gene are widely distributed in *L. monocytogenes* isolates from RTE foods but are less frequent in isolates from human listeriosis cases (Van Stelten et al., 2010). The virulence determinant inlA is necessary for the successful uptake of the pathogen by host cells, a critical factor in the infection cycle of *L. monocytogenes* (Pizarro-Cerdá and Cossart, 2007). As a consequence, despite their frequent occurrence in RTE foods lineage II strains are incapable of achieving a successful systemic infection. On the contrary, the overrepresentation of lineage II strains in the food environment is presumed to be related to the ability to withstand environmental stress (Orsi et al., 2011). Some available evidence shows that stress resistance phenotypes in *L. monocytogenes* are linked genetic lineages with lineage II strains particularly showing a better adaptation to environmental stresses such as salt, acid, and heat (Bergholz et al., 2010; Horlbog et al., 2018).

On the other hand, the near total lack of association of lineage III strains with either foods or human listeriosis cases has provided a need for unraveling the genetic basis of such divergence in ecological niche preferences in *L. monocytogenes*. In one of the few studies to have conducted a pan-genome analysis of 26 strains representing three lineages, Deng et al. (2010), identified 86 disparately distributed genes highly conserved in lineage I and II genomes but highly divergent or absent in lineage III genomes. In their study, the researchers found that among the genes disparately distributed across the lineages were genes involved in carbohydrate metabolism (phosphotransferase system (PTS) genes) and genes for transcription factors. The conclusion the researchers made was that lineage III strains are defective types that evolved by a gradual loss of metabolic function genes. In a more recent

study, Cerutti et al. (2017), further identified genes for small regulatory RNAs that co-evolved with genes for pathogenicity and host interaction present in the genomes of lineage I and II strains but missing in the genomes of lineage III strains thus reinforcing the hypothesis that lineage III strains evolved by loss of virulence functions.

2.2.2   L. monocytogenes *serotypes*

Based on surface markers comprising of cell wall teichoic acid (somatic, O antigens (1/2, 3, 4)) and flagella (H antigens (a, b, c)), *L. monocytogenes* has since the 1970s been classified into thirteen serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7) using the Seeliger/Donker-Voet scheme (Seeliger and Höhne, 1979). Interestingly, these serological differences are also linked to evolutionary lineages. Serotypes 1/2b, 3b, 4b, 4d, and 4e belong to lineage I, while serotypes 1/2a, 1/2c, 3a, and 3c, as well as 4a and 4c, belong to lineages II and III respectively (Orsi et al., 2011). With a predilection for the human host, serotypes belonging to lineage I are the ones generally associated with human infections of which serotypes 1/2b, and 4b are particularly responsible for most (> 95%) of the listeriosis infections with serotype 4b strains associated with the most severe cases of illness (Angelo et al., 2017; Burall et al., 2017; Montero et al., 2015).

2.3   Ecology of *L. monocytogenes*

Although *L. monocytogenes* is best known as a pathogen surviving inside infected human hosts, the organism actually survives for most of its life as a saprophyte in the environment (Renier et al., 2011). As a saprophyte, *L. monocytogenes* is fairly ubiquitous, being abundant in soil, water and vegetation (Linke et al., 2014) where it is believed to utilize nutrients, particularly from decaying vegetation to support its growth (Gray et al., 2006). Through contaminated feed, *L. monocytogenes* also colonizes the gastrointestinal tract of many

animals asymptomatically from where it is extensively disseminated through fecal matter and manure (Warriner and Namvar, 2009). Owing to its ubiquity, raw foods of both plant and animal origin readily carry the pathogen and in turn disseminate it into food processing plants (Castro et al., 2018; Dalzini et al., 2016). A number of longitudinal studies have identified a common occurrence of similar *L. monocytogenes* genotypes from the farm through the entire value chain of many foods (Castro et al., 2018; Dalzini et al., 2016; Martín et al., 2014; Rückerl et al., 2014).

2.4 Listeriosis: The transition from environmental saprophyte to intracellular pathogen

The disease listeriosis is a human infection that results from the ingestion of *L. monocytogenes* cells in contaminated food products. The clinical outcomes of the infection depend on the health status of the infected individual. In healthy individuals, the infection is limited to the gastrointestinal tract and often results in gastroenteritis accompanied by fever (Mclauchlin et al., 2004). In individuals with weakened immunity, the infection results in an invasive form of the disease affecting extra-intestinal tissues with symptoms that include meningitis, endocarditis, meningoencephalitis, and stillbirth (Donovan, 2015). Although the incidence of human listeriosis is often low compared to other food-borne diseases, the invasive form of listeriosis has a very high fatality rate in the major risk groups such as pregnant women, infants, the elderly and the immunocompromised (Warriner and Namvar, 2009).

Central to the pathogenesis of invasive listeriosis is the ability of the pathogen to invade human cells beginning with the epithelial cells of the intestinal tract. This process is mediated by surface expressed proteins internalin A and internalin B that facilitate endocytosis of the organism (Pizarro-Cerdá and Cossart, 2007). Following this internalization, the production of

listeriolysin O (LLO) a cholesterol-dependent cytotoxin, facilitates escape from the vacuole into the cytosol of infected cells (Pizarro-Cerdá and Cossart, 2007). Following a period of intracellular replication inside infected cells, the expression of ActA, a surface protein responsible for an actin-dependent motility system enables *L. monocytogenes* to move from one infected cell to an uninfected neighboring cell, while avoiding the immune system (Pizarro-Cerdá and Cossart, 2007).

As a facultative intracellular pathogen capable of both saprophytic life and life inside infected eukaryotic cells, the transition to life as an intracellular pathogen is facilitated by changes in gene expression patterns from environmental survival related genes to intracellular survival related genes. Central to the transition is the role of the Positive regulatory factor A (PrfA) encoded by the gene *prfA* (Freitag et al., 2009). PrfA is a transcriptional regulator that controls the expression of virulence genes in *L. monocytogenes* (Miner et al., 2007). Among the genes under the transcriptional control of PrfA are; genes that mediate the attachment and invasion of host cells, *InlA* and *InlB* coding for internalins A and B respectively; genes that mediate vacuolar escape, *hly* encoding listeriolysin O *and plcA* and *plcB* encoding phospholipases; genes that mediate bacterial spread from cell to cell such as *actA* encoding an actin polymerization protein ActA for intracellular motility (Freitag et al., 2009; Gray et al., 2006; Miner et al., 2007). Based on whole genome sequencing, the PrfA controlled virulence encoding genes are collectively clustered in one region of the bacterial chromosome as a virulence gene cluster termed *Listeria* pathogenicity island I (LPII) (Vázquez-Boland et al., 2001).

As the master regulator of virulence in *L. monocytogenes*, expressed only during intracellular growth and survival and repressed in saprophytic growth, the transcription of *prfA* responds

to physicochemical signals accompanying environmental transitions (Kuhn and Goebel, 2007; Miner et al., 2007). Although the mechanisms behind the alteration in gene expression are not clearly understood, it has been established that *prfA* expression responds to signals such as temperature, pH, glucose, and salt (Freitag et al., 2009; Kuhn and Goebel, 2007). For instance, PrfA-dependent virulence genes are not transcribed in *L. monocytogenes* cultures growing at 30ºC. However, a temperature shift to 37ºC provides a signal for the transcription of *prfA* and consequently the expression of the PrfA-dependent virulence gene cluster (de las Heras et al., 2011).

2.5   *L. monocytogenes* and RTE foods

Ready-to-eat (RTE) foods are foods that are either eaten in their raw state (unprocessed and minimally processed) or processed foods that are not subjected to any re-processing prior to consumption. This category includes foods such as fresh fruits and vegetables, salads, deli meats, smoked fish, and soft cheeses among others (Luber et al., 2011). The main routes of *L. monocytogenes* entry into the food chain and its subsequent contamination of RTE foods are illustrated in Figure 2.1.

The near-exclusive association of *L. monocytogenes* with RTE food products arises from three factors. The first factor is the lack of lethal processing hurdles that can guarantee pathogen elimination. This is particularly the case with minimally processed fresh fruits and vegetables and processed products whose preservation relies on combinations of many sub-lethal stress hurdles (hurdle technology) (Warriner and Namvar, 2009). The lack of lethal processing steps such as thermal processing presents an opportunity for *L. monocytogenes* survival in such foods (Tiganitas et al., 2009). The second factor is the colonization of food processing plants by persistent strains of *L. monocytogenes* resulting in the re-contamination of even foods whose processing involves lethal steps (Larsen et al., 2014). The third and

perhaps the most significant factor is the psychrotrophic nature of the organism. The universal dependence on refrigeration as a terminal hurdle in the preservation of RTE foods presents a potential for multiplication of the organism during refrigerated storage especially if the other physicochemical hurdles such as a_w and pH are inadequate to prevent growth (Angelidis et al., 2013).

Because of the importance of *L. monocytogenes* growth in RTE food during storage, the probability of growth of the pathogen in different RTE foods is critical to the level of listeriosis risk to consumers (Buchanan et al., 2017). Therefore a risk-based approach to profiling of RTE foods was proposed by the Codex Alimentarius Commission (CAC) with categorization into foods that support growth and those that do not support growth based on pH and a_w as physicochemical hurdles (CAC, 2009). RTE foods with a pH > 4.4, a_w > 0.92, or a combination of pH > 5.0 and a_w > 0.94 are products in which *L. monocytogenes* is capable of growth and therefore are high risk (CAC, 2009). For that reason, such products have a short shelf life under refrigerated storage. Because of the risk of *L. monocytogenes* in RTE foods, the Codex Alimentarius general guidelines recommend a safety threshold of 100 CFU/g of food product over the period of shelf life. In addition to the general guidelines, other countries such as the United States, Canada and regional blocs such as the European Union, have more specific and stricter guidelines (USFDA, 2017; European Commission, 2013; Health Canada Food Directorate, 2010).

Apart from the characteristics of *L. monocytogenes* enabling its growth in RTE foods, the challenge of listeriosis has been exacerbated by socio-economic factors such as the increasing consumer demands for fresh and nutritious foods and increased urbanization which has led to increased consumption of minimally processed and convenient RTE foods (Caggia et al., 2009).
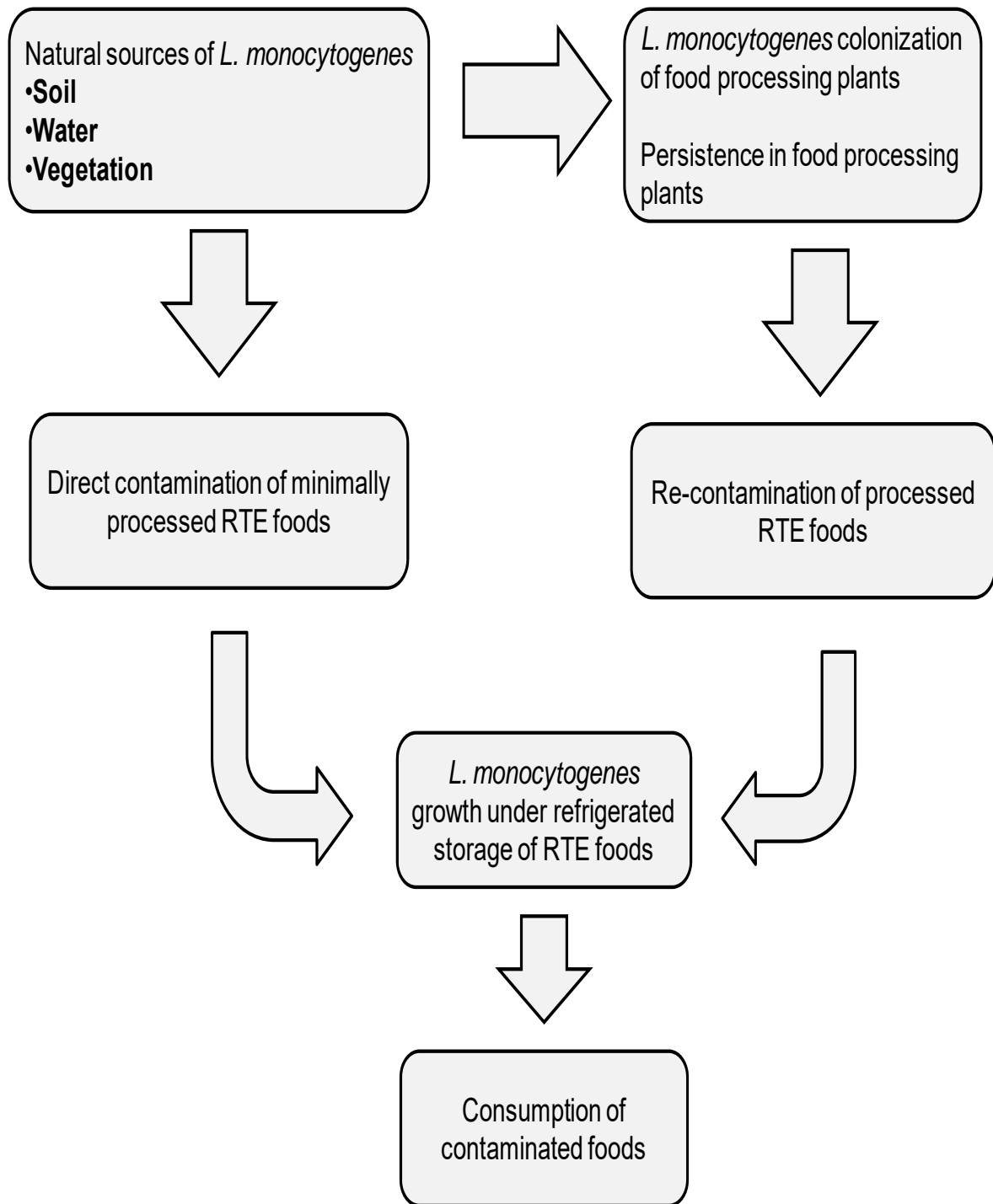
Figure 2.2: The main routes of *L. monocytogenes* entry into the food chain and its subsequent dissemination to RTE foods. (Figure based on personal creation).

2.6   Responses to environmental stresses encountered in the food chain

According to Booth, (2002), stress can be defined as "any change in the genome, proteome or environment that imposes either reduced growth or survival potential" in living organisms. In terms of microorganisms, conditions influencing microbial growth and survival in their habitats are never constant let alone optimum. Physical and chemical parameters such as temperature, pH, water activity (A$_w$), osmotic pressure and nutrient availability are always changing (Yousef and Courtney, 2003). Quite obviously these fluctuations result in destabilization of the homeostatic balance of microbial cells and hence an attempt to adjust cellular processes to a state that allows them to survive and grow under such stressful conditions (Vorob'eva, 2004). *L. monocytogenes* encounters a lot of physical and chemical stresses through the entire food chain from farm to fork (Gandhi and Chikindas, 2007). These range from changing osmotic pressures due to dehydration and use of salts, temperature shifts due to applications of heat and refrigeration, pH extremes due to acid exposure and use of alkaline cleaning agents among others (Gandhi and Chikindas, 2007). This review will give specific attention to the responses of *L. monocytogenes* to acid, heat, osmotic and cold stresses as some of the most pertinent stress factors influencing survival and growth of this pathogen in foods.

2.6.1   General stress response

In general, bacteria possess a coordinated response to environmental changes regardless of the form of stress encountered. This central regulation of response called the general stress response is attributable to global regulators of gene expression called sigma factors. As a regulator of gene expression, sigma factors are subunits of the RNA polymerase holoenzyme that recognize specific promoter sequences of target genes and thus dictate the cellular programme of gene transcription (Llorens et al., 2010). In *L. monocytogenes* and indeed other Gram-positive bacterial species, *Bacillus*, and *Staphylococcus,* in addition to the primary

17

sigma factor that regulates expression of house-keeping genes (sigma factor A, σ^A), an alternative sigma factor (sigma factor B, σ^B), regulates gene expression under conditions of environmental stress (Cetin et al., 2004; Guldimann et al., 2016; Wemekamp-Kamphuis et al., 2004). Initially described in *L. monocytogenes* in the 1990s (Wiedmann et al., 1998), several roles of this sigma factor have since been identified (Cetin et al., 2004; Lee et al., 2013; Sue et al., 2004; Wemekamp-Kamphuis et al., 2004). The regulon of this general stress response regulator has been identified to encompass up to 200 genes that are involved in *L. monocytogenes* environmental survival and some virulence functions (Kazmierczak et al., 2006, 2003). Specifically, genes involved in acid, osmotic, heat, cold, oxidative stress and starvation are under are σ^B-dependent (Abram et al., 2008; Chaturongakul and Boor, 2006; Ferreira et al., 2001; Wiedmann et al., 1998).

The inclusion of some virulence genes under the regulatory control of σ^B, has led to the conclusion that there is an overlap between the role of σ^B as the general stress response regulator and that of PrfA as the regulator of virulence gene expression in *L. monocytogenes* (O'Byrne and Karatzas, 2008). However, a close analysis of virulence genes under the control of σ^B has shown that only the genes needed for gastrointestinal tract survival and invasion are affected. Thus indicating that σ^B is necessary to initiate infection but does not play a role in the intracellular stages of infection (O'Byrne and Karatzas, 2008).

2.6.2 *Stress-sensing and signal transduction*

The regulatory protein σ^B is encoded by the gene *sigB* as part of an operon that includes seven other genes referred to as regulation of sigma B (Rsb) proteins (*rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, and *rsbX*) (Figure 2.2) involved in its transcription in response to environmental signals (Ferreira et al., 2004; Guldimann et al., 2016). Collectively, the Rsb proteins play a

role in the sensing of environmental changes and transduction of signals that result in σ^B activation and subsequent transcription of stress response genes. The proposed model for the activation of the general stress response has been elucidated for *L. monocytogenes* (Chaturongakul and Boor, 2004) (summarized in Figure 2.3). In exponentially growing cells, the σ^B protein exists as an inactive form bound to the anti- σ^B protein, RsbW (Guldimann et al., 2016). In response to environmental changes, the activation of σ^B is achieved by a partner-switching mechanism in which the release of RsbW is mediated by binding of the dephosphorylated form of the anti-anti-σ^B protein, RsbV (Guldimann et al., 2016). All these processes are triggered by environmental stress signals sensed by the sensor kinase RsbT which activates the phosphatase RsbU (Chaturongakul and Boor, 2004).
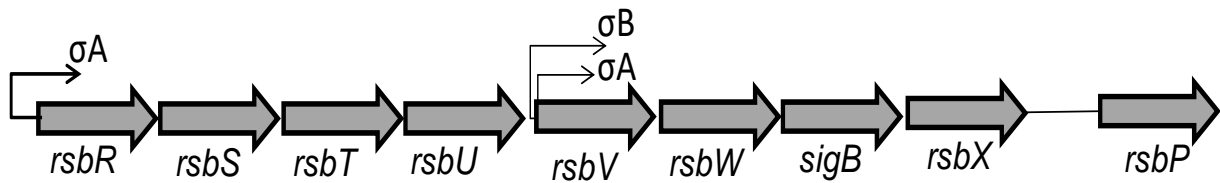
Figure 2.3: Organization of the *sigB* operon in *L. monocytogenes*. Thick grey arrows represent open reading frames. Thin bent arrows indicate promoters regulated by sigma factors, A and B respectively. (Adapted from Ferreira et al. (2004) and Guldimann et al. (2016)).
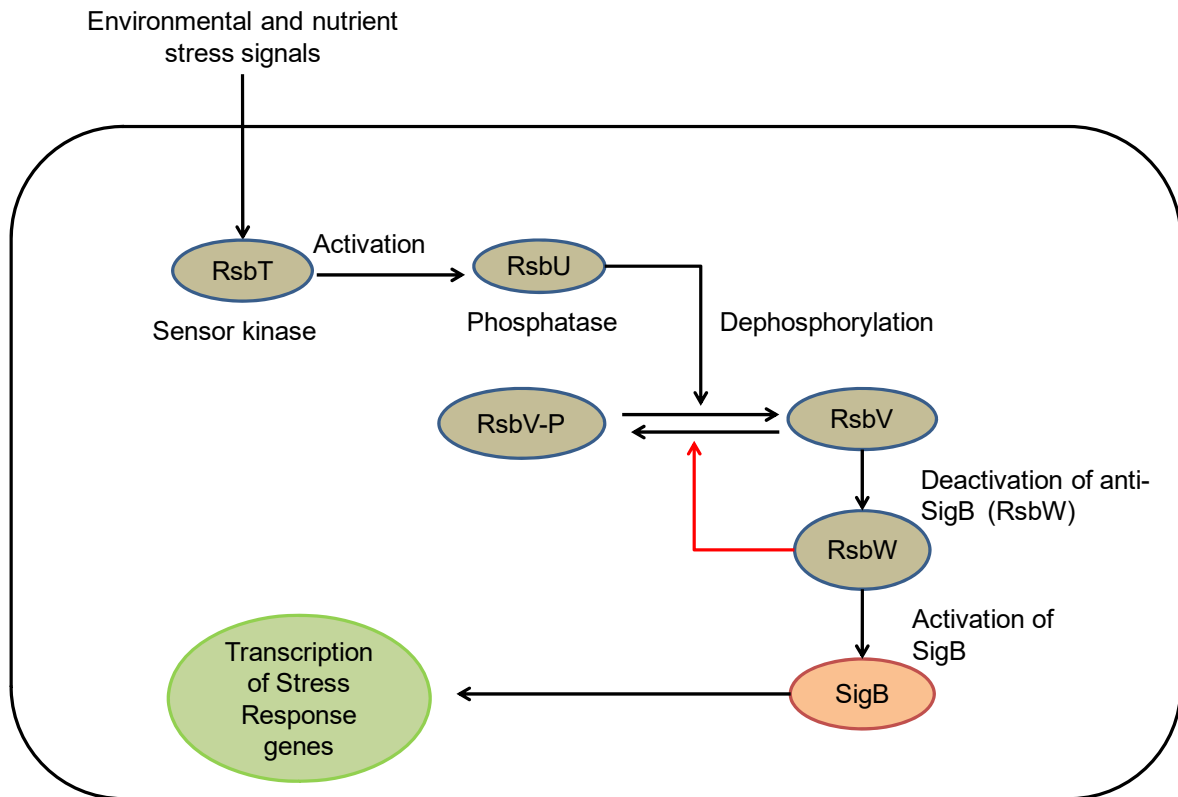
Figure 2.4: Proposed mechanism of Rsb-mediated regulation of SigB expression and activity in *L. monocytogenes*. Black arrows indicate the pathway of signal transduction and the red arrow shows the negative feedback loop. (Adapted from Chaturongakul and Boor 2004 and Guldimann et al. 2016)

2.7 Responses to specific food-related stresses

2.7.1 Osmotic stress response

Bacterial osmotic stress results from changes in the osmolality of the environment in which bacterial cells survive with the main consequence being the disruption of osmotic balance due loss or uptake of water. For *L. monocytogenes*, many conditions that impose osmotic stress occur along the food chain. These include exposure to dry conditions and exposure to hyperosmotic conditions due to the use of salt and sugars in foods. Broadly the effect of such stress is the disruption of osmotic balance in cells as a result of water loss (Burgess et al., 2016). The consequence of water loss is the loss of turgor which in extreme cases can result in cell death due to plasmolysis (Csonka, 1989).

The ability of *L. monocytogenes* to withstand hyperosmotic conditions has been known for a long time (Cole et al., 1990; Faleiro et al., 2003). In response to hyperosmotic conditions, the organism actively accumulates compatible solutes as a way of counterbalancing the negative effect of outward water movement. These solutes, are low-molecular-weight, highly soluble compounds that bear a neutral charge at physiological pH whose accumulation inside the cells helps in restoring turgor, without affecting cytoplasmic function (Angelidis and Smith, 2003) Although several compounds have been identified as potential osmoprotectants, two of these, glycine betaine (*N,N,N*-trimethylglycine) and carnitine (β-hydroxy-γ-*N*-trimethyl aminobutyrate), have been shown to the most potent at conferring osmoprotection. Notably, both compounds are not synthesized by *L. monocytogenes* but are fairly ubiquitous in foods of both plant and animal origin and therefore their intracellular accumulation is achieved by active transport from the environment (Angelidis and Smith, 2003; Ko and Smith, 1999).

Response to osmotic stress is triggered by changes in osmotic pressure as the main signal (Wood, 1999). However, the mechanism of signal sensing and transduction has not been elucidated in *L. monocytogenes* although membrane-bound sensor histidine kinases that are ubiquitous in the bacterial kingdom (extensively studied in *E. coli*) are said to play a role in stress sensing and induction of transporter gene expression (Wood, 1999). The mechanism as presently understood (summarized in Figure 2.4), is based on two glycine betaine transporters (Gbu and BetL) and a single carnitine transporter OpuC that respond to osmotic upshifts (Angelidis and Smith, 2003). Gbu is an ATP dependent transporter encoded by the *gbu* operon that mediates the uptake of glycine betaine in response osmotic and cold stress (Gerhardt et al., 2000). BetL is a non-ATP dependent secondary transporter encoded by *betL* whose uptake of glycine betaine is coupled to Na$^+$ symport (Sleator et al., 1999). Carnitine

transport is mediated by the ATP dependent transporter, OpuC a product of the *opuC* operon

that responds to both osmotic and cold stress (Sleator et al., 2002).
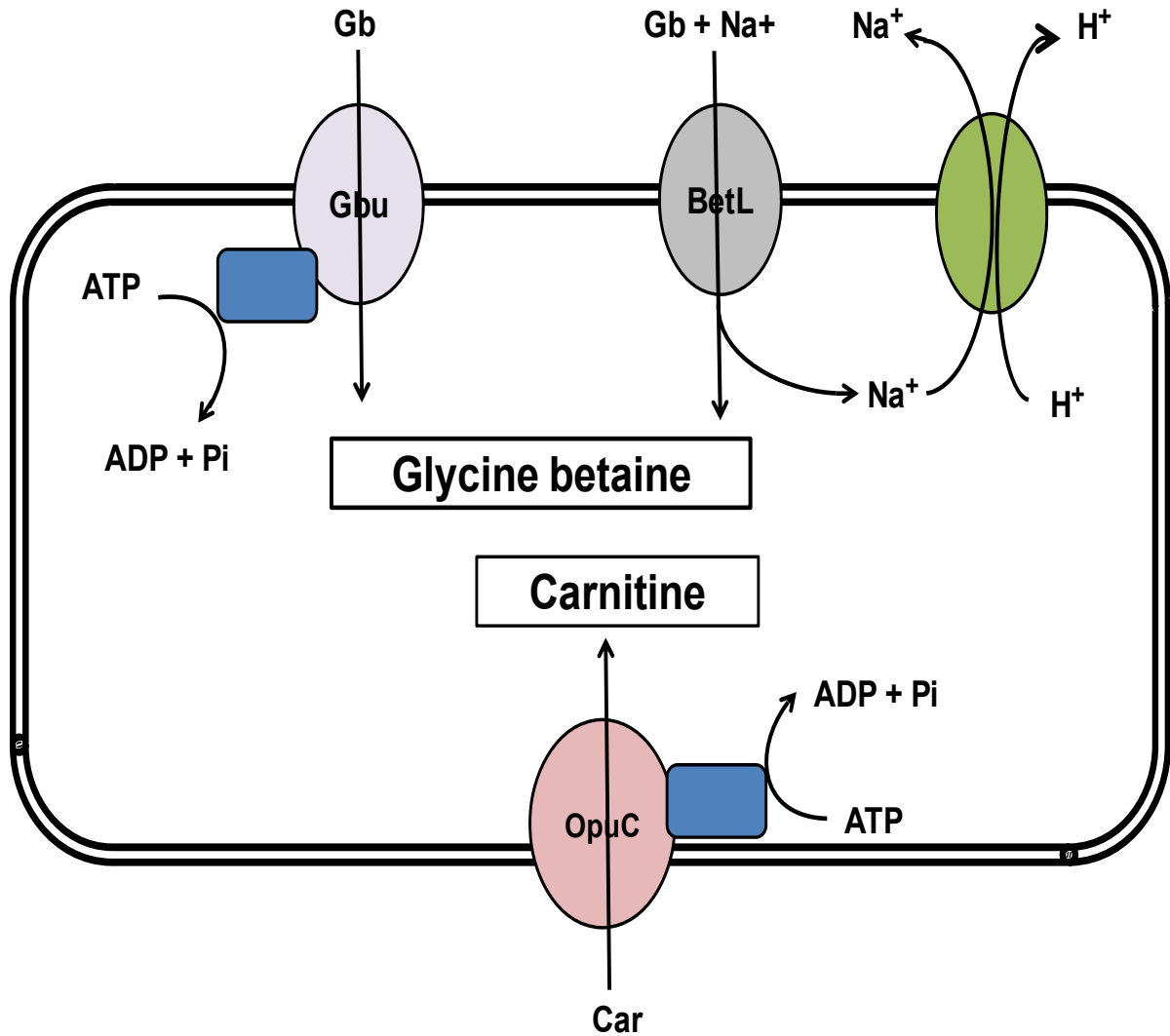
Figure 2.5: Mechanism of *L. monocytogenes* response to osmotic stress. Gb - glycine betaine, car - carnitine. (Adapted from Wood et al. (2001)).

2.7.2   *Acid stress response*

L. monocytogenes is frequently exposed to acidic conditions in foods as weak acids are used

as preservatives in many foods. As a neutralophile, exposure to acid stress disrupts the pH

homeostasis of the cells. At least three systems of the acid stress response, namely, the

arginine deiminase (ADI) system, the F_0F_1-ATPase system, and the glutamate decarboxylase (GAD) system have been identified in *L. monocytogenes* (Cotter et al., 2001a; Ryan et al., 2009, 2008). The collective effect of these systems is to prevent the accumulation of H^+ protons in the cell cytoplasm and disruption of internal pH. Changes in extracellular pH are detected by membrane integrated histidine kinases that induce transcription of stress response genes through two-component signal transduction systems (Ryan et al., 2008). Of these, the first two systems are considered the most specialized in acid stress response and are discussed in detail in the following sections of this review.

2.7.2.1 The GAD system

The GAD system (Figure 2.5) depends on the enzyme glutamate decarboxylase, a product of the *gadD* operon which decarboxylates glutamate to produce γ-amino butyric acid (GABA) while consuming a proton and releasing a bicarbonate anion. The decarboxylation is coupled with an antiporter (GadT) that takes out the produced GABA while taking in glutamate (Cotter et al., 2001a). *L. monocytogenes* produces three glutamate decarboxylase enzymes (GadD1, GadD2, and GadD3) and two antiporters (GadT1 and GadT2) that are encoded as pairs consisting of the *gadD1T1* and *gadD2T2* operons in separate parts of the genome (Cotter et al., 2001b). The genes have distinct functions in the acid stress response of *L. monocytogenes*. The expression of *gadD1T1* is required for mild acid (pH 5.1) survival while *gadD2T2* expression is needed for severe acid stress (pH 2.8), and therefore is necessary for the adaptive acid tolerance response (ATR) (Cotter et al., 2005).
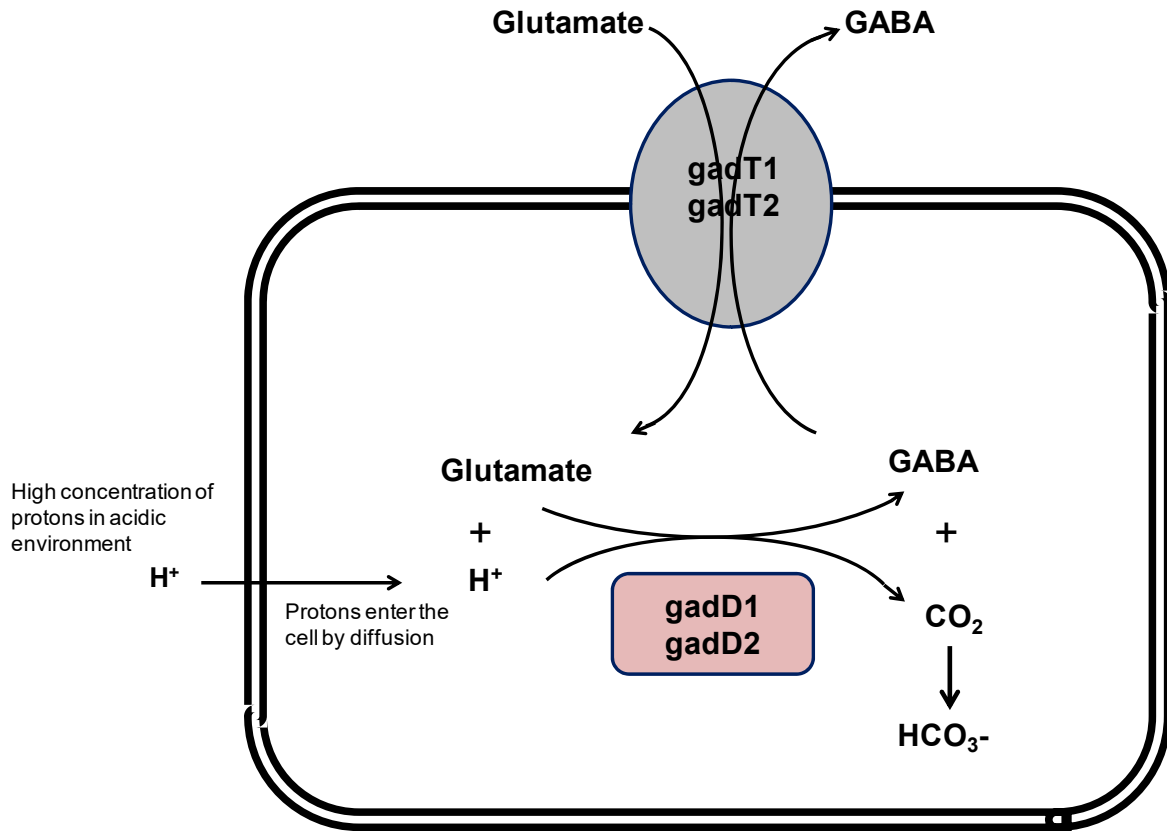
Figure 2.6: The glutamate decarboxylase (GAD) acid stress response system in *L. monocytogenes*. GABA - γ-amino butyric acid. gadT1/T2 are trans-membrane antiporters and gadD1/D2 are cytoplasmic glutamate decarboxylases. Adapted from (O'Byrne and Karatzas, 2008)

2.7.2.2 The ADI system

The arginine deiminase (ADI) system (Figure 2.6) which was previously known in many bacterial genera as an acid stress response mechanism was only identified in *L. monocytogenes* a few decades later than the GAD system (Ryan et al., 2009). It involves the conversion of arginine to ornithine accompanied by the production of ammonia and carbon dioxide (Spano et al., 2004). As part of the system, an arginine-ornithine antiporter (protein ArcD encoded by *arcD*) facilitates the uptake of arginine in exchange for ornithine. Once inside the cell, the deimination of arginine by the enzyme arginine deiminase (encoded by the *arcA*) produces ammonia and citrulline. The latter is then converted to ornithine and

24

carbamoyl phosphate through the enzyme ornithine carbamoyltransferase (encoded by *arcB*).

Carbamoyl phosphate is subsequently converted to ammonia and carbon dioxide through the

activity of carbamate kinase (encoded by *arcC*) (Ryan et al., 2009). This reaction reduces

internal pH through the conversion of ammonia (NH_3) to ammonium ions (NH_4^+) (Ryan et

al., 2008).
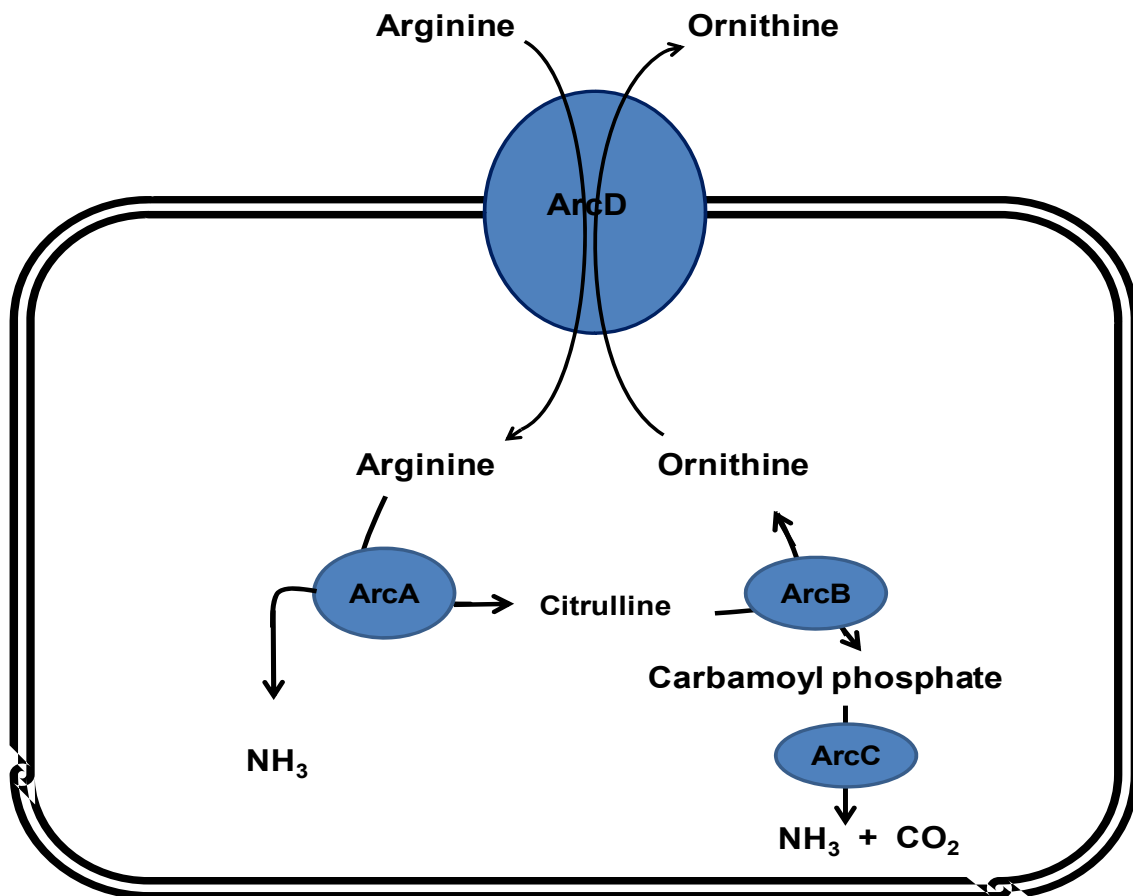
Figure 2.7: The ADI acid stress response system in *L. monocytogenes*. ArcD is a transmembrane arginine-ornithine antiporter, and ArcA/B/C are cytoplasmic proteins involved in the deimination of arginine. (Adapted from Ryan et al. 2009).

2.7.3   *Heat stress response*

Heat application is an established method of food preservation, known for its lethality

especially at elevated temperatures. As a non-spore-former, with a growth temperature range

of 1 - 45°C (Rowan and Anderson, 1998), *L. monocytogenes* is generally susceptible to heat stress although it has been reported to survive temperatures above the growth range (Shen et al., 2014). The heat stress response is universal in all organisms and is triggered by temperature up-shifts above the normal growth range. Its main effect is the protection of cellular proteins and enzymes against heat-induced denaturation that affects their physiological functions (Roncarati and Scarlato, 2017). The universal response involves the increased transcription of heat shock genes coding for heat shock proteins (HSPs) (Lim and Gross, 2011). Most HSPs act as molecular chaperones that protect physiological proteins and enzymes against misfolding and loss of biological functions. In some cases, HSPs also act as proteases that degrade misfolded and damaged proteins (Lim and Gross, 2011). The heat shock response of *L. monocytogenes* consists of three classes of HSPs that are conserved in Gram-positive bacteria. Class I HSPs are chaperones made up of the proteins, dnaK, dnaJ, and groES and groEL encoded in two operons, (the *dnaK* and *groEL-groES* operons) whose expression is controlled by the transcriptional repressor protein hrcA (Gahan et al., 2001; Hanawa et al., 2000). Class III HSPs are both chaperones and proteases whose expression is under the control of the transcriptional repressor CtsR. The major components of this class of HSPs are the Clp proteins (clpP, clpC, clpE, and clpB) involved in the ATP-dependent proteolysis of misfolded proteins as well as acting as chaperones involved in protein folding and assembly (Nair et al., 2000). Class II proteins are general stress response proteins under the regulatory control of σ^B (Roncarati and Scarlato, 2017). Molecular signals that trigger increased temperature stress response can emanate from many sources. These include direct heat sensing by RNA and DNA or indirect signals such as accumulation of denatured proteins (Klinkert and Narberhaus, 2009). In *L. monocytogenes*, the negative regulatory control of the heat shock response by HrcA and CtsR has been extensively described, however, the positive control of this response is not as yet clear. In the model organisms, *B. subtilis* and *E. coli*, the

heat-induced transcription of Class I and Class III heat shock genes is under the control of primary sigma factors σ^A and σ^{32} respectively (Hecker et al., 1996; Roncarati and Scarlato, 2017). Under normal growth temperature, the expression of the genes is prevented by HrcA and CtsR repression. Under conditions of elevated temperatures, derepression is achieved through reduced DNA binding of repressors at elevated temperatures and improved binding of the sigma factors (Figure 2.7) (Roncarati and Scarlato, 2017).
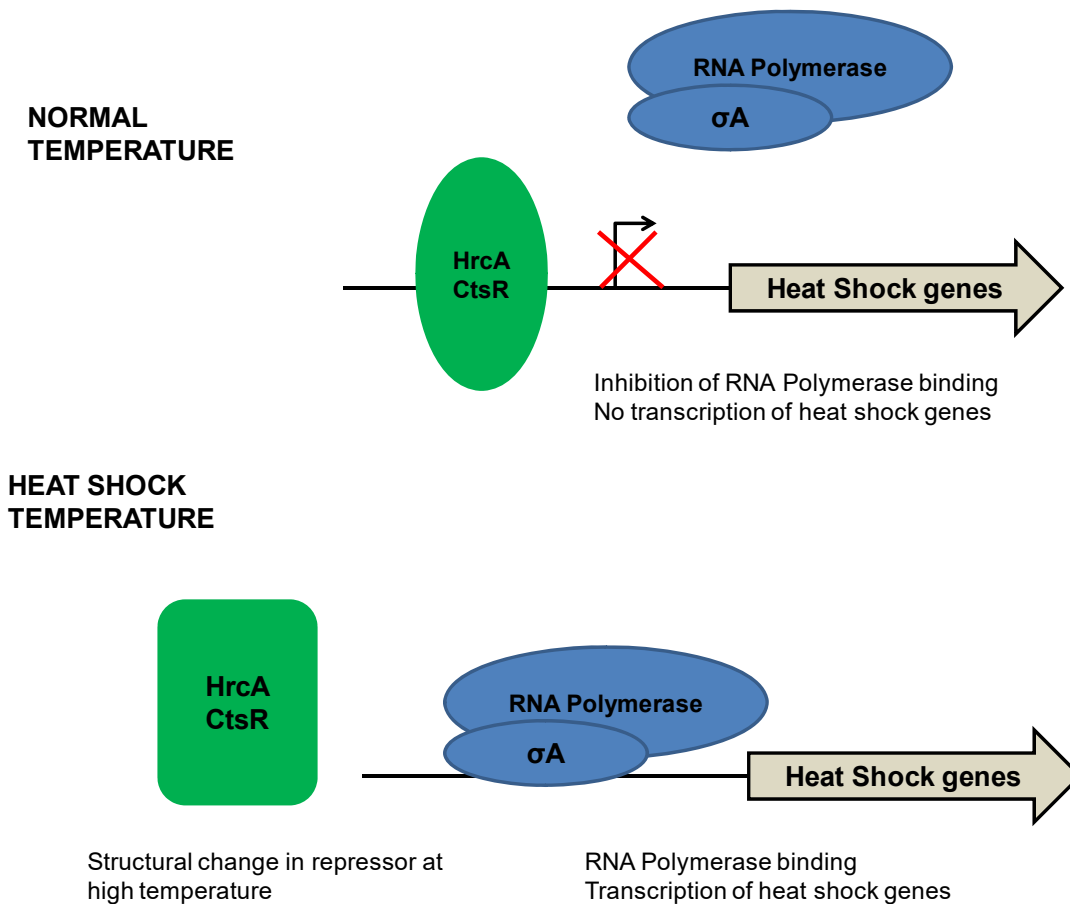
Figure 2.8: Regulation of heat shock response in *L. monocytogenes*. (Adapted from Roncarati and Scarlato, 2017).

2.7.4   Cold stress response

Bacteria exposed to cold stress have to face two major challenges. The first is the loss of fluidity of the cell membrane which affects its physiological functions. The membrane

27

changes from an elastic liquid crystalline state to a gel-phase state upon temperature downshift below the growth temperature range (Phadtare, 2004). The second challenge is the impairment of cellular replication, transcription, and translation processes owing to the negatively supercoiled DNA and stabilized RNA secondary structures (Phadtare, 2004). At least three mechanisms are utilized by *L. monocytogenes* in response to cold stress imposed challenges (Figure 2.8). These include; the adjustment of the fatty acid composition of the cell membranes in order to maintain fluidity; the increased expression of cold shock proteins (CSP) and the accumulation of osmolytes and oligopeptides (Tasara and Stephan, 2006).

Although *L. monocytogenes* is classically a mesophile, with an optimum growth temperature of 30 – 37ºC (Tiwari et al., 2014), the organism has a remarkable ability to multiply under cold temperature conditions (Cabrita et al., 2015). While part of this psychrotrophic growth ability may be intrinsic, a significant part of it is induced by exposure to cold conditions (Bayles et al., 1996). The low-temperature growth is related to the ability to maintain membrane fluidity under cold conditions which allows nutrient transport necessary to support growth (Russell, 2002). A critical aspect of bacterial adaptation to cold temperatures involves the adjustment of membrane fluidity by incorporation of unsaturated fatty acids (BCFA) (Yoon et al., 2015). The profile of fatty acid composition of membranes of *L. monocytogenes* cells growing at 37°C, has revealed that the membranes contain an unusually high (> 95%) of branched-chain fatty acids (BCFA), mostly dominated by both iso- and anteiso- forms $C_{15:0}$ and $C_{17:0}$ fatty acids (Annous et al., 1997). When exposed to cold conditions, the membrane fatty acid composition changes through the incorporation of more anteiso- forms and shorter chain fatty acids (Annous et al., 1997). Anteiso-BCFAs have a lower melting point than analogous iso- BCFAs, hence their incorporation at low temperatures is critical in the maintenance of membrane fluidity (Chan and Wiedmann, 2008). A key determinant in the

adjustment of membrane fluidity is the enzyme β-ketoacyl-acyl carrier protein synthase III (FabH) which catalyzes the initial condensation reaction between iso- and anteiso-branched α-keto acids and acetyl-coenzyme A (Choi et al., 2000). While this enzyme can bind both iso- and anteiso-branched α-keto acid precursors, it is the increased precursor selection specificity that is responsible for the increased synthesis of anteiso-BCFAs at low growth temperatures in *L. monocytogenes* (Singh et al., 2009; Yoon et al., 2015).

The second aspect of *L. monocytogenes* growth ability at low temperatures is associated with the ability to produce cold shock proteins (CSPs). CPSs belong to a family of small highly conserved and structurally related proteins that are widely distributed in the prokaryotic kingdom (Phadtare, 2004). Although CPSs have extensively been studied in the mesophiles *E. coli* and *B. subtilis*, evidence has shown that CSP family protein genes belonging to three families (CspA, CspB, and CspD) are found within the genomes of *L. monocytogenes* (Nelson et al., 2004). The support for the role of these proteins in the cold growth of *L. monocytogenes* has been provided by the observations that these proteins are not necessary for growth at 37°C but are required for growth at 5 – 10°C (Schmid et al., 2009a; Wemekamp-Kamphuis et al., 2002). Using directed mutagenesis, Schmid et al. (2009) observed that CspA is the main CSP required for low-temperature growth of *L. monocytogenes*. Although the exact functions of CSPs are still to be fully elucidated, the current understanding is that these proteins act as nucleic acid chaperones that bind RNA and DNA thus facilitating the control of processes such as replication, transcription, and translation within bacterial cells (Phadtare, 2004). This is presumably necessary to help the organisms overcome the challenges of DNA and RNA supercoiling associated with low-temperature growth (Phadtare, 2004). Coupled with the role of CSPs are RNA helicases, that bind to ribosomes and facilitate RNA maturation challenges at low temperature (Netterling et

al., 2012). Four DEAD-box RNA helicase genes have been found in the genome of *L. monocytogenes* (Markkula et al., 2012). Using knock-out mutants, the helicases have been found to be necessary for *L. monocytogenes* cold growth (Bäreclev et al., 2014; Netterling et al., 2012).

In addition, to changes in membrane composition and production of CSP proteins, *L. monocytogenes* cold stress response also involves the accumulation of compatible solutes, glycine betaine and carnitine as well as oligopeptides as cryoprotectants (Tasara and Stephan, 2006). Hence, the main osmolyte transporters Gbu, BetL and OpuC induced by osmotic stress are also induced by cold stress in *L. monocytogenes* (Liu et al., 2002; Mendum and Smith, 2002). The accumulation of oligopeptides in *L. monocytogenes* is mediated by the oligopeptide permease transporter (OppA) encoded by the *opp* operon (Chan and Wiedmann, 2008). The exact roles of the accumulated osmolytes and oligopeptides are not clear although suggested potential roles include stabilization of enzymatic functions and by osmolytes and synthesis of peptide derivatives required for *L. monocytogenes* cold adaptation (Tasara and Stephan, 2006).
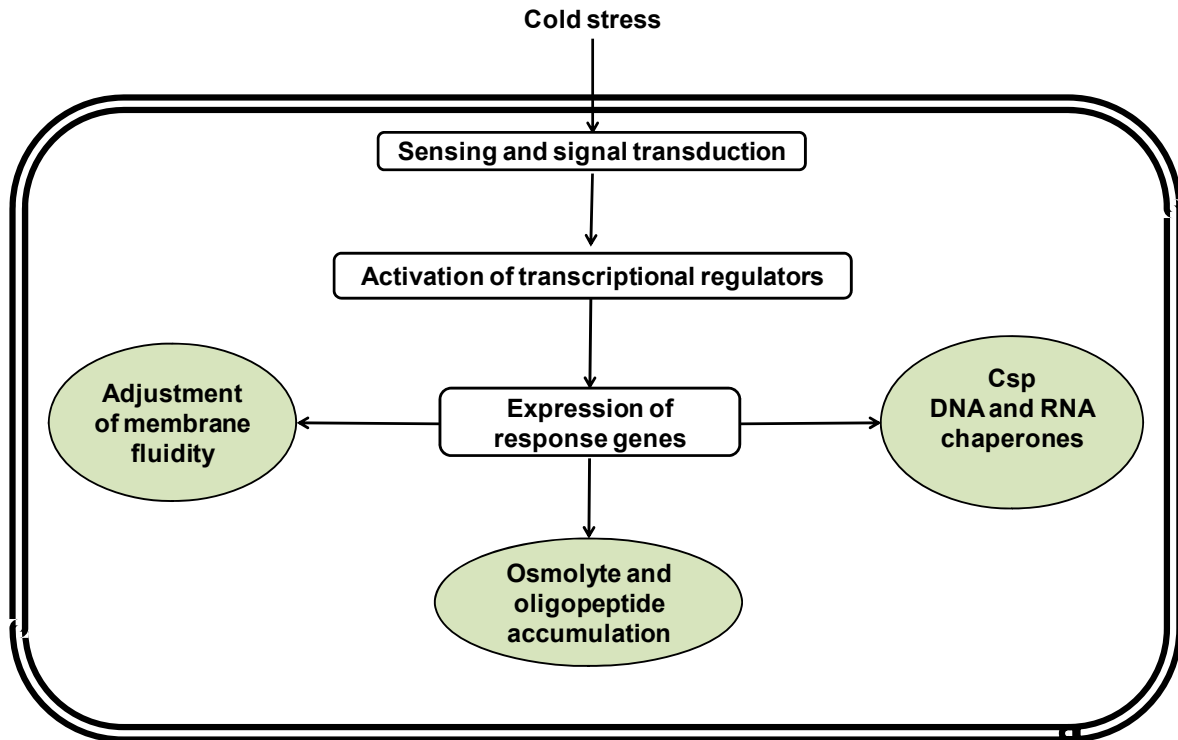
Figure 2.9: A summary of the cold stress response processes of *L. monocytogenes*. (Based on Phadtare, 2004; Phadtare, et al. 1999 and Tasara and Stephan 2006)

2.8   Food safety implications of *L. monocytogenes* stress responses

Although nearly all physical stress conditions such as heat, acid, and reduced a_w are capable of causing loss of viability in microbial cells, the outcome is dependent on the magnitude of stress exposure (Wu, 2008). In many instances, microorganisms are exposed to mild stress levels that only reduce growth without causing loss of viability (Wesche et al., 2009). This is particularly the case with the hurdle approach to food preservation, where foods are treated with many sub-lethal stress hurdles as a way of preserving the natural freshness and nutritional quality of foods by avoiding the negative effects of preservation methods such as high heat and use of synthetic chemical antimicrobials (Singh and Shalini, 2016). Despite the benefits of such approaches, exposure to sub-lethal stresses can result in varying outcomes in microbial cells impacting on survival ability. Some of these outcomes and their implications on food safety are briefly discussed in the following sections of this review.

2.8.1 *Sub-lethal stress and cell injury*

A substantial body of evidence has shown that for any isogenic microbial population in exponential growth or subjected to stress, individual cells never show the same responses (Aspridou and Koutsoumanis, 2015; Bishop et al., 2007; Gao et al., 2011; Koutsoumanis, 2008). For sub-lethal stress exposures, the differences in individual cell responses mean that while a fraction of the cell population will die, the magnitude of stress is not sufficient to cause a loss of viability in most of the cells. The effects of the stress exposure on the surviving fraction are also influenced by individual cell heterogeneity and hence such cells manifest in varying physical and physiological states (Casadesús and Low, 2013; Nyarko and Donnelly, 2015). The surviving cells exhibit physical damage to the structural components such as the cell membrane and cellular proteins which results in a temporary loss of physiological functions. Cells that exhibit this state of impaired physiological functions and damaged structural components without loss of viability are said to be in a state of sub-lethal injury (Wesche et al., 2009). Cells in this state exhibit a temporary and reversible loss of growth ability and are sensitive to lethal agents (Wu, 2008).

The occurrence of sub-lethal injury has been demonstrated in *L. monocytogenes* and many other food-borne pathogens exposed to physicochemical stresses such as heat, acid, osmotic and oxidative stress (Al-Qadiri et al., 2008; Nyarko and Donnelly, 2015; Silva et al., 2015). Considering the realistic situation of pathogen contamination in foods and food processing plants where a continual exposure to stress could impose sub-lethal injury, it is logical to expect that, this is the dominant physiological state occurring under these conditions. However, the fate and impact of injured cells on food safety has not been explored as this is complicated by the occurrence of such cells in mixed populations with un-injured cells and likely in low numbers (Van Nevel et al., 2017). The potential food safety implications of

injured cells include the difficulty in detecting their presence by conventional selective techniques since they are susceptible to selective agents used in selective media as a result of cell damage yet they have been shown to repair the cell damage and re-gain the characteristics of uninjured cells including virulence (Miller et al., 2006; Silva et al., 2015). The detection is further complicated by the need for resuscitation and repair before growth resumption (Wu, 2008).

In order to study the behavior of injured cells, modern single-cell technologies based on fluorescent labeling and cell sorting have proved useful (Veal et al., 2000). The use of fluorescent labels that indicate disruption of cellular components such the cell membrane, the transmembrane potential, and loss of metabolic activity can be used to recover injured cells by fluorescence-activated cell sorting (FACS) (Bridier et al., 2015). Using flow cytometry coupled with fluorescent labeling, many studies have demonstrated the occurrence of stress-induced cell injury in *L. monocytogenes* arising from acid, osmotic, heat and electric fields stresses (Silva-Angulo et al., 2014; Uyttendaele et al., 2008; Xuan et al., 2017). Notably, however, most of these studies only went as far as demonstrating the existence of injured cells as a subpopulation of the surviving population with no specific attention on the fate, re-growth characteristics and behavior of these cells in conditions relating to foods. Only a few studies such as Kennedy et al. (2011), managed to sort and isolate injured *L. monocytogenes* cells using FACS based on membrane integrity indicators. In that study, the researchers showed that about 33% of acid-injured *L. monocytogenes* was capable of re-growth on both selective and non-selective media while heat-injured cells were incapable of growth.

2.8.1.1 Sub-lethal stress and development of adaptive responses

Perhaps the biggest consequence of sub-lethal stress exposure on pathogens is the development of stress tolerance (adaptive) responses. Stress tolerance is understood to be the ability of an organism to survive high and often lethal levels of stress exposure (Brauner et al., 2016). The ability to withstand lethal stress can either be intrinsic or acquired. The mechanisms accounting for the for the development of acquired tolerance responses can result from inheritable genetic changes (resistance) or non-heritable physiological (phenotypic) responses (Abee et al., 2016). Many sub-lethal stress hurdles employed in food preservation have been proven to induce tolerance to lethal stress treatments in *L. monocytogenes* (Chorianopoulos et al., 2011; Davis et al., 1996; O'Driscoll et al., 1996). For instance exposure to sub-lethal acid at pH 5.0 and sub-lethal heat at 46ºC induces tolerance to lethal acid at pH 3.5 and lethal heat at 63ºC respectively (Faleiro et al., 2003; Skandamis et al., 2008). The acid tolerance response (ATR) and heat tolerance response are some of the best known homologous adaptive responses in *L. monocytogenes* (Chorianopoulos et al., 2011; Giaouris et al., 2014; Wen et al., 2009).

The development of adaptive responses in food-borne pathogens has huge implications in food safety as it leads to enhanced survival of pathogens in the food chain. Exposure to mild heat, acid and osmotic stress during pre-processing, for example, can lead to resistance to subsequent heating, acid preservation, and salting respectively (Yousef and Courtney, 2003). An illustration of how stress adapted pathogens emerge as tolerant process survivors through pre-exposure to mild pre-processing steps in a model food chain is shown in Figure 2.9. For a pathogen like *L. monocytogenes*, whose prominence in RTE foods and minimally processed foods is owed to the lack of lethal preservation hurdles, the development of tolerance responses is particularly pertinent. For fermented RTE foods such as cheeses, the

development of adaptive ATR is directly linked to the survival of *L. monocytogenes* in such foods (Cataldo et al., 2007). Beyond survival in foods, the ATR response has been linked to the ability to survive gastric passage, a key virulence determinant for *L. monocytogenes* (Feehily and Karatzas, 2012).
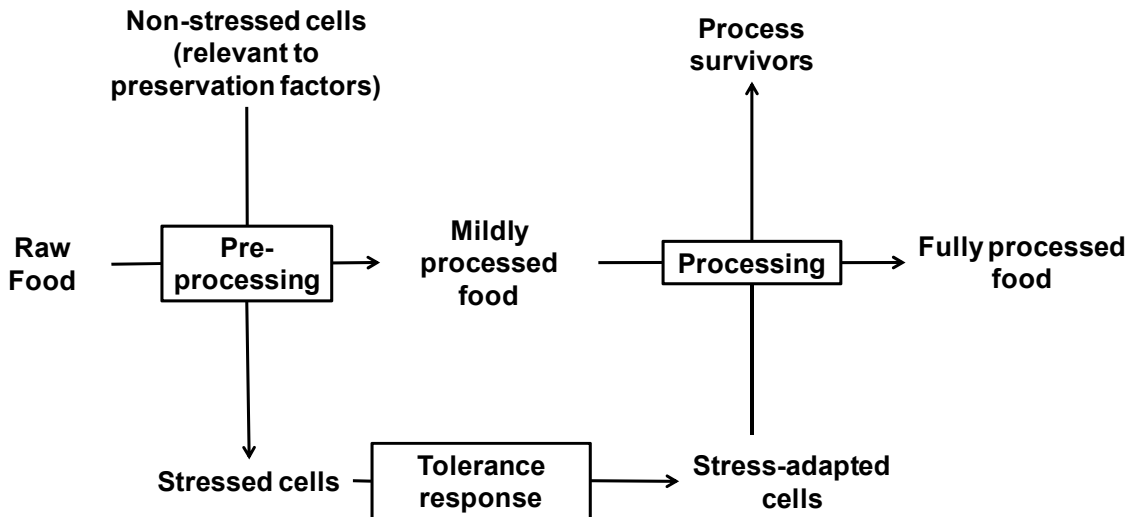
Figure 2.10: Effects of stress adaptation on pathogen survival in the food chain (Yousef and Courtney, 2003)

2.8.2   *Overlap in stress responses and cross-protection in* L. monocytogenes

 Over and above the enhanced tolerance to similar stress factors (homologous tolerance), a lot of cross-tolerance to different stress factors (heterologous tolerance) also occurs (Alvarez-Ordóñez et al., 2015). For example, cross protection to lethal acid and heat stress in *L. monocytogenes* can be induced by a combination of sub-lethal NaCl, heat, and acid stresses (Faleiro et al., 2003; Skandamis et al., 2008). At the center of this cross-tolerance is the role of the general stress response mediated by σ^B, whose role in the transcription of stress response genes is pleiotropic and therefore overlaps among different stress factors (O'Byrne and Karatzas, 2008). Notably, overlaps in responses to specific stress factors occur in *L.*

monocytogenes with the particular case of the overlap between cold and osmotic stresses being the most critical (Tasara and Stephan, 2006).

Considering the practical realities of food processing where pathogens may be exposed to various stress factors sequentially, cross-protection may impose a safety risk as it results in an inadvertent adaptation to subsequent heterologous stress factors. For instance, the role of osmoprotectants in the cold stress response of *L. monocytogenes* has been established (Chan and Wiedmann, 2008; Tasara and Stephan, 2006). The implication of this is that any exposure to sub-lethal osmotic stress during processing prepares *L. monocytogenes* for the survival of refrigerated storage. This is particularly key to the control of *L. monocytogenes* growth in RTE foods as refrigeration is invariably used as a terminal hurdle. Thus implying that even though the organism is psychrotrophic, its survival and growth ability is further enhanced by prior osmotic stress exposure. For a multi-hurdle preservation approach that depends on sub-lethal stresses to be effective, it is desirable that preceding stress factors must sensitize microbial cells to subsequent stress hurdles. Tiganitas et al. (2009) observed that the use of sub-lethal pH followed by NaCl in sequence results in higher inactivation of *L. monocytogenes* than the reverse sequence.

2.8.3 *Stress tolerance and persistence of* L. monocytogenes

Although as a saprophyte, *L. monocytogenes* is found in many raw and unprocessed foods, a close examination of the sources of contamination for processed products has identified the processing environment as an essential reservoir leading to recurrent contamination. This point is supported by the repeated isolation of genetically similar *L. monocytogenes* subtypes from the same plants over months or years (Almeida et al., 2013; Linke et al., 2014). This occurrence of genetically identical strains associated with the same environment over time is

called persistence and the strains exhibiting this phenotype are referred to as persistent strains (Nowak et al., 2017). Because of their association with food processing plants, persistent *L. monocytogenes* strains are the cause of listeriosis outbreaks, unlike transient sporadic non-persistent strains. It is quite remarkable that the persistence phenotype of *L. monocytogenes* occurs regardless of the routine disinfection of food processing environments. Although, the basis of *L. monocytogenes* persistence remains unclear at the moment, what is evident is that the phenomenon is linked to stress survival and stress tolerance given that such strains are continually exposed to antimicrobial agents at concentrations that are sometimes lethal.

In laboratory cultures, bacterial persistence is often demonstrated by exposure of a bacterial population to a stress agent. In this case, while a large fraction of the population is often killed a small sub-population survives resulting in tailing of inactivation curves. The current theories accounting for this survival and persistence are based on both genetic and phenotypic responses as well the ability to form biofilms (Abee et al., 2016; Larsen et al., 2014). The mechanisms are briefly discussed in the following subsections of this review.

2.8.3.1 Genetic mechanisms of persistence and stress resistance

Genetic mechanisms of stress survival result in survivor populations that exhibit stable and heritable stress resistance arising from mutations in part of the genome (Abee et al., 2016; Metselaar et al., 2013). The exact mechanisms accounting for the enhanced tolerance to different stress factors requires the identification of altered genes in stress survivors and their respective functions in stress response. Thus far, this has not been adequately elucidated in many bacterial species. For *L. monocytogenes*, much of what has been established is owed to the work of Abee and co-workers (Abee et al., 2016; Metselaar et al., 2015; Van Boeijen et al., 2011, 2010). In their studies, these researchers observed that persistent survivors of *L.*

monocytogenes after heat, HHP and acid stresses comprised about 20% stable stress resistant variants and that variants resistant to heat and HHP stresses had mutations in the Class III heat shock response regulator gene *CtsR* while and variants resistant to acid stress had mutation in the *rpsU* gene encoding ribosomal protein S21. Because of the central role of *ctsR* as a repressor of heat shock genes, mutations in this gene results in a defect in repression and increased transcription of class III Hsp proteins like clpC which consequently results in enhanced tolerance to heat (Abee et al., 2016; Roncarati and Scarlato, 2017). The occurrence of *rpsU* mutations accompanying acid resistance has been complicated by a lack of any known function for ribosomal protein S21 in the stress response of *L. monocytogenes*. Based on studies on *B. subtilis*, ribosomal protein S21 was shown to be necessary for the correct function of ribosomes and *rpsU* mutants were associated with reduced growth rate (Akanuma et al., 2012; Takada et al., 2014). The suggestion so far is that the acid resistance of *rpsU* mutants could be related to a reduced growth rate (Abee et al., 2016).

2.8.3.2 Phenotypic mechanisms and persister cell formation

Unlike genetic mechanisms that result from mutations in the bacterial genome conferring resistance to stress, phenotypic mechanisms result from phenotypic differentiation of cells into cell types that are more tolerant to stress agents without any change in the bacterial genome (Brauner et al., 2016). Unlike genetically mediated stress resistance, cells re-grown from such persistent populations are susceptible to future stress exposure (Balaban, 2011). Such stress tolerant cells resulting from a phenotypic switch from a susceptible cell state to a tolerant cell state are termed persister cells (Balaban, 2011; Balaban et al., 2004; Norman et al., 2015). Persister cells have for a long time been known in clinical settings as being responsible for the enhanced antibiotic tolerance of many bacterial pathogens resulting treatment failure and infection relapse (Pu et al., 2017, 2016). Although not given as much

attention as a factor in the survival and persistence of foodborne pathogens in processing environments, it is now widely accepted that this cell state is abundant as part of persistent subpopulations following stress exposures (Radzikowski et al., 2016; Stepanyan et al., 2015).

The mechanisms responsible for the phenotypic switch that results in the formation of the persister cells are an ongoing subject of investigation. Based on studies with *E. coli*, the broadly accepted theory is that persister cells result from a transition of cells to a dormant, metabolically inactive state or a state of slow growth (Balaban et al., 2004). In terms of this theory, the switch to a dormant state is due to phenotypic heterogeneity among individual cells in a population, especially variations in the expression levels of stress response genes in individual cells with the attendant effect of producing phenotypically different subpopulations in a genetically identical cell population (Maisonneuve and Gerdes, 2014). Specifically, the switch arises from variations in the expression levels of toxin-antitoxin (TA) modules (encoded by TA operons) of individual cells (Balaban, 2011). TA systems comprise of a protein toxin that inhibits cell growth and a protein antitoxin that regulates the activity of the toxin (Figure 2.10) (Radzikowski et al., 2017). Under conditions of stress, regulatory proteins with pleiotropic effects such as the general stress response regulator σ^B can induce increased expression of the toxin leading to a cessation of growth (Amato et al., 2013; Radzikowski et al., 2017). While TA systems have not yet been fully elucidated in *L. monocytogenes*, Abee et al. (2016) in a search of the *L. monocytogenes* EGDe genome, found eight putative TA modules and observed that two of the modules showed similarity to *E. coli* TA systems.

Although only limited studies have looked at bacterial persister cell formation in response to food-related stresses, there is evidence that this cell state is, in fact, the dominant survival

state (80%) in persistent populations of *L. monocytogenes* subjected to heat and HHP treatments (Van Boeijen et al., 2011, 2010). In addition, Wu et al. (2017) also demonstrated that persister cell formation in *L. monocytogenes* is induced by the food preservative nisin. However, there is still a need to establish any links between persister cell formation and persistence of *L. monocytogenes* (described as the recurrence of genetically identical strains in a single food processing facility over years (Larsen et al., 2014). Specifically, the possibility that persistent strains of *L. monocytogenes* are high persister cell forming mutants.
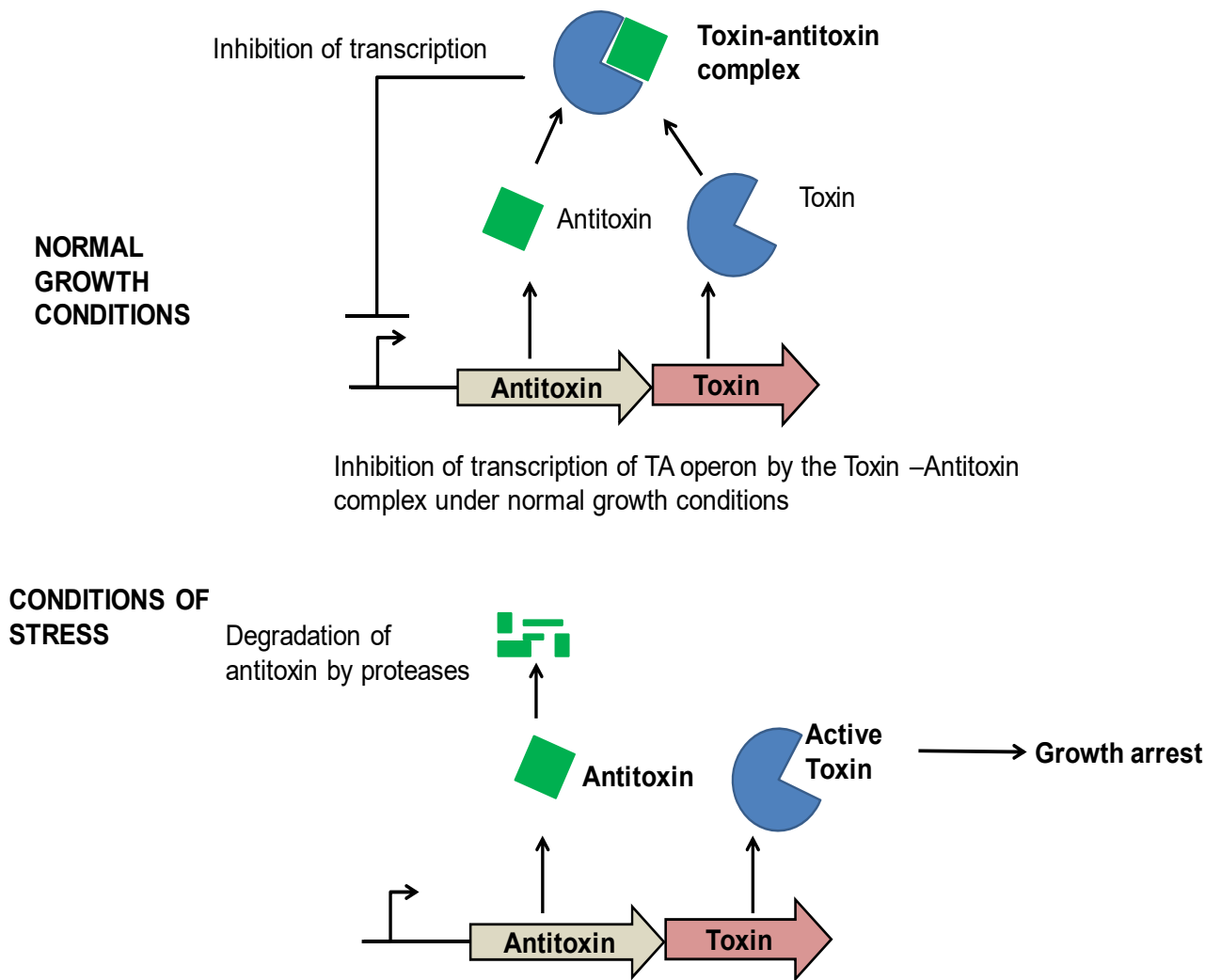
Figure 2.11: Model of induction of growth arrest by toxin-antitoxin systems. Under normal growth conditions, growth arrest is prevented by formation of Toxin-antitoxin complexes. Under stress conditions, growth arrest occurs as a result of degradation of the antitoxin and release of the active toxin. (Adapted from Yamaguchi and Inouye, 2011).

2.8.3.3 Persistence and biofilm formation

As primary sources of *L. monocytogenes* into the food chain, raw foods expectedly harbor both transient and persistent strains (Nowak et al., 2017). However, the near-exclusive association of persistent strains with the food processing environment as a secondary habitat is an indication of a better ability to colonize this habitat. The ability of *L. monocytogenes* to attach onto and colonize different types of materials used for food product contact and non-contact surfaces and thus form biofilms has been confirmed by many previous studies (Bonsaglia et al., 2014; Doijad et al., 2015; Kocot and Olszewska, 2017). What is however not clear is the extent to which biofilm formation contributes to the persistence of *L. monocytogenes*. Attempts to establish a link between persistence and biofilm formation has yielded many conflicting findings. For instance, Costa et al. (2016) concluded that biofilm-forming ability was not linked to persistence of *L. monocytogenes* isolates from cheese processing plants while Wang et al. (2015) and Nowak et al. (2017), concluded that persistent *L. monocytogenes* strains have better adhesion and biofilm formation capacity than non-persistent strains.

As microbial habitats, food processing surfaces are often subject to constant disturbances by routine cleaning and disinfection procedures, which makes bacterial adherence (necessary for colonization and biofilm formation) unlikely. It is, therefore, most likely that biofilm formation is aided by the presence of niches and harborage sites within the processing plant that are difficult to sanitize while at the same time providing conditions, such as availability of nutrients and moisture (Valderrama and Cutter, 2013). It is also likely that even in the state of surface-attached growth, the other mechanisms of *L. monocytogenes* stress survival such as the development of resistant mutants and persister cells also play a role in the long-term survival and persistence of the pathogen in the food processing environments.

2.9 Predictive modeling of microbial responses and the impact of food-related stresses

The ability to predict pathogen responses in foods is central to the quantification of the level of risk they pose. Predictive microbiology makes use of mathematical models that infer on the changes in bacterial populations in foods based on the level of contamination as well as the intrinsic and extrinsic conditions of the food (Pouillot and Lubran, 2011). As an essential tool in quantitative risk assessment, the utility of predictive microbiology models is premised on the established observation that at the population level, microbial responses to environmental factors are reproducible (Ross, 1996), thus allowing for future predictions to environments not previously tested. Several models describing both growth and inactivation responses of pathogens have been developed in the past 30 years (Albert and Mafart, 2005; Baranyi and Roberts, 1994; Cerf, 1977; Geeraerd et al., 2000; Huang et al., 2011; Mafart et al., 2002) and have been used to predict microbial population responses under food relevant conditions. However, as noted in several recent studies, a wide diversity in microbial responses occur within populations and within species (Aryani et al., 2015a; Aryani et al., 2015b; Rodríguez et al., 2016), with potential impacts on the accuracy of risk assessment outcomes. In addition, most food-related stresses have been shown to impose selection pressures on microbial populations (Metselaar et al., 2013; Van Boeijen et al., 2011, 2013) resulting in the presence of stress resistant variants whose presence could potentially alter the population responses. Metselaar et al. (2016) showed the implications of stress adaptation in modeling the growth and inactivation behavior of *L. monocytogenes* stress resistant variants that exhibited a lower inactivation and growth rates compared to wild-type cells. While strain variability is often incorporated in the development of prediction models by use of mixed strain challenge tests, there are still challenges with respect to predicting responses of individual cells within populations (Koutsoumanis and Aspridou, 2017).

With respect to the risk associated with *L. monocytogenes* in RTE foods, microbial modeling has provided the basis of the current Codex guidelines of growth/no-growth categorization. However, based on a workshop that was hosted by the Joint Institute of Food Safety and Applied Nutrition (JIFSAN) in 2015, whose outcomes were reviewed by Buchanan et al. (2017), the general consensus is that the current models for predicting *L. monocytogenes* responses in foods may not be adequate. This highlights the need for new empirical data incorporating the ecological behavior of stress resistant and stress tolerant persister cells.

2.10 Conclusions

What is clear from available literature is that the survival of *L. monocytogenes* in foods and processing environments and its subsequent ability to cause human disease are complex and multi-factorial phenomena. While a significant volume of literature is already available on the organism's ability to survive environmental stress, a lot more still needs to be done to better understand and control the pathogen. Issues such as the fate and behavior of stress-injured cells in food systems, and the role of stress-induced persister cells in aiding persistence in food processing environments still need to be elucidated.

3 CHAPTER 3: HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

1. *L. monocytogenes* strain variations, and form of stress exposure will determine the degree of cell injury, resuscitation and growth kinetics of injured cells.

In addition to genetic differences among strains, phenotypic heterogeneity among individual cells in isogenic bacterial populations has recently been seen to influence microbial responses to stress (Aspridou and Koutsoumanis, 2015; Koutsoumanis and Lianou, 2013). The dynamic responses to stress in bacterial populations creates a mixture of cells in different physiological states with surviving populations consisting of both stress tolerant non-damaged cells and sub-lethally injured cells with damage to their cell components (Kennedy et al., 2011). While sub-lethally injured cells are known to resuscitate and repair the injury under growth permitting conditions (Wesche et al., 2009), the resuscitation behavior is probably multi-factorial and is likely influenced by the extent and form of cell damage.

2. *L. monocytogenes* strains exposed to acid and osmotic stress will exhibit different rates of inactivation in lactic soft cheese.

Bacterial stress exposures result in the development of stress tolerance responses in part of their surviving cell populations which enhance the survival of such populations to future stress encounters (Giaouris et al., 2014). For a lactic soft cheese

whose preservation depends on acidity as the main hurdle, acid stress exposure is likely to result in enhanced survival than osmotic stress treatment.

3. *L. monocytogenes* cells exposed to lethal acid stress will form persister cells and the cells regrown from persister survivors will have a different stress response gene expression profile from their parental generation when revived under conditions of mild acid and cold stress.

The reason for this argument is that persister cells are highly tolerant cells that aid bacterial survival of bactericidal concentrations of antimicrobials through phenotypic differentiation into a dormant state (Gefen and Balaban, 2009). Although persister cells are genetically identical to their non-persister parental generation, available evidence now shows that phenotypic differences between the two cell states can be vertically transmitted through non-genetic materials such as gene expression regulators (Day, 2016).

3.2   Objectives

1. To determine the degree of cell injury induced by acid, osmotic and heat stress on *L. monocytogenes* strains.

2. To determine the resuscitation and growth kinetics of FACS sorted stress injured cells.

3. To determine the effect of stress exposure on the inactivation rates of stressed *L. monocytogenes* strains in lactic soft cheese.

4. To elucidate the potential target of acid stress-induced cell damage in *L. monocytogenes* using FT-IR microspectroscopy.

5. To determine the potential role of persister cells in enhancing *L. monocytogenes* persistence.

6. To investigate the gene expression profiles of cells re-grown from persister survivors.

4   CHAPTER 4

RESUSCITATION AND GROWTH KINETICS OF SUB-LETHALLY INJURED *LISTERIA MONOCYTOGENES* **STRAINS FOLLOWING FLUORESCENCE ACTIVATED CELL SORTING (FACS)**

4.1 Abstract

This study investigated the effect of acid (pH 4.2), osmotic (10% NaCl) and heat (55 °C for 30 min) stress-induced injury on *Listeria monocytogenes* strains ATCC19115, 69, 159/10 and 243 using differential plating and flow cytometry coupled with membrane integrity indicators, thiazole orange (TO) and propidium iodide (PI) staining. Growth kinetics of injured cells sorted by fluorescence activated cell sorting (FACS) were studied at 4, 25 and 37°C. The percentage of cell injury detectable by both flow cytometry and differential plating varied significantly among strains and stress treatments ($p < 0.0001$). Based on flow cytometry and TO/PI staining, acid stress caused the highest level of injury followed by heat and osmotic stress. Following cell sorting, acid and osmotic stress injured cells were capable of resuscitation and re-growth while heat-injured cells (except for strain 69) were incapable of re-growth despite having a high level of membrane intact cells. The lag phase duration (λ) of sorted stress injured cells resuscitated in brain heart infusion (BHI) broth was significantly influenced by strain variations ($p < 0.0001$), stress treatments ($p = 0.007$) and temperature of resuscitation ($p \leq 0.001$). Following repair, the maximum specific growth rate (μ_{max}) of resuscitated cells was not different from untreated control cells regardless of strain differences and stress treatments. Only temperature had a significant effect ($p < 0.0001$) on growth rate. Sorted cells were also capable of growth at 4 °C, with the time to detectable growth (≥ 1.40 Log_{10} CFU ml^{-1}) ranging from 3 to 15 d. Overall, re-growth potential of sorted cells showed that while membrane integrity was a good indicator of cell injury and viability loss for acid and osmotic stress, it was not a sufficient indicator of heat stress injury. Once injured cells repair the cellular damage, their growth rate is not different from non-injured cells regardless of form of stress and strain differences. Thus highlighting the potential food safety risks of stress injured *L. monocytogenes* cells.

Keywords

Listeria monocytogenes, Stress, Injury, Flow Cytometry, Cell Sorting, Resuscitation.

4.2 Introduction

Listeria monocytogenes is a Gram-positive anaerobic facultative non-spore forming rod that is widely distributed in nature. The bacterium is the causative agent of the human disease listeriosis, an infection commonly associated with the consumption of contaminated minimally processed and processed ready to eat (RTE) foods (Aureli et al., 2000; Olsen et al., 2005). Owing to its ubiquity, the organism is easily associated with raw foods of both plant and animal origin (Jamali et al., 2013; Wang et al., 2013). From raw food materials, *L. monocytogenes* readily colonizes and establishes persistence in food processing equipment, and associated environment (Muhterem-Uyar et al., 2015; Pagadala et al., 2012) forming a major reservoir for the subsequent contamination of finished products (Bolocan et al., 2015; Strydom et al., 2013).

The persistence of *L. monocytogenes* in food processing environments and its subsequent ability to overcome preservation hurdles associated with RTE foods is key to its pathogenesis (Jensen et al., 2008; Kastbjerg, et al., 2010; Silva et al., 2015). Cell populations surviving in such environments are chronically exposed to sub-lethal physical and chemical stresses such as reduced a_w, pH, and temperature shifts as well as nutrient stress (Dalzini et al., 2015; Giaouris et al., 2014; Sadeghi-Mehr et al., 2016). Due to individual cell heterogeneity, response to such chronic sub-lethal stress exposure varies among sensitive and resistant cell subpopulations resulting in a mixture of bacterial cells in various physiological states (Casadesús and Low, 2013; Ryall et al., 2012). Stress sensitive cell populations exhibit a partial and reversible damage to the structural and functional cellular components and hence exist a sub-lethally injured state (Wesche et al., 2009; Wu, 2008).

The occurrence of sub-lethally injured cells of pathogenic bacteria has huge implications in food safety since such cells require a period of repair in non-selective media before the pathogen can be detected by conventional selective techniques (Wu, 2008). Detection, therefore, is influenced by the extent of injury and rate of repair. Following a sub-lethal stress exposure, the occurrence of injured cells in mixed populations with live undamaged cells makes it difficult to study their resuscitation and growth behavior as their response is often masked by the latter. This problem is made worse by the occurrence of such cells in low numbers and often in complex microbial communities (Waage et al., 1999).

Flow cytometry and Fluorescence Activated Cell Sorting (FACS) allows for the quantitative measurement of optical characteristics of individual cells passing through a focused light beam (Veal et al., 2000). When coupled with fluorescent labeling, the technique allows for the differentiation of cell subpopulations in various physiological states (Kennedy et al., 2011). As an indicator of cell viability, injury or death, bacterial cells are often subjected to a double staining protocol using nucleic acid binding dyes excitable with laser but with different emission spectra (Joux and Lebaron, 2000; Veal et al., 2000). A combination of membrane-permeant dyes such as acridine orange, thiazole orange, ethidium bromide, 4',6-diamidino-2-phenylindole (DAPI) and SYBR green (Martin et al., 2005; Perfetto et al., 2006; Shapiro, 2001) with impermanent dyes such as propidium iodide, TO-PRO-3, SYTOX green (Mortimer et al., 2000; Novo et al., 2000) allows for the differentiation of bacterial cell physiological states on the basis of membrane integrity (da Silva et al., 2009; Díaz et al., 2010). Because of their compromised membranes, injured cells are distinguished by their ability to take up both stains and therefore show strong fluorescence of both permeant and impermeant dyes. The addition of FACS allows the differently stained cells to be recovered as separate subpopulations. This is a microfluidics-based cell separation technique used to

separate cells with rare phenotypes from complex ecological communities and heterogeneous populations (Bhagat et al., 2010; Gossett et al., 2010) allowing for the subsequent study of such unique features in isolation.

While the stress response of *L. monocytogenes* has been extensively studied, models for the behavior of stressed cells in food systems are often based on heterogeneous mixtures of live and injured cells with the response taken to represent the average for the whole population. Given the heterogeneity of such populations, the response of sub-lethally injured cells is often underestimated thus impacting on the safety of RTE foods where *L. monocytogenes* has a potential of resuscitation and growth. In this study, the effect of sub-lethal acid, heat and NaCl stress response of food-borne *L. monocytogenes* strains was investigated using flow cytometry. Sub-lethally injured subpopulations based on a double fluorescent staining were recovered by FACS and their resuscitation and growth kinetics in broth were studied under different temperatures.

4.3   Materials and methods

4.3.1   Bacterial strains

The strains of *L. monocytogenes* used in this study included three isolates (69, 159/10 and 243) obtained from the Department of Food Science and Biotechnology, University of Free State, Bloemfontein, South Africa, having been isolated from an avocado processing plant (Strydom et al., 2013) as well as strain ATCC19115. The strains were positively confirmed as *L. monocytogenes* by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (Bruker, Germany) with Spectral Archiving and Microbial Identification System (SARAMIS™) (Anagnostec, Potsdam, Germany) database. Stock cultures of the strains were maintained in cryovials of 25% glycerol at -80ºC. The cultures were activated by

inoculating 100 µl of stock culture into 10 ml of brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) and incubation at 37ºC for 24 h.

4.3.2 Stress treatments and induction of injury

Cultures of test organisms were subjected to acid, salt and heat stress in order to induce cell injury. Working cultures were prepared by transfer of 100 µl of activated culture into 10 ml of BHI broth and incubation at 37ºC for 18 h which was repeated twice before exposure to stress. For stress exposure, cultures were centrifuged at 5000 g for 10 min in 2 ml microcentrifuge tubes (Eppendorf, Hauppauge, USA). Harvested cells were washed twice with sterile phosphate buffered saline (PBS, pH 7.3) (Oxoid) and re-suspended at a cell density of approximately 10^8 CFUml^{-1} (McFarland 0.5) in the following solutions in 10 ml volumes; (i) sterile acidified saline solution (pH 4.2) adjusted with 0.1 M lactic acid (Sigma-Aldrich, Steinheim, Germany) for acid stress; (ii) sterile 10% NaCl (Merck, Darmstadt, Germany) solution (pH 7.0) for osmotic stress; (iii) sterile PBS pre-warmed to 55ºC for heat stress. Treatment conditions were chosen to represent the potential sub-lethal stresses encountered by *L. monocytogenes* in foods and food processing environments. Acid and osmotic stress cell suspensions were incubated in a static incubator (Labcon, Mogale City, South Africa) at 25ºC for 24 h while heat stress suspensions were immediately heated to 55ºC in a water bath (Labotec, Midrand, South Africa) for 30 min. Untreated overnight cultures of each test organism were used as positive controls.

4.3.3 Determination of sub-lethal injury by plate counts

The percentage injury after each stress treatment was determined by comparison of growth of treated cells on selective and non-selective agar. Each sample of stressed cells was serially diluted in sterile 0.1% peptone water and plated on BHI agar (Oxoid) as non-selective media

and PALCAM agar (Oxoid) as selective media. As positive controls, untreated overnight cultures of each test organism were similarly plated on both selective and non-selective media. Plates were incubated at 37ºC for 48 h and viable cell counts were used to calculate percentage injury using the following equation (Busch and Donnelly, 1992).

$$\% \ Injury = \left[1 - \left(\frac{Counts \ on \ selective \ Agar}{Counts \ on \ non - selective \ Agar} \right) \times 100 \right]$$

$$(2.1)$$

4.3.4 Preparation of cell suspensions and staining

After each stress treatment, cell suspensions were centrifuged at 5000 g for 10 min and washed twice with sterile PBS. The cell pellets were re-suspended in PBS and diluted to a cell density of 10^6 to 10^7 cells/ml. A 0.5 ml of diluted cell suspension in sterile flow cytometry tubes was then stained with 5 µl of 0.42 µM thiazole orange (TO) (BD Biosciences, San Jose, USA) solution in dimethyl sulfoxide (DMSO) and 5 µl of 4.3 mM propidium iodide (PI) (BD Biosciences) solution in water. Stained cells were incubated at room temperature in the dark for 10 min. Positive controls consisted of untreated overnight cultures of each test organism. The cultures were centrifuged at 5000 g for 10 min, washed twice with sterile PBS and stained with TO/PI. As negative controls, cells from fresh overnight cultures were suspended in 70% ethanol for 30 min at room temperature. The ethanol killed cells were re-centrifuged at 5000 g for 10 min, washed twice and re-suspended in sterile PBS followed by staining with TO/PI.

4.3.5 Flow cytometry analysis

Flow cytometry analysis was performed using a flow cytometer (BD FACSAria™, BD Biosciences) equipped with a 20 mW argon laser emitting at 488 nm. For each cell crossing the focus point of the laser, two light-scattering signals, forward scatter (FSC) and side scatter

(SSC) and two fluorescence signals for red and green fluorescence were recorded. The fluorescence of thiazole orange was collected in the FL1 photomultiplier with a band pass filter of 525 nm. The fluorescence of propidium iodide was recorded in the FL3 photomultiplier with a short pass filter of 620 nm. For each sample, 10,000 events were measured at a flow rate of approximately 800 events per second (eps). The recorded light-scattering and fluorescence signals were collected as logarithmic signals and the obtained data was analyzed using a BD FACSDiva v8.0.1 software. Data were presented as density dot plots of Red vs Green Fluorescence. Gates representing, live intact (TO-positive), and dead (PI-positive) cells were drawn using control samples consisting of live and dead ethanol-treated TO/PI stained cells. The injured cell subpopulation was defined as cells with a combined high fluorescence of both stains (TO-positive, PI-positive) (Arku et al., 2011). To avoid overlap in the emission spectra of the two fluorochromes, controls consisting of live unstained, live TO-stained and dead ethanol-treated PI-stained cells were used for fluorescence compensation. In order to exclude cell doublets and clumps, a plot of FSC-H vs FSC-A was done for every analysis (Wersto et al., 2001). Cells that were not along the diagonal cluster of the FSC-H vs FSC-A plot were gated out and excluded in the analysis. The percentage of cells for each subpopulation was determined as the number of events in each gate as a proportion of the total number of events.

4.3.6 Sorting of subpopulations

The cell sorting process was done using a BD FACSAria™ cell sorter (BD Biosciences), following the procedure described by Le Roux et al. (2015a) with conditions and parameters as previously described by Kennedy et al. (2011). Before sorting, the cell sorter was calibrated using the standard protocols of BD BioSciences. The stream was calibrated using the Accudrop delay function. This was followed by checking the sort accuracy and purity

using the purity function. Accuracy and purity were tested by using internally labeled fluorescent beads (cytometer setup and tracking (CST) beads, BD BioSciences) excitable with 488 nm laser. After sorting approximately 40,000 beads for each subpopulation, the sorted beads were re-analyzed to determine the sort efficiency which was confirmed to be > 99% at all times. Sorting of membrane-damaged cells was based on drawing defined gates of the three main subpopulations (Intact, TO-positive/PI-negative; membrane damaged, TO-positive/PI-positive; dead, TO-negative/PI-positive). The population of interest was defined as cells with fluorescence signal of both TO and PI. Due to partial cell membrane damage, such cells exhibit an incomplete fluorescence resonance energy transfer (FRET) between thiazole orange and propidium iodide resulting in the cells emitting high fluorescence of both red and green fluorescence in both FL1 and in FL3 channels (Tamburini et al., 2013). Cells were sorted using a two-way sort into separate sterile 5 ml flow cytometry tubes with filter-sterilized PBS as sheath fluid, a 70 µm nozzle, a drop delay of 52 - 56 and a sort rate of 500 eps. Sorting was stopped after achieving a target of 10,000 events for the membrane damaged cell subpopulation. A sort of untreated intact cells was used as a positive control.

4.3.7 *Resuscitation of injured cells and growth kinetics*

After sorting 10,000 events (10^4 cells) in about 200 – 500 µl of sterile sheath fluid, the volume was adjusted to 1 ml using sterile PBS (pH 7.3) to give a suspension with a cell density of 10^4 cells/ml. An aliquot of 100 µl was used to inoculate 10 ml of BHI broth which was subsequently incubated at 37ºC, 25ºC and 4ºC. Resuscitation and growth of injured cells was monitored by viable counts at intervals of 1, 2, 4, 8, 12, 18, 24, 30 h for tubes incubated at 37ºC and 25ºC. Recovery at 4ºC was monitored at intervals of 3, 5, 10 and 15 d. At each sampling time, dilutions were made in maximum recovery diluent (0.1% peptone (Biolab, South Africa) and 0.85% NaCl (Merck) in distilled water), plated on BHI agar and incubated

at 37ºC for 48 h. The Log_{10} transformed growth data were fitted to the growth model of Baranyi and Roberts (1994) using DMFit version 3.5 Excel add-in software to determine specific growth rate (μ_{max}) and lag phase duration (λ). The limit of detection was 1.40 Log_{10} CFU ml^{-1}.

4.3.8   Recovery of sorted cells by plate counts

In order to check the re-growth ability of sorted cells, 100 µl of the sorted cell suspension was diluted with 900 µl of sterile maximum recovery diluent and inoculated onto BHI agar immediately after sorting. Inoculated plates were incubated at 37ºC for 48 h.

4.3.9   Data analysis

All experiments were repeated three times. Analysis of variance (ANOVA) ($\alpha = 0.05$) was used to determine the effect of strain, stress treatment, and temperature factors on percentage injury, lag phase duration and growth rates of *L. monocytogenes*. For multiple comparisons, ANOVA with Tukey's HSD test for correction in GraphPad Prism 6.0 was used to test for any significant differences between strains and stress treatments. A significance level of 0.05 with adjusted *P* values was used in each case. A heat map exploring the relationship between percentage injury, lag phase duration, and growth rate was constructed using Xlstat 2016 statistical package version (Addinsoft, New York, USA) after normalization of the data.

4.4   Results

4.4.1   Stress-induced injury by differential plating

The percentage of *L. monocytogenes* injury after exposure to osmotic, acid and heat stress based on differential plating on BHI agar and PALCAM agar is shown in Figure 4.1. Stress treatments had a significant effect ($p < 0.0001$) on percentage injury of *L. monocytogenes*

strains (Table 4.1). The degree of injury to acid, and heat stress ranged from 21.29 - 100%, 50.76 - 100% and 44.05 - 100% respectively (Figure 4.1). With the exception of strain 69, which showed a consistent lack of ability to grow on PALCAM agar after exposure to all forms of stress and therefore had an injury percentage of 100%, significant differences ($p <$ 0.0001) in the degree of injury were observed among *L. monocytogenes* strains in response to the three forms of stress for strains ATCC19115, 159/10 and 243 (Table 4.1).
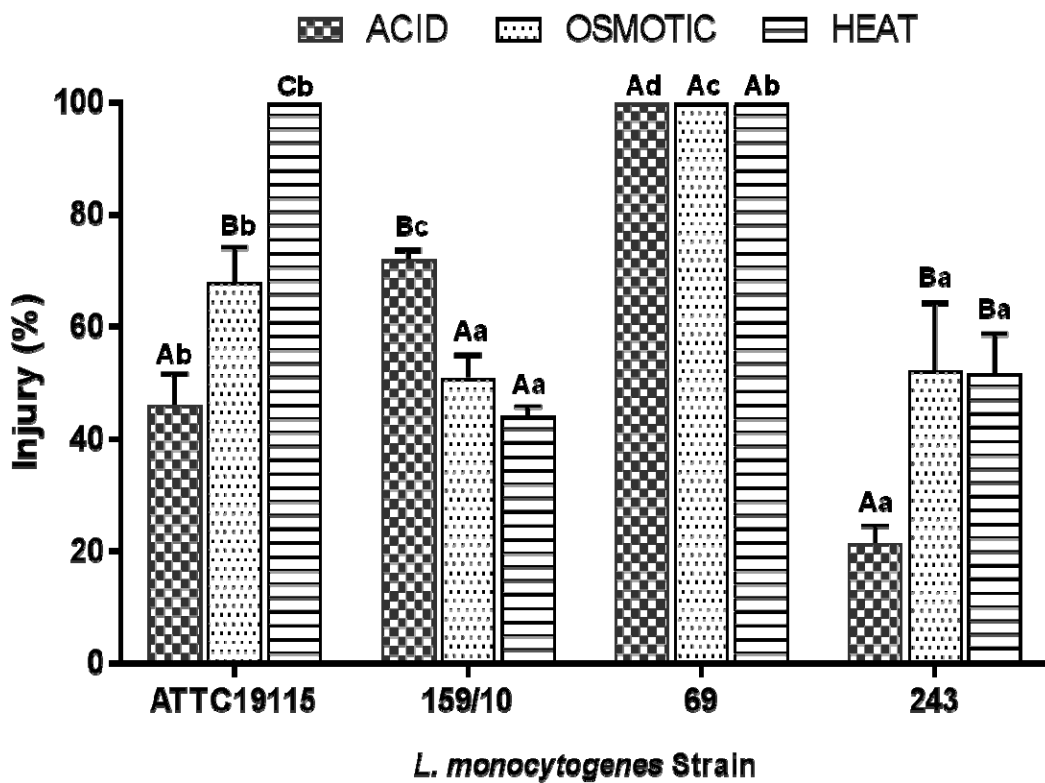
Figure 4.1: Percentage of sub-lethal injury of *L. monocytogenes* after osmotic, acid and heat stress exposure based on differential plating. Bars with different uppercase letters for each strain indicate significant differences (p ≤ 0.05). For each stress treatment, bars with different lowercase letters indicate significant differences (p ≤ 0.05) (Tukey's HSD).

4.4.2 Stress-induced injury by flow cytometry and differential staining

Figure 4.2 shows representative fluorescence density dot plots indicating the gates for the three subpopulations of live intact, injured and dead *L. monocytogenes* cells. Responses to each stress treatment are shown as fluorescence density dot plots in Figure 4.3 (A – C). In general, exposure to the three forms of stress increased the intensity of the red fluorescence resulting from PI. The proportion of injured cells showing the fluorescence signal of both stains was highest for acid treated cells (Figure 4.3A), followed by heat (Figure 4.3C) and osmotic stress (Figure 4.3B). The summary of the percentage proportion of injured cells (TO positive, PI positive) and intact cells (TO positive) for each of the stress treatments is shown in Figure 4.4. As observed with differential plating, the degree of cell injury by flow cytometry was significantly influenced ($p < 0.0001$) by stress treatment and strain of *L. monocytogenes* (Table 4.1). Osmotic stress resulted in the lowest degree of cell injury for all strains (17.5 – 26.6%) (Figure 4.4) with most of the surviving cells in the intact cell gate (Figure 4.3B). Strains ATCC19115 and 243 displayed a high susceptibility to acid stress with 79.1% and 71.6% injury respectively (Figure 4.4) which included some of the cells in the dead cell gate (Figure 4.3A).

Contrary to the high degree of injury observed for strain 69 with differential plating, flow cytometry showed that the same strain had consistently the highest proportion of membrane intact cells and low percentage injury (Figure 4.3 and Figure 4.4).
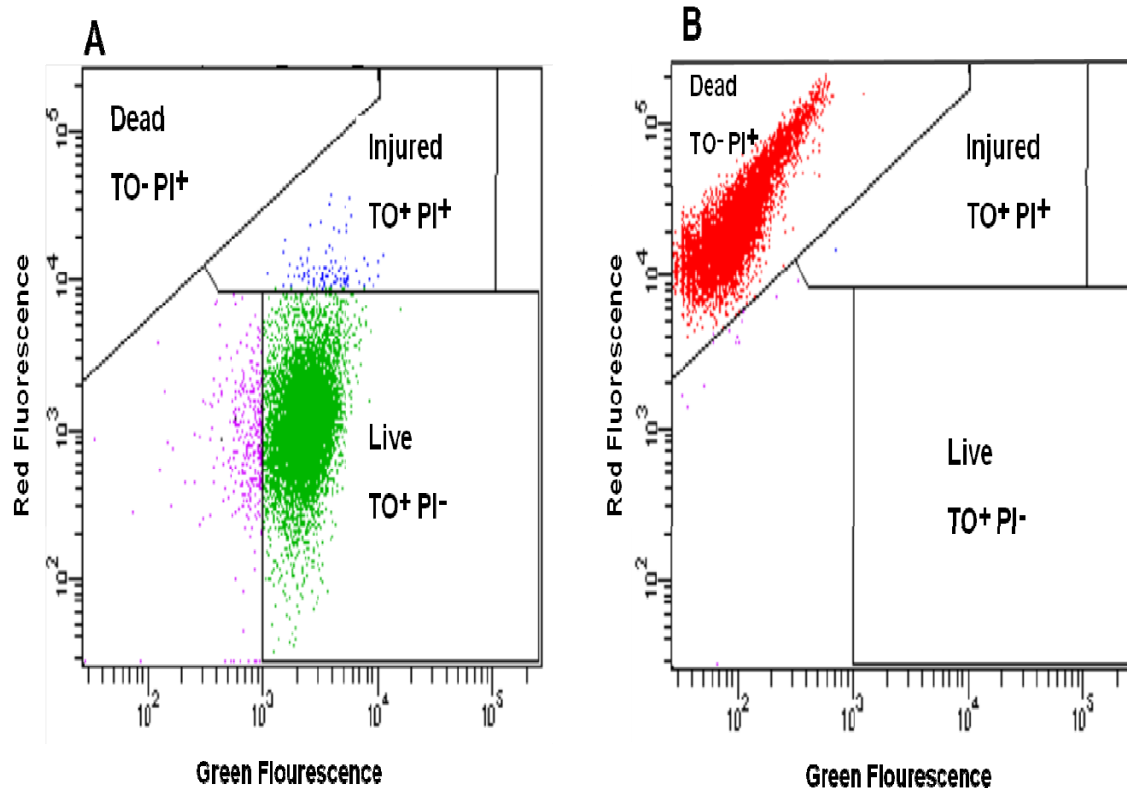
Figure 4.2: Representative fluorescence density dot plots of live *L. monocytogenes* cells stained with TO/PI (A) and ethanol-treated cells stained with TO/PI (B) showing gates of live, injured and dead cells.
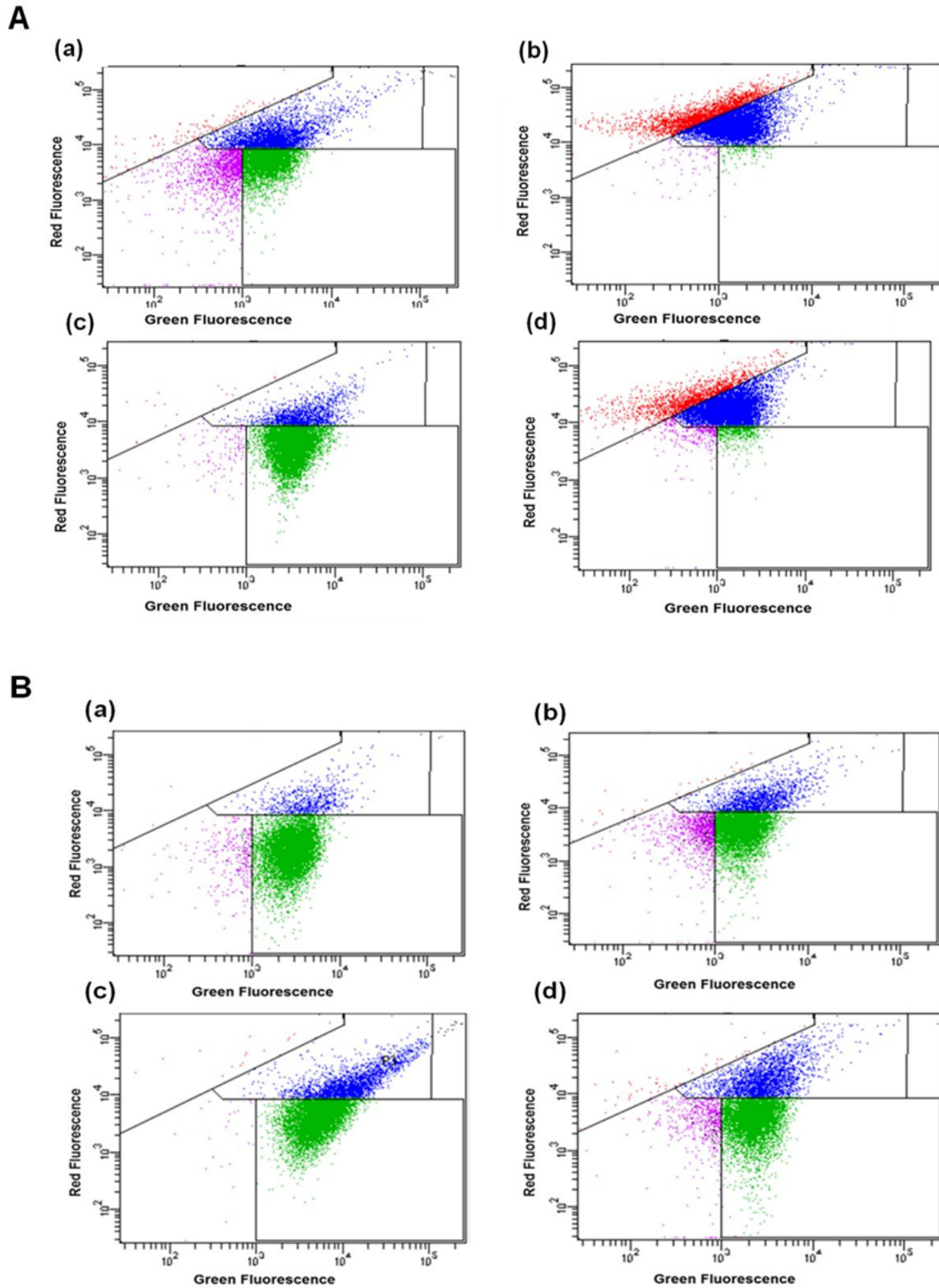
Figure 4.3: Fluorescence density dot plots of acid (A), osmotic (B) and heat (C) stress-treated *L. monocytogenes* cells stained with TO/PI. (a) - 159/10, (b) - ATCC19115, (c) - 69, (d) – 243.
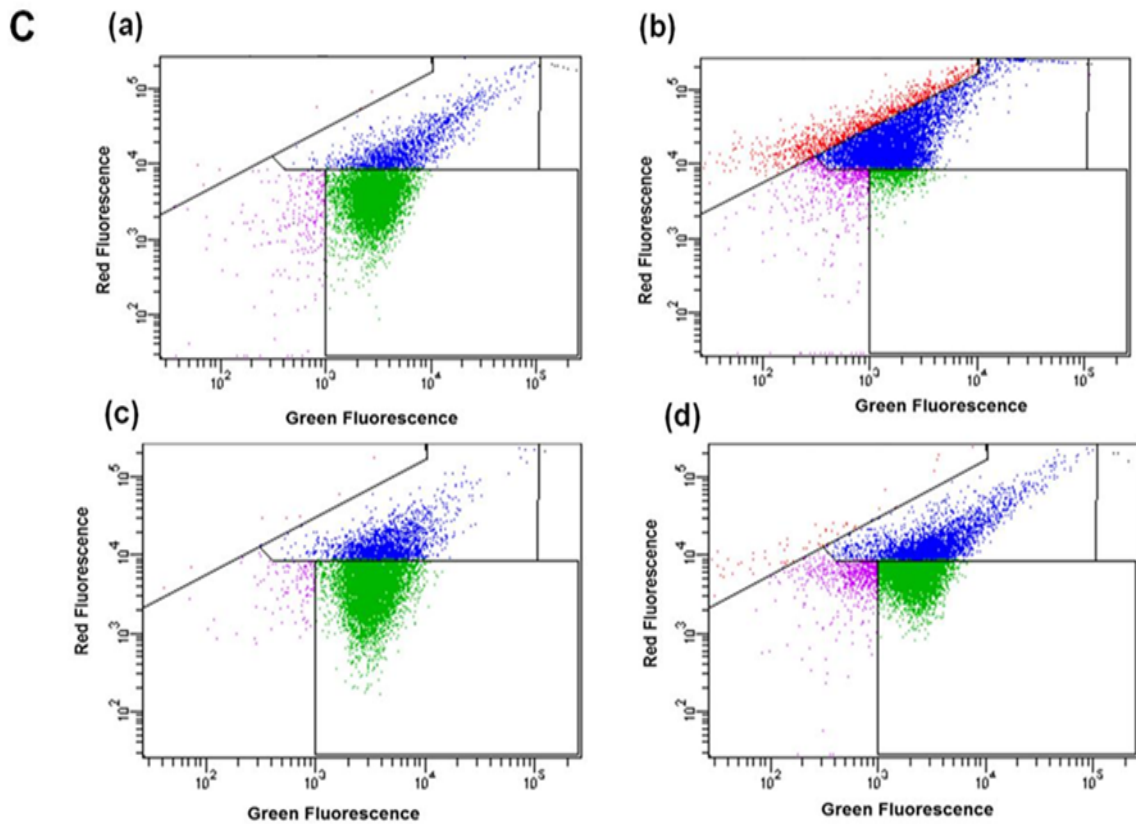
Figure 4.3 Cont': Fluorescence density dot plots of acid (A), osmotic (B) and heat (C) stress-treated *L. monocytogenes* cells stained with TO/PI. (a) - 159/10, (b) - ATCC19115, (c) - 69, (d) – 243.

Table 4.1: Analysis of variance showing the effect of strain and stress factors on percentage injury of *L. monocytogenes* based on differential plating and flow cytometry

Factor	DF	*P* value	
		Differential Plating	Flow Cytometry
Strain (ATCC19115, 159/10, 69, 243)	3	< 0.0001	< 0.0001
Stress (Acid, Osmotic, Heat)	2	< 0.0001	< 0.0001
Strain×Stress	6	< 0.0001	< 0.0001
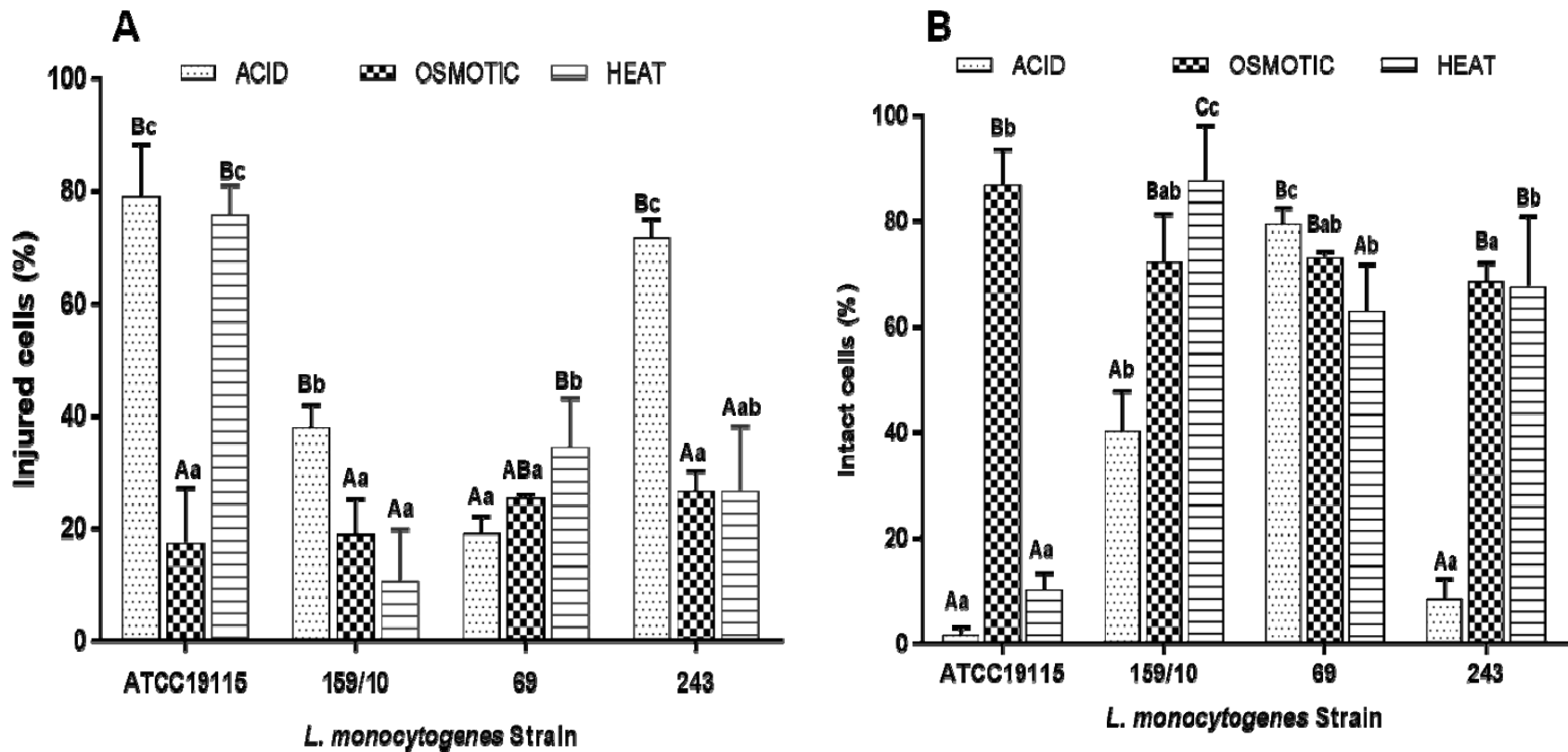
Figure 4.4: Percentage of sub-lethally injured (A) and intact (B) *L. monocytogenes* cells after exposure to osmotic, acid and heat stress based flow cytometry and TO/PI differential staining. For each strain, bars with different uppercase letters indicate significant differences ($p \leq 0.05$). For each stress treatment, bars with different lowercase letters indicate significant differences ($p \leq 0.05$) (Tukey's HSD).

Table 4.2: Re-growth potential of FACS sorted injured *L. monocytogenes* cell subpopulations re-grown on BHI agar at 37°C for 48 h (sorted cells = 10,000 cells per ml)

L. monocytogenes Strain	Acid		Osmotic		Heat	
	Log_{10} cells/ml	% Re-growth	Log_{10} cells/ml	*% Re-growth	Log_{10} cells/ml	*% Re-growth
ATTC19115	$2.81^a \pm 0.09$	6.6 ± 1.4	$3.04^a \pm 0.06$	10.9 ± 1.5	ng	ng
159/10	$3.98^c \pm 0.03$	96.29 ± 5.3	$3.86^c \pm 0.08$	70.0 ± 11.3	ng	ng
69	$3.96^c \pm 0.34$	92.9 ± 11.3	$3.91^c \pm 0.03$	81.5 ± 4.9	3.46 ± 0.02	29.0 ± 1.4
243	$3.58^b \pm 0.05$	44.0 ± 15.2	$3.46^b \pm 0.08$	29.2 ± 5.3	ng	ng

Values are means ± standard deviations of three replicate experiments. Means with different letters in the same column indicate significant differences ($p \leq 0.05$). ng – no detectable growth after sorting. *% Re-growth as a proportion of sorted cells.

4.4.3 Re-growth potential of sorted cells on BHI agar

After cell sorting, the growth potential of injured cells was assessed by their ability to form colonies on BHI agar (Table 4.2). Sorted acid and NaCl treated cells were all capable of re-growth although the proportion of cells capable of re-growth as a percentage of sorted cells was significantly lower ($p \leq 0.05$) for strains ATCC19115 and 243. Among heat-injured, only strain 69 cells were capable of re-growth.

4.4.4 Lag phase duration (λ) of resuscitating injured cells

The lag phase duration for resuscitating injured cells was significantly influenced by variations among *L. monocytogenes* strains ($p < 0.0001$), form stress treatment ($p \leq 0.007$), and resuscitation temperature ($p \leq 0.001$) (Table 4.5). At 25ºC, λ values were 4.51 – 9.14 and 3.26 – 9.22 days for acid and osmotic stress injured cells respectively (Table 4.3). When resuscitated at 37ºC, the same cells had λ values of 1.96 – 7.15 and 0.50 – 7.14 days respectively. When individual strains were compared, *L. monocytogenes* strain 69 had the shortest lag phase duration ($p < 0.05$) at 37ºC for its acid and osmotic stressed cells (Table 4.4) while strains with a high susceptibility to stress (ATCC19115 and 243) had longer periods of injury repair. λ values for unstressed control cells could not be determined by the DMfit software owing to their quick commencement of growth (Tables 4.3 and 4.4).

4.4.5 Maximum specific growth rate (μmax) of resuscitated cells

The temperature of incubation had a significant effect ($p < 0.0001$) on the maximum specific growth rate of resuscitated *L. monocytogenes* cells (Table 4.5). At 37ºC, μ_{max} values were in the range of 0.47 – 0.69 Log_{10} $CFUml^{-1}h^{-1}$ (Table 4.3) compared to 0.38 – 0.54 Log_{10} $CFUml^{-1}h^{-1}$ at 25ºC (Table 4.3). *L. monocytogenes* strain variations ($p \leq 0.330$) and stress treatments ($p \leq 0.407$) had no significant effect on growth rate (Table 4.5). μ_{max} values were 0.38 - 0.69

and $0.42 - 0.69$ Log_{10} $CFUml^{-1}h^{-1}$ for acid and NaCl injured cells respectively (Tables 4.3 and 4.4). However, when compared to their respective untreated controls, μ_{max} values for injured cells at 25ºC were significantly higher ($p < 0.05$) for strains 69, 159/10 and 243 (Table 4.3).

Table 4.3: Lag phase duration (λ) and growth rate (μ_{max}) of FACS sorted osmotic, acid and heat-injured *L. monocytogenes* cells resuscitated in BHI broth at 25°C

L. monocytogenes Strain	Treatment	λ (h)	μ_{max} (Log$_{10}$ CFUml^{-1}h^{-1})	R^2
ATCC19115	Acid	6.48^{Aa}±0.74	0.40^{Aa}±0.08	0.98
159/10		9.14^{Ba}±1.46	0.46^{Aab}±0.08	0.94
69		4.98^{Aa}±0.60	0.49^{Ab}±0.09	0.99
243		4.51^{Aa}±0.80	0.38^{Ab}±0.01	0.99
ATCC19115	Osmotic	7.65^{Ba}±0.77	0.47^{Ba}±0.02	0.99
159/10		7.61^{Ba}±0.23	0.54^{Bb}±0.01	0.97
69		3.26^{Aa}±0.05	0.42^{Ab}±0.03	0.97
243		9.22^{Bb}±0.10	0.50^{Bc}±0.03	0.99
ATCC19115	Heat	ng	ng	
159/10		ng	ng	
69		5.98^{ab}±0.22	0.47^{b}±0.01	0.99
243		ng	ng	
ATCC19115	Control	nd	0.50^{Ba}±0.01	0.97
159/10		nd	0.38^{BAa}±0.01	0.99
69		nd	0.25^{Aa}±0.04	0.99
243		nd	0.27^{Aa}±0.01	0.98

λ and μ_{max} values are means ± standard deviations of three replicate experiments. Means with different uppercase letters in the same column for each treatment indicate significant differences ($p \leq 0.05$). For each strain, means with different lowercase letters indicate significant differences ($p \leq 0.05$). ng – no growth after sorting. nd – lag phase duration not detectable. R^2 – Goodness of fit for the Baranyi and Roberts (1994) model (Coefficient of determination).

Table 4.4: Lag phase duration (λ) and growth rate (μ_{max}) of FACS sorted osmotic, acid and heat-injured *L. monocytogenes* cells resuscitated in BHI broth at 37°C

L. monocytogenes Strain	Treatment	λ (h)	μ_{max} (Log$_{10}$ CFUml^{-1}h^{-1})	R^2
ATCC19115	Acid	7.15^{Ca}±0.07	0.69^{BCb}±0.01	0.99
159/10		4.14^{Ba}±0.01	0.47^{Aa}±0.03	0.98
69		1.96^{Aa}±0.19	0.65^{ABb}±0.04	0.97
243		5.12^{Ba}±0.29	0.54^{ABb}±0.02	0.99
ATCC19115	Osmotic	5.39^{Ba}±0.05	0.62^{Cb}±0.04	0.99
159/10		6.52^{Bb}±0.09	0.69^{Cc}±0.01	0.97
69		0.50^{Aa}±0.16	0.52^{Ba}±0.01	0.97
243		7.14^{Ba}±0.59	0.49^{Ab}±0.06	0.99
ATCC19115	Heat	ng	ng	
159/10		ng	ng	
69		5.31^{b}±0.01	0.69^{b}±0.00	0.97
243		ng	ng	
ATCC19115	Control	nd	0.42^{Aa}±0.05	0.95
159/10		nd	0.58^{Bb}±0.01	0.99
69		nd	0.66^{Bb}±0.03	0.99
243		nd	0.44^{Aa}±0.04	0.98

λ and μ_{max} values are means ± standard deviations of three replicate experiments. Means with different uppercase letters in the same column for each treatment indicate significant differences ($p \leq 0.05$). For each strain, means with different lowercase letters indicate significant differences ($p \leq 0.05$). ng – no growth after sorting. nd – lag phase duration not detectable. R^2 – Goodness of fit for the Baranyi and Roberts (1994) model (Coefficient of determination).

Table 4.5: Analysis of variance showing the effect of strain, stress and temperature factors on lag phase duration, maximum specific growth rate of *L. monocytogenes*

Factor	DF	*P* value	
		λ	μ_{max}
Strain (ATCC19115, 159/10, 69, 243)	3	< 0.0001	0.330
Stress (Acid, Osmotic, Heat)	2	0.007	0.407
Temperature (25ºC, 37ºC)	1	0.001	< 0.0001

4.4.6 Recovery of injured cells at 4ºC

Table 4.6 shows the growth potential of injured *L. monocytogenes* resuscitated in BHI broth at 4ºC. No growth was detectable in the first 3 days of incubation with a detection limit of 1.40 Log_{10} CFU ml^{-1}, for all strains except for *L. monocytogenes* 69 whose population had risen to greater than 3.0 Log_{10} CFU ml^{-1} in the same period. The population of acid and osmotic stress injured strain 159/10 reached levels of 2.72 Log_{10} CFU ml^{-1} and 2.92 Log_{10} CFU ml^{-1} respectively after 10 days. Acid and osmotic stress injured *L. monocytogenes* strains ATCC19115 and 243 took 15 d to reach levels ≥ 2.30 Log_{10} CFU ml^{-1}. With the exception of strain 69, heat-injured cells could not recover after 15 days of incubation.

.

Table 4.6: Recovery of injured *L. monocytogenes* cells in BHI broth at 4°C and time to detection of ≥ 1.40 Log_{10} $CFUml^{-1}$

L. monocytogenes Strain	Osmotic		Acid		Heat	
	Time (d)	Log_{10} $CFUml^{-1}$	Time (d)	Log_{10} $CFUml^{-1}$	Time (d)	Log_{10} $CFUml^{-1}$
ATCC19115	15	2.30±0.06	15	3.16±0.16	nd	
159/10	10	2.92±0.44	10	2.72±0.33	nd	
69	3	3.06±0.08	5	6.19±0.02	3	2.30±0.06
243	15	3.44±0.46	15	3.25±0.07	nd	

nd – Not detectable

4.4.7 *Heat map clustering of cell injury, lag phase duration and growth rate*

Heat map clustering of cell injury, lag phase duration and growth rate (Figure 4.5) showed that the response clustered into three main groups indicating an association between percentage injury of individual strains and lag phase duration. Strain 69 which had a low percentage cell injury tended to have a short lag phase duration at both 25ºC and 37ºC. In addition, strains with a high degree of cell injury particularly related to acid stress tended to have long lag phase durations.
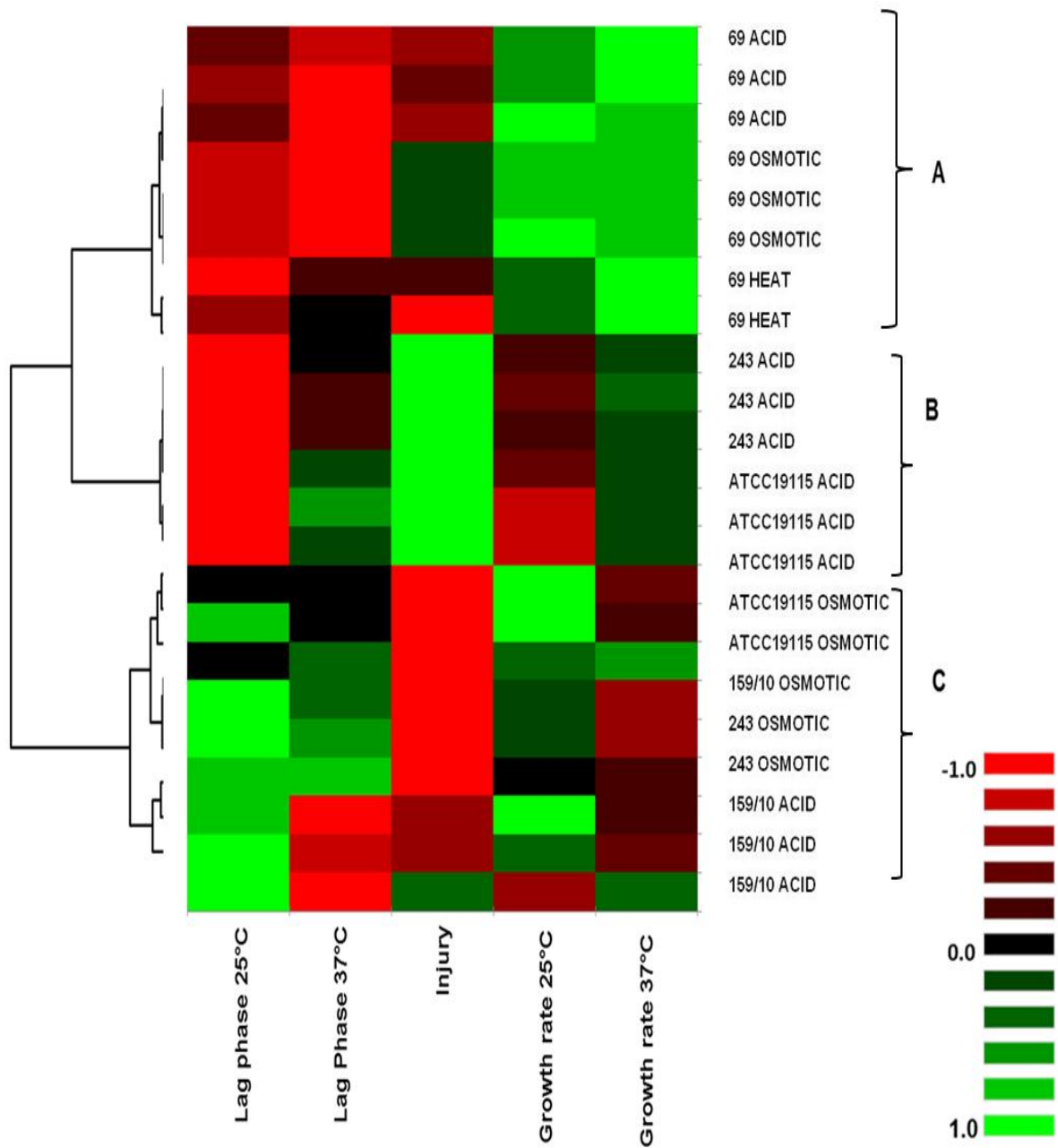
Figure 4.5: Heat map of the relationship between cell injury and growth parameters of FACS sorted osmotic, acid and heat-injured strains of *L. monocytogenes* resuscitated in BHI broth. Green (+1.0) represents high values of cell injury and growth parameters, red (-1.0) - low values and black (0.0) - median values. Response clustered into three main groups. A - Strain 69 with short lag phase, B - High acid injury with long lag phase, C - Low osmotic and acid injury with short lag phase.

4.5 Discussion

The objective of this study was to examine the effect of stress-induced cell injury on the resuscitation and growth behavior of resuscitated *L. monocytogenes* cells following cell sorting. The initial step in the study was to assess the degree of stress-induced injury based on differential plating and flow cytometry. This was followed by recovery of injured cells by FACS sorting and their re-growth in BHI broth.

On the analysis of stress imposed injury, both methods showed significant differences in response among *L. monocytogenes* strains and also among stress treatments. No clear patterns of response were observed with the plate count method with the most conspicuous observation being the high percentage of injury (100%) recorded for strain 69 (for all stress treatments) and heat stressed ATCC19115, which manifested as a complete lack of growth on selective media after stress exposure. In contrast to flow cytometry and differential staining, a clear pattern of response was observed in which acid stress caused the highest degree of cell membrane damage with osmotic stress causing the least, regardless of strain. The complete cell injury of strain 69 (based on plate counts) was unexpected given that $\leq 40\%$ of the cells (for all forms of stress) were identified as membrane damaged by flow cytometry. Possible reasons for this observation could be that stressed non-injured cells may be susceptible to selective agents incorporated in selective media. The presence of acriflavine, nalidixic acid and lithium chloride in PALCAM agar has been shown to be inhibitory to some stressed *L. monocytogenes* strains (Liamkaew et al., 2014). This result emphasizes the drawbacks of culture-based methods for detection of sub-lethal injury as it implies a potential for over-estimation of injury and under-estimation of live cells (Van Nevel et al., 2017). However, in some cases, the two methods showed a good agreement in detection of injury. For instance, the 100% injury for strain ATCC19115 (lack of growth on selective media) was strongly

supported by cytometric data which revealed that 73% of the cells were injured with about 12% of the population dead. A positive correlation between the two methods has been reported in previous studies (Massicotte et al., 2017).

Differences in percentage injury among the three stress treatments based on flow cytometry and differential staining are likely an indication of differences in mechanism of antimicrobial action and extent to which the stress exposures affect cell membrane integrity. The high degree of injury arising from acid stress is likely related to the antimicrobial mechanism of lactic acid. While acid stress is not associated with direct degradation of cell membrane structural components (Wood, 1999), the increased influx of protons (associated with acid exposure) disrupts the trans-membrane potential thereby affecting the proton motive force and membrane permeability (Alakomi et al., 2000). This explanation has been supported by previous studies using flow cytometry and membrane permeability markers which showed that lactic acid can increase membrane permeability in Gram-negative bacteria (Alakomi et al., 2000). The resultant effect on membrane permeability could be the cause of the increased uptake of PI by acid-stressed *L. monocytogenes* cells.

The low percentage of cell injury resulting from NaCl stress is a reflection of the intrinsic salt resistance of *L. monocytogenes*. Previous studies have reported the ability of *L. monocytogenes* strains to survive and grow in high NaCl containing media (Bergholz et al., 2010). The effect of osmotic stress on bacterial cells arises from changes in hydrostatic pressure that results in cell membrane tension and bilayer deformations (Wood, 1999). The low level of injury implies that the effect of the osmotic stress was not sufficient to cause any significant influence on membrane permeability resulting in a low level of PI uptake by stressed cells.

Except for *L. monocytogenes* ATCC19115, the level of heat-induced injury was ≤ 40%. Heat stress response of *L. monocytogenes* has been shown to vary considerably among strains from different serotypes (Aryani et al., 2015a). Apart from the cell membrane, heat stress affects several other cellular targets such as enzymes, RNA and DNA (Kramer and Thielmann, 2016). Using a multi-method approach, Kramer and Thielmann, (2016) concluded that several cellular targets such as the respiratory pathway are affected by heat stress before loss of membrane integrity. This highlights the fact that membrane integrity indicators like PI may not be good indicators of cell injury and viability loss following heat exposure.

Following cell sorting, the percentage of re-growth of injured cells showed a link to the extent of cell damage recorded by PI uptake of stressed cells. While PI staining has been reported to be a sensitive marker of cell damage, it is a poor indicator of cell viability loss (Novo et al., 2000) thus some TO positive/PI positive cells may not be viable. It is likely that a high degree of cell damage for stress-sensitive strains, ATCC19115 and 243 could have resulted in loss of viability for some sorted injured cells resulting in low percentage of re-growth. Amor et al. (2002) observed a similar reduction in the re-growth potential of sorted injured *Bifidobacterium* cells following bile salt stress.

The lag phase duration of FACS sorted injured cells, resuscitated in BHI broth varied significantly with temperature, stress treatment and strains of *L. monocytogenes*. In bacterial growth kinetics, the lag phase duration is a measure of the time a bacterial population takes to commence exponential growth following a transition to a new environment (Swinnen et al., 2004). The duration of this period is influenced by the physiological state of the cell population prior to environmental change (Augustin et al., 2000). The shorter lag phase at 37 °C could be a result of a quicker rate of repair which is associated with the rate of initiation of

protein synthesis, in particular, the enzymes necessary for the synthesis of membrane lipids needed for repair of damaged cell membranes (García et al., 2006). With the exception of strain 69, the lag phase duration of resuscitating acid and osmotic stress injured *L. monocytogenes* was at least 4.5 h at 25 °C and 4.1 h at 37 °C which was expectedly higher than non-injured control cells. The extended length of the lag phase for injured cells results from the need to resolve cell damage before cells can commence division. In addition to cell membrane damage, which was the basis of cell sorting, stress-induced cell injury can result in damage to other functional components of the cell such as DNA, RNA and membrane potential (Chilton et al., 2001). Resuscitation of such injured cells has been shown to depend on the degree of damage which is influenced by the magnitude of stress (Mellefont and Ross, 2003). The short lag phase of strain 69 was particularly unique from the other three strains. The quick recovery of this strain appeared to be consistent with its low percentage of membrane damage after stress exposure.

Unlike acid and osmotic stress, heat-injured cells of strains ATCC19115, 159/10 and 243 were unable to re-grow under any conditions when sorted into BHI broth. This indicates that in addition to membrane damage, heat stress results in damage to other cellular targets such as proteins, DNA, rRNA, ribosomes, respiratory processes and electron transport chain (Kramer and Thielmann, 2016). The fact that membrane intact cells were detectable after heat exposure is an indication that heat-induced disruption of cellular processes occurs before membrane damage (Kramer and Thielmann, 2016). This signifies that membrane integrity alone is not a sufficient indicator of injury and viability of heat-stressed cells.

The long lag phases of resuscitating *L. monocytogenes* cells have huge implications for the detection of injured cells in food systems. For effective detection, bacterial concentration

must reach about 10^2 to 10^4 CFU ml^{-1} in the enrichment broth (Dupont and Augustin, 2009). In this study, the inoculation level of injured cells was meant to achieve a cell density of 10^2 cells/ml in the growth medium. However, in the case of acid injured cells, the proportion of cells capable of growth dropped below the detection limit of 1.40 Log$_{10}$ CFU ml^{-1} for the first 4 h. This initial response is perhaps due to the fact that individual cell variation influences the probability of growth post stress exposure. Dupont and Augustin (2009), observed that stress exposure reduces the growth probability of individual cells of *L. monocytogenes* in half-fraser broth. This highlights the importance of including a step of injured cell repair in a non-selective enrichment broth before selective enrichment in pathogen detection methods.

The growth rate of resuscitated *L. monocytogenes* showed no variation among strains and was expectedly lower at 25 °C than at 37 °C. Growth rate differences between 25 °C and 37 °C, reflect temperature dependence of growth processes in exponential phase. Within the growth-permitting temperature range, bacterial growth rate is influenced by the rate of biological reactions which follow first-order kinetics, a situation that has been observed for *L. monocytogenes* growth in foods (Lee et al., 2014). In addition, at temperatures below optimum, reduction in cell membrane fluidity lowers nutrient affinity which subsequently reduces growth rate (Nedwell, 1999).

The importance of *L. monocytogenes* as a pathogen in RTE foods arises from the fact that refrigeration is invariably used as a terminal hurdle in the preservation of such foods and yet the organism is a psychrotroph capable of growth at refrigeration temperatures (Schmid et al., 2009b). After inoculating 100 cells/ml of injured cells into BHI broth at 4 °C, the number of cells capable of growth dropped below the detection limit of 1.40 Log10 CFU ml^{-1} in the first 5 days except for strain 69. Considering that the stress treatments and subsequent sorting and

inoculation of cells were done at room temperature, the transfer of injured cells to a 4 °C environment could have induced cold shock to which some of the injured cells were susceptible. Differences in the cold growth behavior of strains indicated a link to the percentage of cell damage observed in flow cytometry analysis with strain 69 showing the fastest growth while strains ATCC19115 and 243 had extended growth periods. Depending on the physicochemical properties of the food, the control of *L. monocytogenes* in RTE foods is largely based on limiting its cold growth by extension of the lag phase period (Angelidis et al., 2013). The findings of this study show that cold stress may be insufficient to prevent recovery of injured *L. monocytogenes* cells. However, resuscitation of these cells was done in a broth model without any physicochemical hurdles to limit growth. It is likely that in a food matrix with additional hurdles to prevent growth, the response of such injured cells could be different. García, et al. (2005) observed that sub-lethally injured *E. coli* cells after pulsed electric fields treatment were sensitive to refrigerated storage in apple juice, thus stressing the need to study the resuscitation of sorted cells in different food matrices.

4.6   Conclusions

This study underscored the importance of flow cytometry in the study of stress imposed cell injury and the potential of FACS cell sorting in the study of injured cells. Generally, flow cytometry and fluorescent staining with membrane integrity indicator probes showed a clear pattern of response with acid stress causing the highest level of injury followed by heat and osmotic stress. Lack of re-growth of FACS sorted heat-injured cells showed that while membrane integrity was a good indicator of cell injury and viability loss for acid and osmotic stress, it was not a sufficient indicator of heat stress injury. The length of the lag phase duration (indicating repair period) is the major difference in the resuscitation behavior of *L. monocytogenes* strains. Once injured cells repair the cellular damage, their growth rate is not

different from non-injured cells regardless of form of stress and strain differences. Thus highlighting the potential food safety risks of stress injured *L. monocytogenes* cells.

5   CHAPTER 5

MODELLING POPULATION DYNAMICS OF *LISTERIA MONOCYTOGENES* IN LACTIC SOFT CHEESE FOLLOWING ACID AND OSMOTIC STRESS EXPOSURE

5.1 Abstract

The survival response of *Listeria monocytogenes* strains in lactic soft cheese stored at 4ºC was studied following acid and osmotic stress exposures. Survival data of individual and mixed strains were fitted to four primary inactivation models (log-linear with tail, biphasic, Weibull and Weibull with tail). Lack of fit (LoF) analysis, showed the Weibull and log-linear with tail models provided the best fit of the data and were used to determine kinetic parameters. Analysis of inactivation kinetics showed that the susceptibility of individual strains ($p < 0.0001$) and the form of stress exposure ($p < 0.05$) have a significant effect on survival response. Both acid and osmotic stress exposures resulted in enhanced survival for stress tolerant strains and enhanced inactivation for the susceptible strain indicating that stress exposure results in the development of tolerance responses or cell injury depending on the susceptibility of individual strains. Typing of surviving populations of *L. monocytogenes* in mixed strain challenge tests revealed that one strain out-lived other strains and remained the only survivor after 15 days. Variations in the persistence of strains existing as co-contaminants in soft cheese imply that models for *L. monocytogenes* survival in foods must be based on such persistent strains.

Keywords: *Listeria monocytogenes*, modeling, survival, stress, lactic soft cheese.

5.2 Introduction

Listeria monocytogenes is the etiologic agent of the human disease listeriosis, an invasive infection resulting from ingestion of contaminated foods. Among dairy products, soft cheeses are leading causes of listeriosis outbreaks in many parts of the world (Makino et al., 2005; Melo et al., 2015; Schoder et al., 2012). As ready to eat (RTE) foods with no post-process lethality step, contamination of soft cheese with *L. monocytogenes* during processing provides an ideal condition for pathogen survival. According to the Codex Alimentarius Commission (CAC, 2009) guidelines, RTE foods are distinguished into two categories based on the physicochemical limits that influence their ability or inability to support the growth of *L. monocytogenes*. RTE products with a pH $\leq$ 4.4, a_w $\leq$ 0.92 or pH $\leq$ 5.0 and a_w $\leq$ 0.94 are considered unable to support *L. monocytogenes* growth. In both product categories, the safety threshold for *L. monocytogenes* is 100 CFU/g throughout the product shelf life. However, regardless whether a product supports or does not support growth, it must have no detectable *L. monocytogenes* in a 25 gram sample if it is meant for infants, and other high risk groups. Owing to their low pH (3.8 – 4.4) (Rogga et al., 2005), lactic soft cheeses are classified as RTE foods that do not support the growth of *L. monocytogenes*. However, cases of listeriosis outbreak related to acid curd cheeses have been reported. The most recent being the Germany and Austrian outbreak related to quargel acid curd cheese (Fretz et al., 2010; Koch et al., 2010).

An important element of *L. monocytogenes* risk assessment is the use of accurate and reliable kinetic data obtained from models reflecting the behavior of the pathogen in foods (Drosinos et al., 2006). Due to its continued exposure to environmental stresses in the food processing environment, *L. monocytogenes* cells may develop stress tolerance responses that enhance survival ability (Giaouris et al., 2014), cross-protection to subsequent stress exposures in the

food product (Mataragas et al., 2008) and cell injury (Wesche et al., 2009). While *L. monocytogenes* survival models have been done in several RTE foods (Mataragas et al., 2015a; Mataragas et al., 2015b; Tiwari, et al., 2014), the effect of stress adaptation, cross-protection and injury on the growth or inactivation of the pathogen has been limited to broth models (Schvartzman et al., 2010; Tiganitas et al., 2009). Survival models that do not incorporate the physiological state of the cells at the point of product contamination, the food product structure and composition are likely to lead to prediction errors and output uncertainty in risk assessment studies (Schvartzman et al., 2010).

An additional challenge with *L. monocytogenes* contamination is that food processing environments can harbor several different strains (Strydom et al., 2013) resulting in food products contaminated by multiple strains as has previously been demonstrated with contaminated cheese and cantaloupe (Danielsson-Tham et al., 1993; Laksanalamai et al., 2012; Rychli et al., 2014). The occurrence of multiple *L. monocytogenes* strains in co-cultures can trigger competition among strains which influences growth ability and detectability of less competitive strains (Zilelidou et al., 2015; Zilelidou et al., 2016b). However, the concept of *L. monocytogenes* strain competition has been studied under growth permissive conditions. The survival ability of mixed strains in food products that do not support the growth of the organism has not been studied. The aim of the study was to model survival response of *L. monocytogenes* strains individually and as mixed strains in a lactic soft cheese after exposure to acid and osmotic stress and to identify the surviving populations in cheese samples co-inoculated with mixed strains.

5.3   Materials and methods

5.3.1   *Bacterial strains and stress treatments*

The strains of *L. monocytogenes* used in this study and their growth and stress treatment conditions were as described in Section 4.3.1 – 4.3.2 (Chapter 4) of this thesis. Because of the need to resolve strain survival in mixed strain studies, strains 69, ATCC19115 and 159/10 were used in this study while strain 243 was left out as it had a DNA fingerprint profile similar to strain 159/10 based on GTG_5 repetitive elements PCR (REP-PCR).

5.3.2   *Cheese preparation and inoculation*

Soft cheese was produced following the method used for the industrial production of South African cottage cheese. Commercial fat-free pasteurized milk with a solid content of 14% (m/v) was inoculated with a starter culture comprising of a mixture of *Lactococcus lactis* subspp. *lactis* and *Lactococcus lactis* subspp. *cremoris* (Cape Food Ingredients, South Africa) and left to ferment for 16 h at 25°C to a pH of 4.6. After coagulation and cutting, the coagulum was heated slowly to a temperature of 45°C over a period of 2 h with constant stirring and held at the same temperature for 10 min. After cooling to 25°C, the coagulum was drained and the solid curds were washed with cold and then ice water. The soft cheese was left to drain the excess whey on a cheesecloth for 8 h and analyzed for pH, titratable acidity and lactic acid bacterial counts (pH - 4.17±0.06; titratable acidity (%) – 0.63±0.06; lactic acid bacterial count (Log_{10} $CFUg^{-1}$) – 9.16±0.21). One hundred gram samples of the cheese were packaged in sterile 100 ml polypropylene tubs and inoculated with 100 µl of standardized bacterial suspensions to a final density of approximately 10^5 CFU/g and thoroughly mixed to achieve uniform distribution of the inocula. Inoculated samples were stored at 4°C for 15 days.

5.3.3 Enumeration of survivors

The number of surviving *L. monocytogenes* in cheese samples was monitored by viable counts at 0, 2, 4, 6, 8, 10, 12 and 15 days. Five gram samples were diluted with 45 ml of a maximum recovery diluent (MRD) (0.85% NaCl (Merck) and 0.1% peptone (Oxoid) in sterile stomacher bags and homogenized for 30 s in a laboratory stomacher. Serial dilutions (1 ml) of the homogenate were plated out in duplicates on PALCAM agar (Oxoid). Plates were incubated at 37°C for 48 h. The detection limit was 5 CFU/g. Samples that showed no detectable *L. monocytogenes* survivors after 15 days were subjected to an enrichment process following the standard ISO method (EN-ISO 11290-1) for the detection of *L. monocytogenes* in foods. A 10 g sample was pre-enriched in 90 ml Half-Fraser broth (Oxoid) and incubated at 30ºC for 24 h followed by an enrichment of 0.1 ml of the pre-enrichment broth in 10 ml of Fraser broth (Oxoid) and incubation at 37ºC for 24 h.   This was followed by streaking of the enrichment broth on PALCAM agar (Oxoid).

5.3.4 Survival models

A plot of inactivation data (Log_{10} CFU/g vs Time in days) was prepared in Microsoft Excel 2013 (Microsoft, USA). The experimental data were fitted to four mathematical models (Equations 5.1 - 5.4) by non-linear regression using GInaFiT version 1.7 (Geeraerd et al., 2005).

Log-linear with tail model (Geeraerd et al., 2000).

$$Log_{10}N(t) = Log_{10}\left(\left(10^{Log_{10}(N_0)} - 10^{Log_{10}(N_{res})}\right) \times \exp\left(-K_{max} \times t\right) + 10^{Log_{10}(N_{res})}\right)$$

$$(5.1)$$

where *N(t)* is the cell concentration in CFU/g at time *t* (d), *N₀* is the initial cell density (CFU/g), k_{max} is the maximum inactivation rate (per day). N_{res} - resistant cell fraction.

Weibull model (Mafart et al., 2002).

$$Log_{10}N(t) = Log_{10}N_0 - \left(\frac{t}{\delta}\right)^p$$

(5.2)

where $N(t)$ is the cell concentration in CFU/g at time t (d), N_0 is the initial cell density (CFU/g), δ is the time to the first-decimal reduction (d) and p is a shape parameter.

Weibull with tail model (Albert and Mafart, 2005).

$$Log_{10}\,N(t) = Log_{10}\left(\left(10^{Log\,10\,(N_0)} - 10^{Log\,10\,(N_{res})}\right) \times 10^{\left(\left(\frac{t}{\delta}\right)^p\right)} + 10^{Log\,10\,(N_{res})}\right)$$

(5.3)

where $N(t)$ is the cell concentration in CFU/g at time t (d), N_0 is the initial cell density (CFU/g), δ represents the first-decimal reduction time (d), p is a shape parameter. N_{res} - resistant cell fraction.

Biphasic model (Cerf, 1977).

$$Log_{10}\,N(t) = log_{10}(N_0) + log_{10}(f \times exp(-k_{sens} \times t) + (1 - f) \times exp\,(-k_{res} \times t))$$

(5.4)

where $N(t)$ is the cell concentration in CFU/g at time t (d), N_0 is the initial cell density (CFU/g), f is the fraction of sensitive subpopulation, $(1 - f)$ is the fraction of the resistant subpopulation, k_{sens} and k_{res} are the inactivation rates of the sensitive subpopulation and resistant subpopulations respectively.

5.3.5 *Statistical evaluation of models*

To select the best models, survival models were analyzed for their adequacy in describing the experimental data using the LoF statistic. The residual mean square error of the model was

compared to the residual mean square error of the data using the F test ($\alpha = 0.05$), according to the method of Zwietering et al. (1990).

$$F = \frac{MSE_{Model}}{MSE_{Data}}$$

(5.5)

where MSE_{Model} and MSE_{Data} are the mean square errors of the model and data respectively. Model adequacy was accepted if the calculated F ratio was less than the tabulated F value (F_{Table}), where the degrees of freedom for the model (DF_{Model}) was the total number of data points (number of replicates × number of sampling points) minus the number of sampling points while the degrees of freedom for the data (DF_{Data}) was the number of data points minus the number of replicates (Drosinos et al., 2006).

In addition to the LoF test, the root mean square error (RMSE), accuracy factor (Af), bias factor (Bf) and coefficient of multiple determination (R^2) (Drosinos et al., 2006; Mataragas et al., 2008; Ross, 1996) were utilized to further analyze the selected models.

$$RMSE = \sqrt{\frac{\sum(Observed - Predicted)^2}{n}}$$

(5.6)

$$Accuracy\ Factor = 10^{\left(\Sigma\, Log \left|\frac{Predicted}{Observed}\right| / n\right)}$$

(5.7)

$$Bias\ Factor = 10^{\left(\Sigma\, Log \left(\frac{Predicted}{Observed}\right) / n\right)}$$

(5.8)

where n is the number of data points and observed and predicted values represent the experimental data and the values predicted by the model at each respective data point.

5.3.6 Typing of L. monocytogenes survivors from mixed strain challenge tests

A total of 80 colonies from four different plates representing both acid and osmotic stress-treated survivors from mixed strain challenge tests after 15 days were typed by GTG$_5$ REP-

PCR fingerprinting. Genomic DNA was extracted from cultures grown in BHI broth at 37°C overnight using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Irvine, USA) following the manufacturer's instructions. DNA concentration in ng/µl was determined by measuring optical density (OD_{260nm}) using a nano spectrophotometer (Bibby Scientific, Staffordshire, UK).

Fingerprinting of survivors was done using the primer GTG_5 (5´-GTGGTGGTGGTGGTG-3´) following the method described by Zunabovic et al. (2012) with modifications. The reaction was performed in a total volume of 20 µl consisting of a 2 × Mastermix (Kapa Biosystems, Massachusetts, USA) containing 0.4 U Taq DNA polymerase, 0.2 mM deoxyribonucleotide phosphate, 1.5 mM $MgCl_2$, 35 pmol of primer (WhiteSci, Cape Town, South Africa) and 5 ng template DNA with the following PCR conditions; initial denaturation at 95ºC for 7 min; 34 cycles of denaturation at 92°C for 30 s, annealing at 38°C for 1 min and extension at 65°C for 8 min; final extension at 65°C for 16 min in a thermal cycler (Bio-Rad CFX96 Deep Well™ Real-Time System, Bio-Rad, Hercules, USA). GelRed™ (Biotium, Fremont, USA) stained amplification products were separated by electrophoresis (BayGene, Beijing, China) with 1.5% agarose (Benchmark Scientific, Edison, USA) gel in 1 × Tris-acetate EDTA (TAE) buffer (AMRESCO, Ohio, USA) at 4 V/cm for 2 h and viewed under UV light in a gel documentation system (Gel Doc™, BioRad). Banding patterns were analyzed using the Image Lab™ software (Biorad). Each survivor was compared to parental strains using a visual comparison of banding patterns.

5.3.7   *Data analysis*

A two-way analysis of variance (ANOVA) with the Tukey's HSD test for multiple comparisons was used to determine the effect of strain and stress treatment, factors on the

inactivation kinetic parameters and also for differences among the individual strains and treatments. Analysis was performed using GraphPad Prism (GraphPad Software, Inc., USA). All experiments were analyzed in duplicates and each experiment was repeated two times.

5.4   Results

5.4.1   Statistical evaluation of inactivation models

The LoF results for the four models are shown in Table 5.1. The weibull model was accepted in 66.7% of the cases followed by the log-linear with tail model (58.3%). Consequently, the two models were used to fit experimental data to obtain kinetic parameters.

Table 5.1: Statistical evaluation (LoF analysis) of models used to describe the inactivation of *L. monocytogenes* in lactic soft cheese

Strain	Treatment	Model			
		Biphasic	**Weibull**	**Log-Linear + Tail**	**Weibull + Tail**
ATCC19115	Control	Yes	Yes	Yes	Yes
	Acid	Yes	Yes	Yes	Yes
	Osmotic	Yes	Yes	Yes	Yes
159/10	Control	No	No	No	No
	Acid	Yes	Yes	Yes	No
	Osmotic	No	No	No	No
69	Control	No	Yes	Yes	No
	Acid	Yes	Yes	Yes	Yes
	Osmotic	No	No	No	No
Mixed strains	Control	No	Yes	No	No
	Acid	Yes	Yes	Yes	Yes
	Osmotic	No	No	No	No
	Percentage	**50.0**	**66.7**	**58.3**	**41.7**

The two models were further assessed using *Af* and *Bf* analysis (Table 5.2). Differences were observed in the accuracy of prediction between stressed and non-stressed cells as well among strains. In the case of acid and salt-stressed strains ATCC19115, 159/10 and mixed strains,

both models showed a good agreement between experimental data and model predictions with *Bf* and *Af* values equal to or close to 1.00. However, both models displayed a slight over-estimation with an 11 - 20% prediction error in the case of unstressed cells. A completely different response pattern was observed for strain 69 when subjected to stress. *Bf* values showed an average of 15 – 42% higher predictions than the experimental data and a 56 - 79% prediction error for both models.

Table 5.2: Bias factor (*Bf*) and Accuracy factor (*Af*) analysis of the log-linear with tail and Weibull models for the prediction of *L. monocytogenes* in lactic soft cheese

Strain	Treatment	Log-linear with tail		Weibull	
		Bf	*Af*	*Bf*	*Af*
ATCC19115	Control	1.01	1.14	0.98	1.20
	Acid	1.00	1.02	1.00	1.02
	Osmotic	1.00	1.04	1.00	1.03
159/10	Control	1.01	1.12	1.01	1.11
	Acid	1.00	1.01	1.00	1.01
	Osmotic	1.00	1.03	1.00	1.03
69	Control	1.01	1.08	1.00	1.08
	Acid	1.24	1.79	1.42	1.62
	Osmotic	1.15	1.73	1.42	1.56
Mixed strains	Control	1.01	1.12	1.01	1.13
	Acid	1.00	1.03	1.00	1.03
	Osmotic	1.00	1.02	1.00	1.02

5.4.2   *Weibull model fitted inactivation curves*

Weibull model fitted survival curves are shown in Figures 5.1 - 5.3. Non-stressed cells were generally more susceptible to inactivation in lactic soft cheese. From an initial cell population of 4 – 5 Log_{10} CFU/g, surviving populations were less than 2.0 Log_{10} CFU/g after 15 days. However, exposure to acid and salt stress prior to cheese inoculation resulted in reduced inactivation with surviving populations greater than 2.5 Log_{10} CFU/g for strain ATCC19115, 159/10 and mixed strains. *L. monocytogenes* 69 was the only strain to exhibit an increased sensitivity to the soft cheese after acid and osmotic stress treatments. There were no detectable levels of the organism in the soft cheese after 10 days using the enrichment approach.
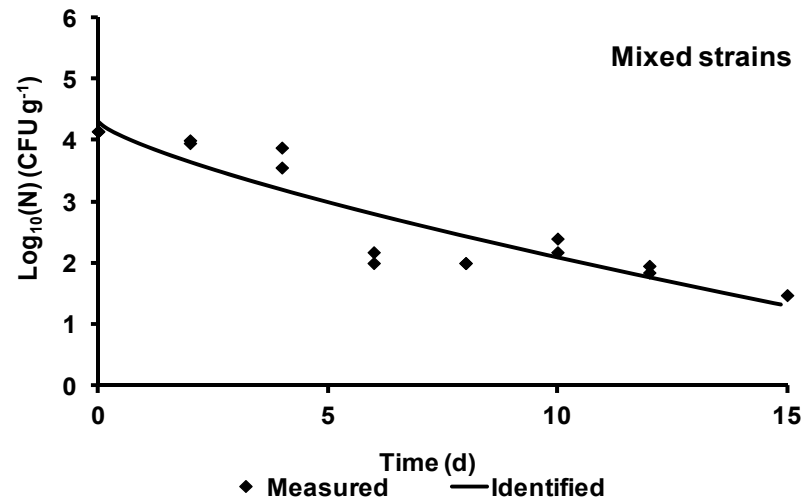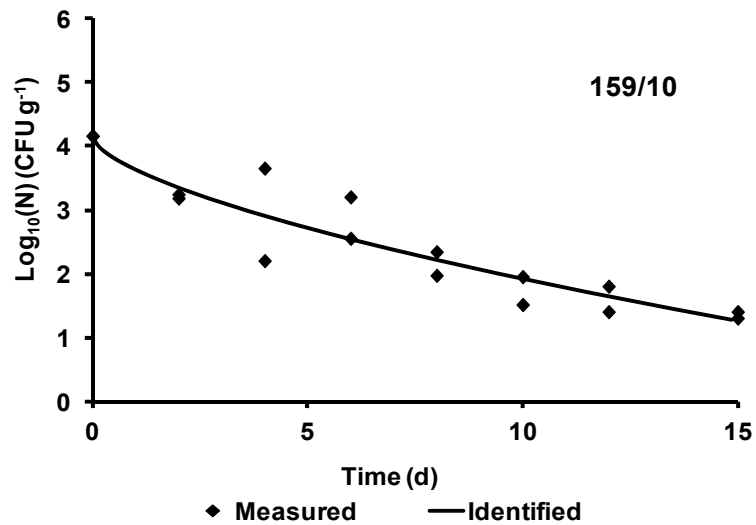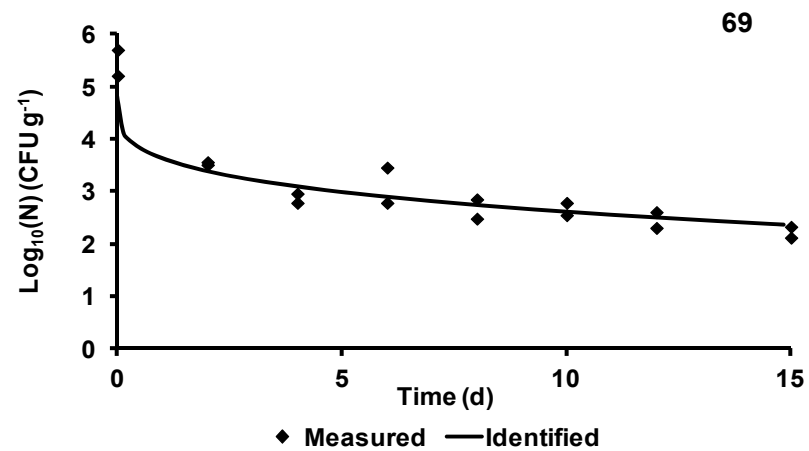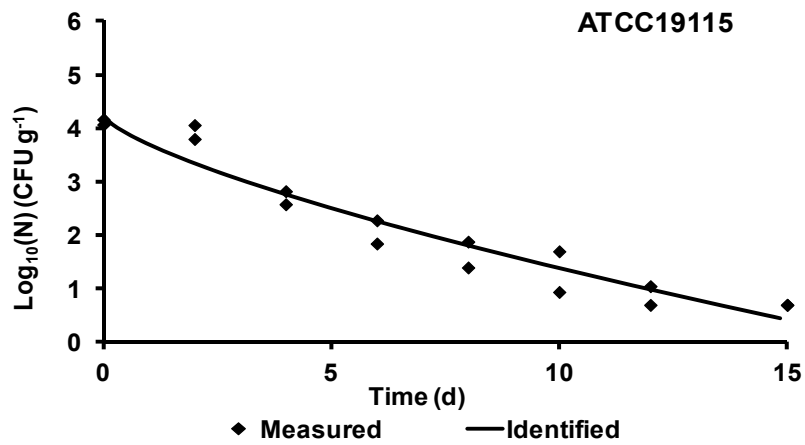
Figure 5.1: Weibull model fitted inactivation curves of control *L. monocytogenes* cells in lactic soft cheese stored at 4°C.
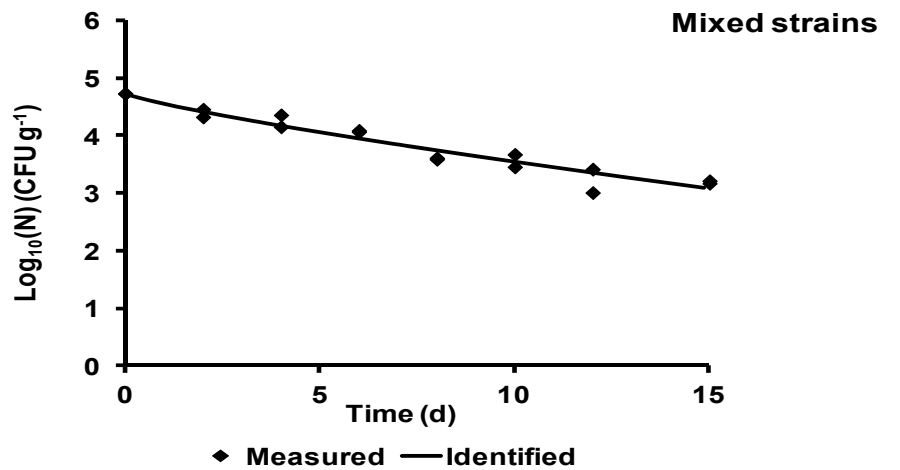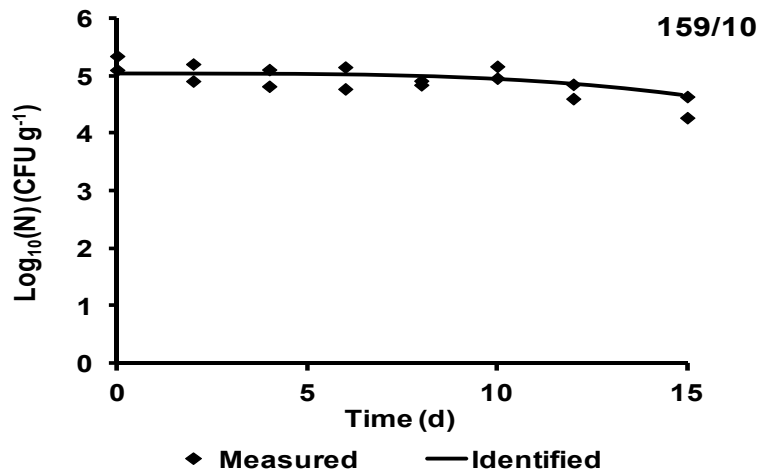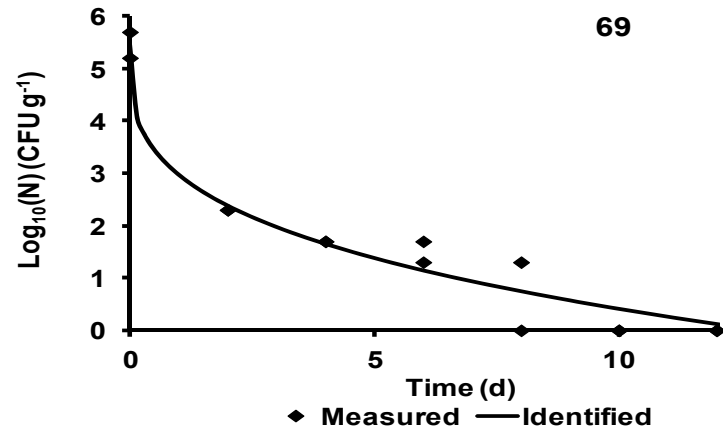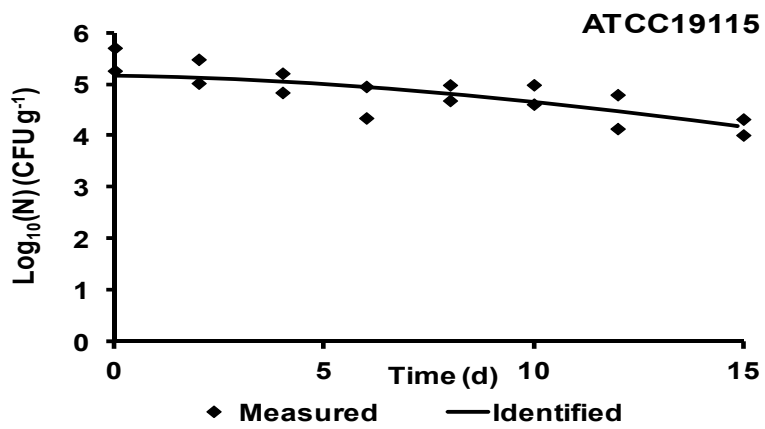
Figure 5.2: Weibull model fitted inactivation curves of acid-stressed *L. monocytogenes* cells in lactic soft cheese stored at 4°C.
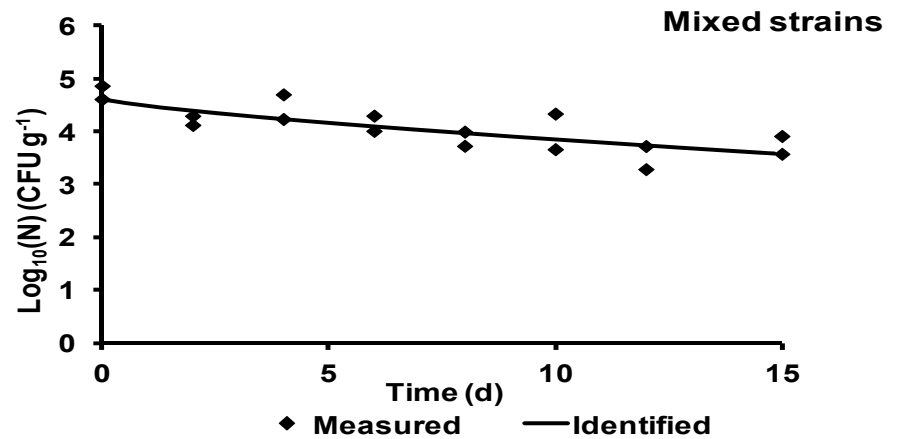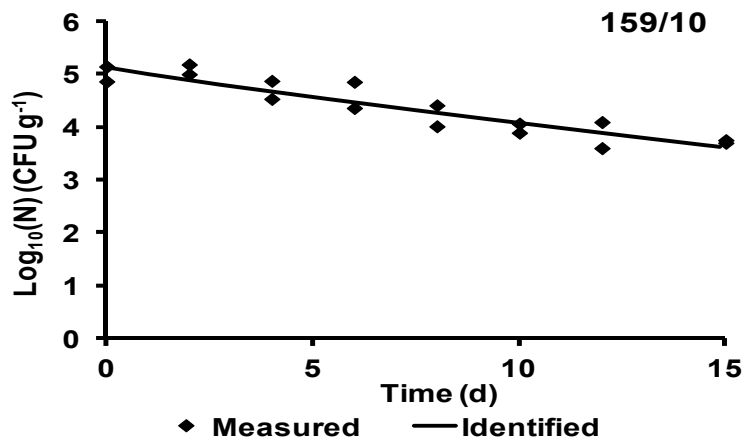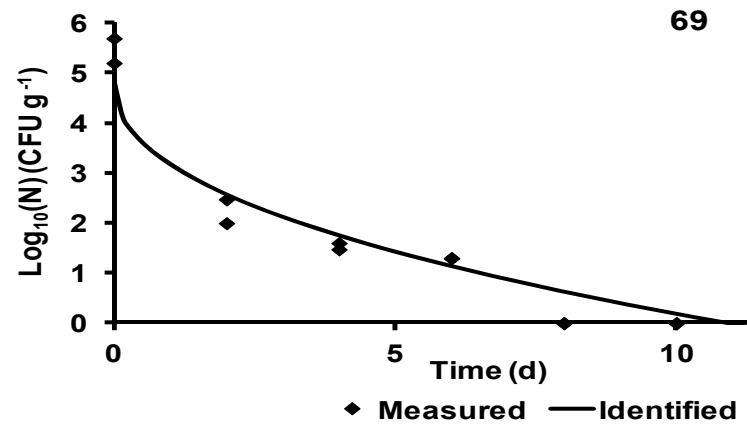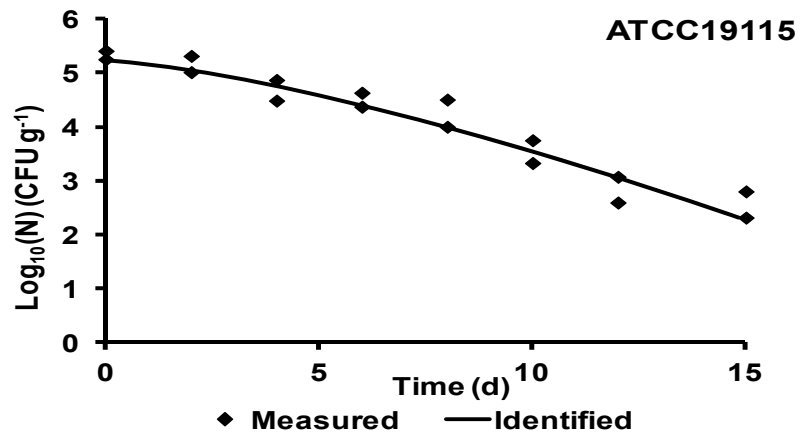
Figure 5.3: Weibull model fitted inactivation curves of osmotically -stressed *L. monocytogenes* cells in lactic soft cheese stored at 4°C.

5.4.3 Inactivation kinetic parameters

Inactivation kinetic parameters (Table 5.3) showed significant differences ($p < 0.05$) between stressed and non-stressed *L. monocytogenes* cells for individual and mixed strains (Table 5.4). The rate parameter of the log-linear with tail model (k_{max}) values were significantly higher for non-stressed cells. k_{max} values for strains ATCC19115, 159/10 and the mixed strains ranged from $0.53 - 0.77$ Log_{10} CFU/g/d for non-stressed cells and $0.05 - 0.46$ Log_{10} CFU/g/d for both acid and osmotically -stressed cells. Similarly, the rate parameter of the Weibull model (δ) values were significantly lower ($p < 0.05$) for non-stressed cells. For strains ATCC19115, 159/10 and mixed strains, δ values were $2.34 - 3.38$ days for non-stressed cells and $6.84 - 19.81$ days for both acid and osmotically -stressed cells. A very high rate of inactivation of *L. monocytogenes* strain 69 was observed during the first two days of cheese storage for both stressed non-stressed cells ($p > 0.05$), resulting in a sharp decrease in the surviving populations. However, for non-stressed cells, the remaining cells were slowly inactivated thereafter consequently leading to severe tailing.

Goodness of fit indicators for the two models were also supported by the root mean square error (RMSE) and coefficient of multiple determination (R^2) indices (Table 5.3). RMSE values ranged from $0.09 - 0.69$ and $0.05 - 0.44$ for the log-linear with tail and Weibull models respectively for all experimental trials. R^2 values for both models were > 0.81 for all experimental trials except for acid-stressed 159/10. Although no tailing was observable from the inactivation curves except for non-stressed cells of strain 69, the log-linear with tail model still able to estimate residual population (N_{res} parameter) except for acid and salt-stressed strain ATCC19115 and acid-stressed 159/10.

Table 5.3: Estimation of kinetic parameters of inactivation of stressed and non-stressed *L. monocytogenes* in lactic soft cheese stored at 4°C based on the log-linear with tail and Weibull models

Experimental Strain	Treatment	Model/Parameters								
		Log-Linear with tail					Weibull				
		N_o (Log$_{10}$ CFU/g)	N_{res} (Log$_{10}$ CFU/g)	k_{max} (Log$_{10}$ CFU/g/d)	R^2	RMSE	N_o (Log$_{10}$ CFU/g)	δ (d)	p	R^2	RMSE
ATCC19115	Control	4.22 (0.16)	0.76 (0.17)	0.77^C (0.07)	0.95	0.28	4.22 (0.25)	2.34^A (0.83)	0.72 (0.12)	0.92	0.36
	Acid	5.28 (0.07)	-	0.16^A (0.05)	0.87	0.13	5.16 (0.55)	14.91^C (0.70)	1.65 (0.29)	0.92	0.10
	Osmotic	5.46 (0.10)	-	0.46^B (0.04)	0.96	0.21	5.23 (0.09)	6.84^B (0.54)	1.38 (0.13)	0.98	0.15
159/10	Control	3.95 (0.21)	1.29 (0.32)	0.53^F (0.09)	0.84	0.39	4.14 (0.26)	2.92^D (1.32)	0.64 (0.15)	0.85	0.38
	Acid	5.11 (0.05)	-	0.05^D (0.08)	0.62	0.09	5.03 (0.02)	19.81^F (1.68)	3.46 (0.94)	0.82	0.06
	Osmotic	5.17 (0.09)	3.45 (0.30)	0.29^E (0.05)	0.91	0.16	5.14 (0.11)	9.39^E (1.41)	0.90 (0.18)	0.89	0.17
69	Control	5.44 (0.24)	2.65 (0.10)	2.22^G (0.41)	0.89	0.34	4.84 (0.25)	0.23^H (0.33)	0.27 (0.09)	0.82	0.44
	Acid	4.60 (0.41)	0.14 (0.26)	1.45^G (0.27)	0.85	0.69	5.45 (0.27)	0.05^G (0.04)	0.31 (0.04)	0.96	0.38
	Osmotic	4.97 (0.39)	0.23 (0.20)	2.16^G (0.36)	0.89	0.61	4.81 (0.36)	0.03^H (0.02)	0.27 (0.04)	0.92	0.52
Mixed Strains	Control	4.41 (0.22)	1.78 (0.19)	0.71^I (0.13)	0.86	0.38	4.30 (0.30)	3.38^I (1.58)	0.72 (0.20)	0.81	0.44
	Acid	4.66 (0.05)	2.91 (0.16)	0.30^H (0.03)	0.94	0.13	4.67 (0.07)	8.53^J (0.80)	0.83 (0.09)	0.93	0.14
	Osmotic	4.64 (0.05)	3.43 (0.14)	0.25^H (0.04)	0.87	0.13	4.65 (0.07)	12.79^K (1.27)	0.76 (0.12)	0.87	0.14

Values for model parameters N_o, N_{res}, k_{max}, δ and p are means with standard error in parenthesis of two replicate experiments. For each strain, k_{max} and δ values with the same letter are not statistically different from each other (p < 0.05). N_0 - initial cell density (CFU/g), k_{max} - maximum inactivation rate (per day). N_{res} - resistant cell fraction. δ - time to the first-decimal reduction (d) and p - shape parameter. R^2 (Coefficient of determination) and RMSE (Root mean square error) are Goodness of fit indicators.

Table 5.4: Analysis of variance of the rate parameter of the Weibull model (δ) showing the effect of strain and stress factors on the inactivation of *L. monocytogenes* in lactic soft cheese ($n = 4$)

Factor	DF	*P* value
Strain (ATCC19115, 69, 159/10, Mixed)	3	< 0.0001
Stress (Control, Acid, Osmotic)	2	0.028
Strain×Stress	6	0.480

DF- Degrees of freedom

5.4.4 Typing of *L. monocytogenes* survivors from mixed strain challenge tests

The GTG$_5$-REP-PCR fingerprint patterns of some acid treated *L. monocytogenes* survivors compared to the parental generation of the inoculated strains is shown in Figure 5.4. From a total of 80 colonies, representing surviving populations from mixed strains of both acid and osmotically-stressed cells, the majority of survivors with the exception of one isolate (Isolate 10 in Figure 4.5) showed similar fingerprint patterns that were identical to parental strain 159/10. Although the isolate contained most of the bands identified with parental strain 159/10, it had an additional two bands that made it unique.
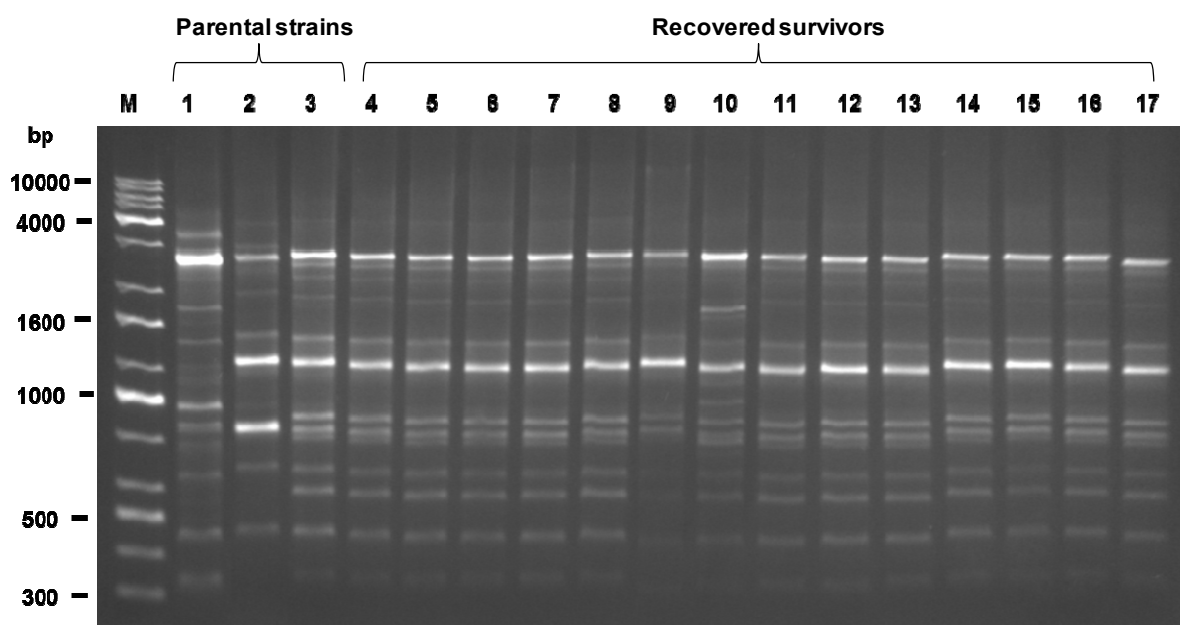
Figure 5.4: Representative agarose gel image of GTG$_5$-REP-PCR fingerprint patterns of acid-stressed *L. monocytogenes* survivors and parental strains. M – Molecular weight marker. Lane 1- ATCC19115; Lane 2 - 69; Lane 3 - 159/10; Lanes 4 – 17 represent survivor isolates.

5.5 Discussion

This work quantified the influence of stress exposure on the survival ability of *L. monocytogenes* strains in a lactic soft cheese using predictive models. The value of such

investigations is that they provide the fundamental parameters for estimation of risk arising from the consumption of contaminated RTE foods. In order to accommodate strain diversity in real situations of product contamination, three strains were used to model the survival of *L. monocytogenes* as individual strains and as mixed strains.

Inactivation response varied among individual strains and also among stressed and non-stressed cells. Inter-strain variations in response to stress among *L. monocytogenes* have been observed in several studies (Adrião et al., 2008; Francis and O'Beirne, 2005; Komora et al., 2017; Lianou et al., 2006). This phenotypic variation in stress resistance is attributed to genetic variation among strains (Bergholz et al., 2010) indicating adaptation to different environments (Lianou and Koutsoumanis, 2013). Bergholz et al. (2010) observed that genetically different strains of *L. monocytogenes* exhibit differences in salt stress response phenotypes. The diversity in phenotypic stress response among strains of *L. monocytogenes* emphasizes the importance of using more than one strain in the development of models for risk assessment (Lianou and Koutsoumanis, 2013).

In general, the two inactivation models chosen to fit survival data (log-linear with tail and Weibull) exhibited high accuracy in describing the response of acid and salt-stressed *L. monocytogenes* in lactic soft cheese. The sharp decrease in the population of survivors during the first few days of cheese storage observed for non-stressed cells of both individual and mixed strains is an indication that non-stressed cells comprise of both sensitive and resistant cell subpopulations. The upward concavity of inactivation curves indicates the quick inactivation of the sensitive subpopulation (Peleg, 2003; Peleg and Penchina, 2000). For cells subjected to acid and osmotic stress prior to cheese inoculation, the lack of a sharp decrease is an indication that stress exposure results in the death of susceptible subpopulation and

selection of the sturdy resistant fraction (Bishop et al., 2007; Booth, 2002) which upon cheese inoculation is inactivated slowly.

While exposure to stress led to an enhanced survival for strains ATCC19115, 159/10 and mixed strains, the opposite was observed for strain 69. Intrinsic differences in stress resistance among *L. monocytogenes* strains have been reported (Adrião et al., 2008; Francis and O'Beirne, 2005; Komora et al., 2017). Such variations likely influenced the survival ability in the soft cheese. In addition, variations among strains also influence the extent of cell injury after stress exposure. Previous findings from Chapter 4 of this thesis on strain 69 showed a high level of cell injury occurs following exposure to acid, osmotic and heat stress. Bacterial cell damage induced by ethanol has been shown to result in sensitization of such injured cells to subsequent acid and osmotic stresses (Barker and Park, 2001). The fast inactivation of this strain in the soft cheese could be related to its level of cell injury after stress which enhanced its susceptibility to the food stress.

With the exception of strain 69, the rate of cell reduction (δ and k_{max} values), was significantly lower for cells subjected to stress exposure. This indicates that pre-exposure to stress resulted in enhanced tolerance to the food stress. The enhanced tolerance arises from the ability of stressed cells to maintain a homeostatic balance in terms of intracellular pH and osmotic potential respectively (Bayles and Wilkinson, 2000; Beumer et al., 1994; Skandamis et al., 2008). The ability of stressed cells to survive a second stress exposure has been seen to be influenced by the type and magnitude of the first stress exposure (Skandamis et al., 2008; Tiganitas et al., 2009). *L. monocytogenes* cells surviving acid stress exposure develop an acid tolerance response (Davis et al., 1996; O'Driscoll et al., 1996) which protects such cells against subsequent lethal acid stress exposure thus accounting for the reduced rate of

inactivation for acid-stressed cells in the lactic soft cheese, with an acidic pH (pH 4.2). In the case of osmotically-stressed cells, pre-exposure to a hyper-osmotic environment results in the development of osmotolerance arising from the intracellular accumulation of compatible solutes (Bayles and Wilkinson, 2000; Duché et al., 2002). While both stress exposures resulted in the development of stress tolerance responses, osmotically stressed *L. monocytogenes* cells for strains, ATCC19115, 159/10 and mixed strains had significantly higher rates of inactivation than their acid-stressed counterparts. This emphasizes the fact that the acid tolerance response enhances the survival of *L. monocytogenes* in lactic soft cheese more than the osmotolerance response. The transfer of osmotically stressed cells to soft cheese imposes a further homeostatic burden on the already stressed cells (Skandamis et al., 2008; Tiganitas et al., 2009), hence survival in the soft cheese might require an extra energy expenditure to counter the acid exposure in the food which is less so for the already adapted acid-stressed cells.

After inoculating lactic soft cheese with three strains of *L. monocytogenes*, the surviving population belonged to one strain. Detection of strain 159/10 as the dominant survivor in the mixed strain challenge appears to be related to its consistently higher survival rate after exposure to both acid and osmotic stress than the other two strains when inoculated into the cheese individually. The lack of strain 69 in the mixed strain challenge was expectedly related to its individual response which showed an inability to survive for 15 days in the soft cheese. However, strain ATCC19115 showed a high survival ability when inoculated in soft cheese alone. The total absence of this strain in the cocktail challenge after 15 days indicates a potential interaction among the strains. *L. monocytogenes* strains have been shown to exhibit competition in co-cultures (Gorski et al., 2006; Zilelidou et al., 2016b, 2015) resulting in reduction or total lack of growth for outcompeted strains. However, it is important to

observe that the competition phenomenon has largely been demonstrated for *L. monocytogenes* co-cultures in growth supporting media where the growth of the dominant strain suppresses that of the outcompeted strain. It is likely that a similar interaction may occur when the mixed strains are subjected to a growth restrictive environment as was the case in this study. One hypothesis proposed by Cornforth and Foster (2013) is that there is a link between stress response and bacterial competition. Although no specific link has been reported for *L. monocytogenes*, it is likely that the ability of strain 159/10 to survive stress could be related to its ability to out-live other strains when used as mixed strains. Observations of this study have implications in predictive microbiology as it indicates the importance of using strains that survive better in the development of models for *L. monocytogenes* survival in foods.

5.6   Conclusions

The survival response of *L. monocytogenes* in lactic soft cheese following stress pre-exposure differs among strains and appears to be influenced by the susceptibility of individual strains. In stress-susceptible strains, stress pre-exposure sensitizes the stressed cells to the subsequent food stress thus accelerating their inactivation. However, in the tolerant strains, pre-exposure to stress enhances survival of stressed cells in the cheese owing to the development of stress tolerance response and cross-protection. While both acid and osmotic stress tolerance responses protect *L. monocytogenes* against the acidity of soft cheese, the acid tolerance response imposes a better protection. Profiling of surviving populations from mixed strain challenge tests shows that one persistent *L. monocytogenes* strain out-lives the other strains. Therefore it is important that survival models for predicting *L. monocytogenes* in foods must incorporate the aspect of strain differences, stress tolerance responses and persistence in order to obtain more representative estimates of pathogen behavior for risk assessment.

6   CHAPTER 6

FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPY DETECTION OF ACID STRESS-INDUCED CELLULAR CHANGES IN *LISTERIA MONOCYTOGENES* STRAINS

6.1 Abstract

The effects of acid stress exposure (pH 4.2) on the biomolecular changes in *L. monocytogenes* strains 69 and 159/10 cells were studied using Fourier Transform Infrared (FT-IR) spectroscopy. Based on spectral comparisons of stress-treated and non-treated control cells, significant alterations were observed in the spectral regions of proteins, nucleic acids, lipids, and polysaccharides indicating a complex response to acid stress involving disruptions of secondary structure of proteins, denaturation and conformational changes in DNA/RNA as well as membrane and cell wall disruptions. The distinguishing feature of response between the two strains was in the alterations in spectral regions of membrane lipid acyl chains, related to membrane fluidity changes that were observed only for *L. monocytogenes* 69. Evidence from scanning electron microscopy showed that a physical damage to the cell wall and cell membrane were likely manifestations of the acid-induced biomolecular disruptions. Along with cell surface changes, the acid stress treatments induced the formation of extracellular membrane vesicles in strain 69 that were not evident in the tolerant strain 159/10. This study shows that differences in membrane fluidity are an important determinant of *L. monocytogenes* stress resistance and that the more tolerant and persistent strains probably survive stress by maintaining a rigid membrane.

Keywords: *Listeria monocytogenes*; acid stress; Fourier Transform Infrared (FT-IR) spectroscopy; vibration.

6.2   Introduction

Listeria monocytogenes is a food-borne pathogen commonly associated with refrigerated RTE foods (Luber et al., 2011). The absence of lethal preservation hurdles and dependence on sub-lethal stresses in the production of RTE foods, creates conditions for the survival of stress tolerant and stress injured cells (Abee et al., 2016; Mir et al., 2018). Owing to its psychrotrophic nature, *L. monocytogenes* stress survivors have been known to slowly multiply in chilled RTE foods (Kataoka et al., 2017; Skalina and Nikolajeva, 2010).

Acid stress is a common method of controlling microbial growth in RTE foods (Hill et al., 2002). Depending on pH and the amount of undissociated acid (Wemmenhove et al., 2016), the effects of acid stress on microbial cells include the disruption of membrane potential, electron transport chain and enzyme inhibition among the physiological targets (Brul and Coote, 1999). In order to study the physiological and biochemical cellular changes accompanying stress survival, a variety of biochemical and fluorescence-based methods are traditionally used (Bridier et al., 2015). However such approaches rely on the use of invasive techniques and often toxic fluorescent labels (Le Roux et al., 2015b).

Fourier Transform Infrared (FT-IR) spectroscopy is a non-invasive label-free technique that measures the vibrational behavior of molecules exposed to infrared radiation (Alvarez-Ordóñez et al., 2011). The technique has been applied in the study of microbial stress response (Alvarez-Ordóñez and Prieto, 2010; Nyarko and Donnelly, 2015; Wu et al., 2013), based on alterations of cellular constituents such as lipids, carbohydrates, proteins and nucleic acids reflecting the structural and physiological changes in stressed bacterial cells (Wu et al., 2013).

In Chapter 4 of this thesis, it was shown using flow cytometry and membrane integrity indicators that *L. monocytogenes* cells exposed to acid, osmotic and heat stress exhibit varying levels of cell damage. However, while membrane integrity was identified as a common target of stress, it was evident that other cellular targets were also affected. The objective of the study, therefore, was to elucidate the potential targets of stress-induced cell damage in *L. monocytogenes* using FT-IR microspectroscopy with the aim of identifying the potential markers of stress susceptibility and tolerance in the response of *L. monocytogenes* strains.

6.3   Materials and methods

6.3.1   *Bacterial strains and stress treatments*

The strains of *L. monocytogenes* used in this study and their growth and stress treatment conditions were as described in Section 4.3.1 – 4.3.2 (Chapter 4) of this thesis. Specifically, in this Chapter, only the acid stress treatments of strains 69 and 159/10 were done. Strains 69 and 159/10 were used because of their contrasting susceptibilities to acid stress (Chapter 4 and 5).

6.3.2   *FT-IR microspectroscopy analysis*

Cell preparation for FT-IR analysis was done following the method described by Alvarez-Ordóñez et al. (2010) with modifications. Treated and control cells were centrifuged at 8 000 g for 5 min, washed twice and re-suspended in normal saline solution. A 10 µl aliquot of washed cells was placed on a CaF_2 window (Crystan, UK) and left to dry under a laminar flow hood for 2 h to form a thin dry film of bacterial cells. FT-IR spectra were obtained using a Bruker V70x spectrometer with an attached 15× IR objective Hyperion microscope (Bruker, Germany). Spectra, in transmission mode, were collected in the range of 4000 - 850

cm^{-1} and a resolution of 4 cm^{-1}. Each spectrum represented a total of 32 scans. The final spectra were an average of three replicate experiments.

6.3.3   Spectral processing and data analysis

Spectral processing was done using OPUS ver 6.0 software (Bruker). Spectra were baseline corrected using the rubberband method and vector normalized. The normalized spectra were subjected to second derivative transformation using the Savitsky-Goly function with 9 smoothing points. The effect of stress treatment was analyzed on the basis of comparison of peak intensities of spectral regions associated with cell membrane lipids, secondary protein structure, carbohydrates, and DNA conformational changes (Hlaing et al., 2017). The pick peak function in OPUS was used to identify peak positions (wavenumber shifts) and intensities of normalized spectra. Differences in normalized peak intensity values between treated and control cells were analyzed using the Student t-test ($p < 0.05$) (Rak et al., 2014) in GraphPad Prism 7.0 (GraphPad Software Inc, USA).

6.3.4   Scanning Electron Microscopy (SEM)

Following acid stress treatment, cells were centrifuged at 5 000 × g for 10 min and washed twice with PBS and re-suspended in a pre-fixing solution comprising of 2.5% (v/v) glutaraldehyde solution and 2.5% (v/v) formaldehyde solution in PBS (pH 7.4) for 1 h. The cells were washed three times with 75 mM sodium phosphate buffer (pH 7.4) for 10 min each and fixed with 1% aqueous osmium tetroxide solution (Merck, Germany) for 1 h. Fixed samples were washed three times and dehydrated in graded doses of ethanol (30%, 50%, 70%, 90%, and three times at 100%) each for 10 min. Dehydrated samples were dried in a 50:50 mixture of hexamethyldisilazane solution (HMDS) (Merck, Germany) and ethanol for 1 h followed by HMDS for 1 h. Cell pellets suspended in HDMS were spotted on a glass

coverslip and left to dry overnight. Samples were then mounted and coated with carbon. The samples were examined using a scanning electron microscope, (Cryo-SEM JEOL 840, Zeiss Gemini 2).

6.4 Results

6.4.1 Normalized FT-IR spectra

Figure 6.1 shows a representative normalized FT-IR spectra (wavenumber 3800 – 850) (*L. monocytogenes* strain 69 control cells), indicating the main regions of interest. As expected of any bacterial spectrum, four main regions were identified. The window around wavenumber 3000 – 2800 cm^{-1} with two identifiable peaks attributed to membrane fatty acids; the region between wavenumber 1800 – 1500 cm^{-1} attributable to Amide I and Amide II bands of proteins; the region between wavenumber 1500 – 1200 cm^{-1} with at least three noticeable peaks attributed to lipids and nucleic acids and the region between wavenumber 1200 – 900 cm^{-1} attributed to polysaccharides and nucleic acids (Alvarez-Ordóñez and Prieto, 2010).
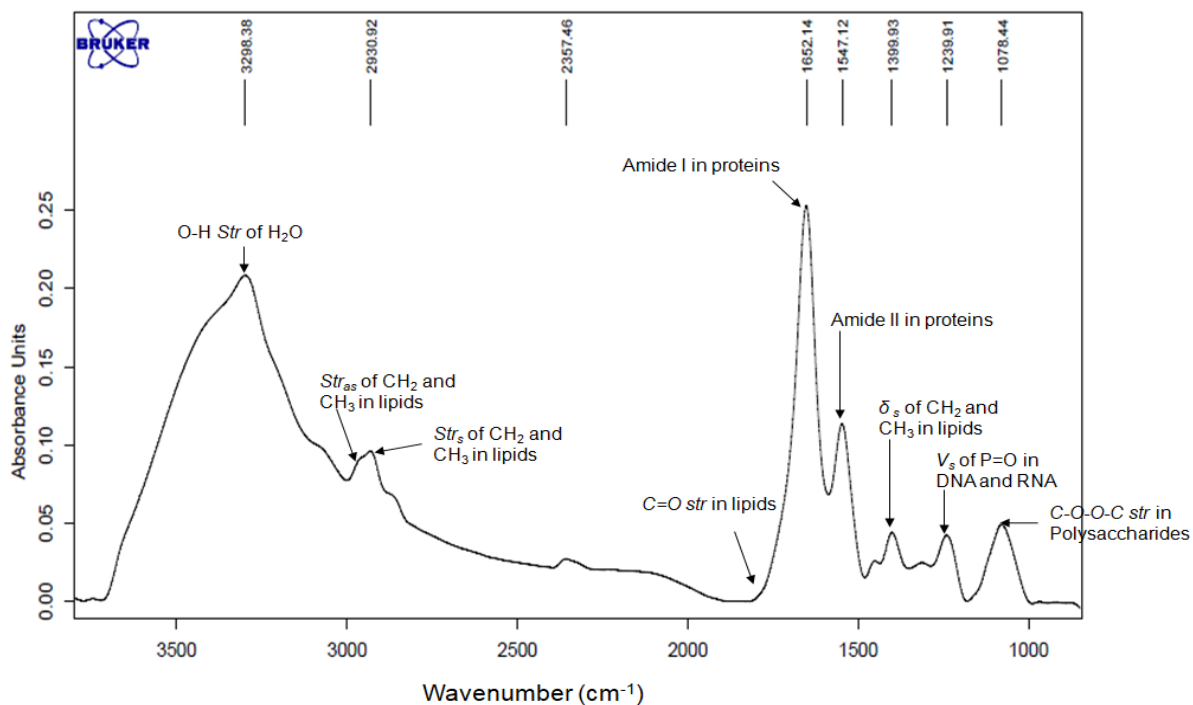
Figure 6.1: Representative FT-IR spectra of control cells for *L. monocytogenes* 69 indicating the major peaks of attention. *Str* – stretching; *Str_{as}* – asymmetric stretching; *Str_s* – symmetric stretching; δ_s – symmetric deformation; V_s – symmetric vibration. Peak assignments based on Al-Qadiri et al. (2008); Alvarez-Ordóñez et al. (2011); Davis and Mauer (2010); Hlaing et al. (2017); Wu et al. (2013) and Zoumpopoulou et al. (2010).

6.4.2 Spectral comparison of treated and control cells

The average second derivative spectra of control and acid treated cells are shown in Figure 6.2, from which differences in peak intensities were visible in the spectra, of both strains. Peak intensities of treated cell spectra were generally lower than control cells for both strains with the exception of a few peaks such as the C=O stretching vibrations in lipids and nucleic acids peak at approximately 1710 wavenumber and C-O and C-C stretching vibrations of DNA backbone at around 900 – 1000 cm^{-1} that showed increased intensities in stress-treated cells (Figure 6.2).
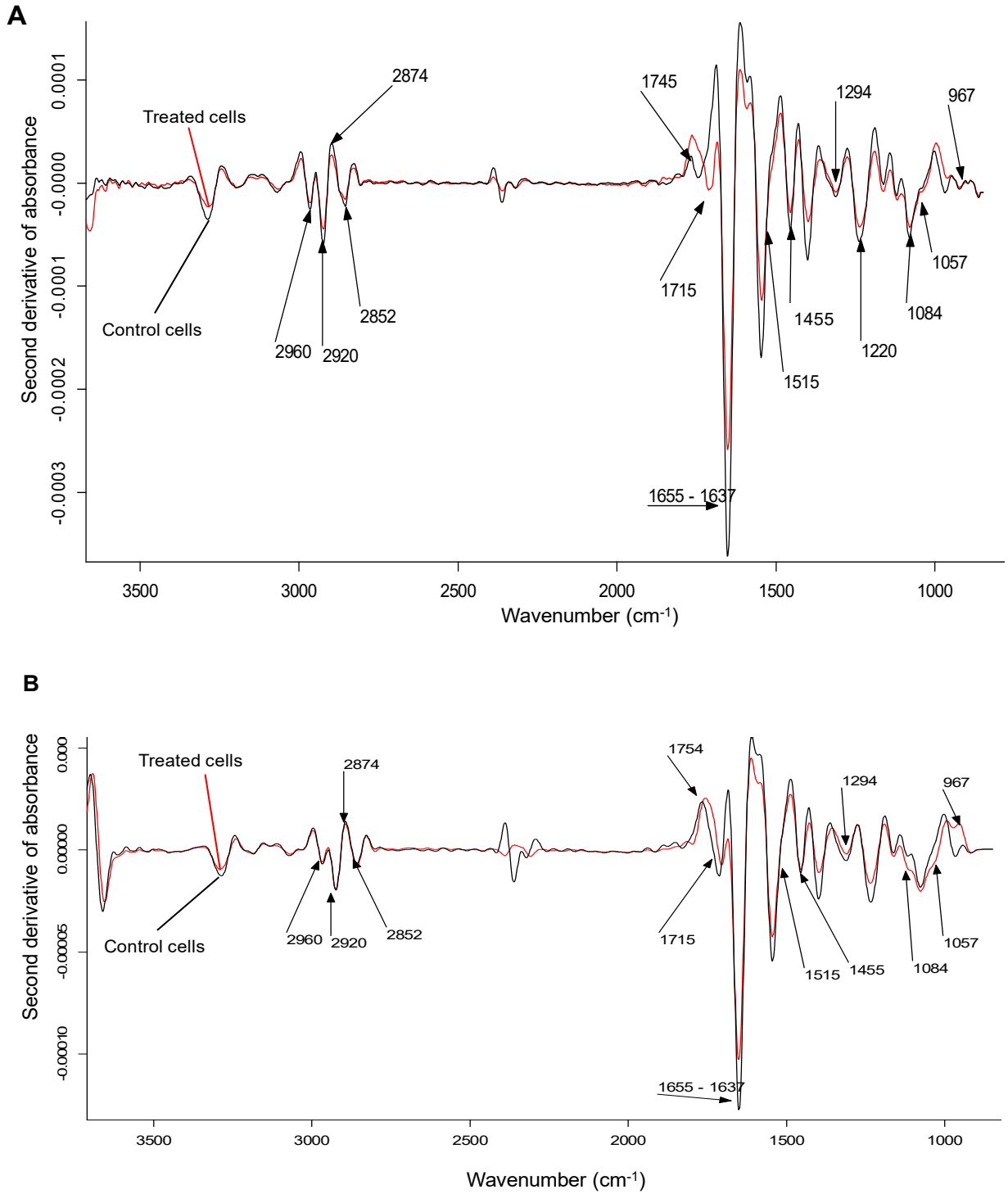
Figure 6.2: Second derivative spectra of acid treated and control cells of *L. monocytogenes* strain 69 (A) and strain 159/10 (B). Annotations indicate the bands for the main biomolecules analyzed.

The effect of acid treatment was resolved by statistical comparison of peak intensities at specific wavenumbers representing spectral signatures of cellular proteins, carbohydrates, nucleic acids and lipids following a second derivative transformation of the normalized spectra of control and acid treated cells and identification of any peak shifts. The summary of significantly altered peaks as a result of acid treatment is shown in Table 6.1. While only marginal changes were observed in peak shifts, significant alterations were discernible in peak intensities between treated and control cells. For *L. monocytogenes* 69, significant alterations ($p = 0.0001$) were observed at $2960 – 2852$ cm^{-1}, a region representing symmetric and asymmetric stretching vibrations of C-H in CH$_2$ and CH$_3$ groups of membrane fatty acids. Interestingly, *L. monocytogenes* 159/10 showed no significant alterations ($p > 0.05$) in the same region. In addition to changes in the acyl side chain, significant alterations ($p < 0.0001$) were also observed in the PO$_2^-$ asymmetric stretching vibrations of phosphodiesters in membrane phospholipids (~ 1221 cm^{-1}). Similar to the acyl chain, no significant differences ($p = 0.93$) were observed in the asymmetric stretching vibrations of PO$_2^-$ groups in membrane phospholipids.

Significant changes ($p < 0.0001$) were observed in Amide I bands ($1655.- 1637$ cm^{-1}) vibrations attributed to α-helical and β-pleated sheet structures of proteins, asymmetric deformation vibrations of CH$_3$ and CH$_2$ groups in proteins and lipids (~ 1455), PO$_2^-$ symmetric stretching in DNA, RNA and phospholipids (~ 1084 cm^{-1}) and C-O-O-C stretching of polysaccharides (~ 1057 cm^{-1}) in both organisms. Both strains showed no significant alterations ($p = 0.82$ for *L. monocytogenes* 69; $p = 0.69$ for *L. monocytogenes* 159/10) (Table 1) in C-O and C-C stretching vibrations of DNA backbone (~ 967 cm^{-1}).

Table 6.1: Spectral alterations and peak intensity comparisons (Student *t*-test) between control and acid treated *L. monocytogenes* 69 and 159/10

L. monocytogenes 69			*L. monocytogenes* 159/10			Peak assignment
Peak (wavenumber, cm^{-1})		*P* - value	Peak (wavenumber, cm^{-1})		*P* - value	
Control	Treated		Control	Treated		
2960.5±0.4	2960.2±2.1	0.0001	2960.9±1.5	2959.7±1.8	0.75	C-H asymmetric stretching of -CH_3 in lipids
2921.6±1.1	2920.9±0.4	0.0001	2921.3±0.5	2920.0±0.8	0.89	C-H asymmetric stretching of -CH_2 in lipids
2874.2±1.3	2873.9±1.3	0.0001	2877.6±6.5	2874.8±1.7	0.93	C-H symmetric stretching of -CH_3 in lipids
2852.2±0.5	2852.0±0.7	0.0002	2851.6±0.7	2851.4±1.1	0.93	C-H symmetric stretching of -CH_2 in lipids
1745.8±0.7	1746.6±0.2	0.38	1746.4±0.4	1745.7±0.7	<0.00001	C=O stretching of lipid esters
1715.5±0.4	1715.5±0.2	<0.00001	1715.8±0.3	1714.9±0.5	<0.00001	C=O stretching in lipids and nucleic acids
1655.5±1.5	1654.8±1.7	<0.00001	1653.9±2.0	1656.1±0.2	<0.00001	Amide I band composed of C=O and C-N stretching vibrations of the protein backbone (α-helical structures)
1637.7±0.3	1637.5±0.2	<0.00001	1637.1±0.3	1637.7±0.4	<0.00001	Amide I composed of β-pleated sheets
1455.2±1.0	1455.6±0.3	<0.00001	1455.6±0.5	1453.3±4.6	<0.00001	asymmetric deformation of CH_3 and CH_2 in proteins and lipids
1221.8±0.6	1216.6±2.3	<0.00001	1216.9±5.0	1215.3±2.5	0.93	PO_2^- asymmetric stretching of phosphodiesters in phospholipids
1084.1±0.0	1083.9±0.9	<0.00001	1084.1±0.2	1083.8±0.3	<0.00001	PO_2^- symmetric stretching in DNA, RNA and phospholipids
1057.3±0.2	1057.5±0.4	<0.00001	1057.1±0.4	1057.3±0.9	<0.00001	C-O-O-C stretching of polysaccharides
967.7±0.6	967.3±0.8	0.82	966.8±1.1	967.4±1.1	0.69	C-O and C-C stretching of DNA backbone

Peak assignments based on Al-Qadiri et al. (2008); Alvarez-Ordóñez et al. (2011); Davis and Mauer (2010); Hlaing et al. (2017); Wu et al. (2013) and Zoumpopoulou et al. (2010).

6.4.3   SEM morphology of treated cells

In addition to FT-IR spectral analysis, physical and morphological changes in acid-stressed *L. monocytogenes* cells were also observed by scanning electron microscopy. Figure 6.3 shows the micrographs of stress-treated cells compared to untreated control cells. While control cells had smooth and intact cell surfaces, some treated cells showed evidence of cell surface deformations indicating a collapse of the cell wall and possible the cell membrane and in a few cases, evidence of cell rupture. The most striking changes reflecting the effect of acid stress treatment were observed in *L. monocytogenes* strain 69 where its treated cells showed an extensive formation of extracellular vesicles.
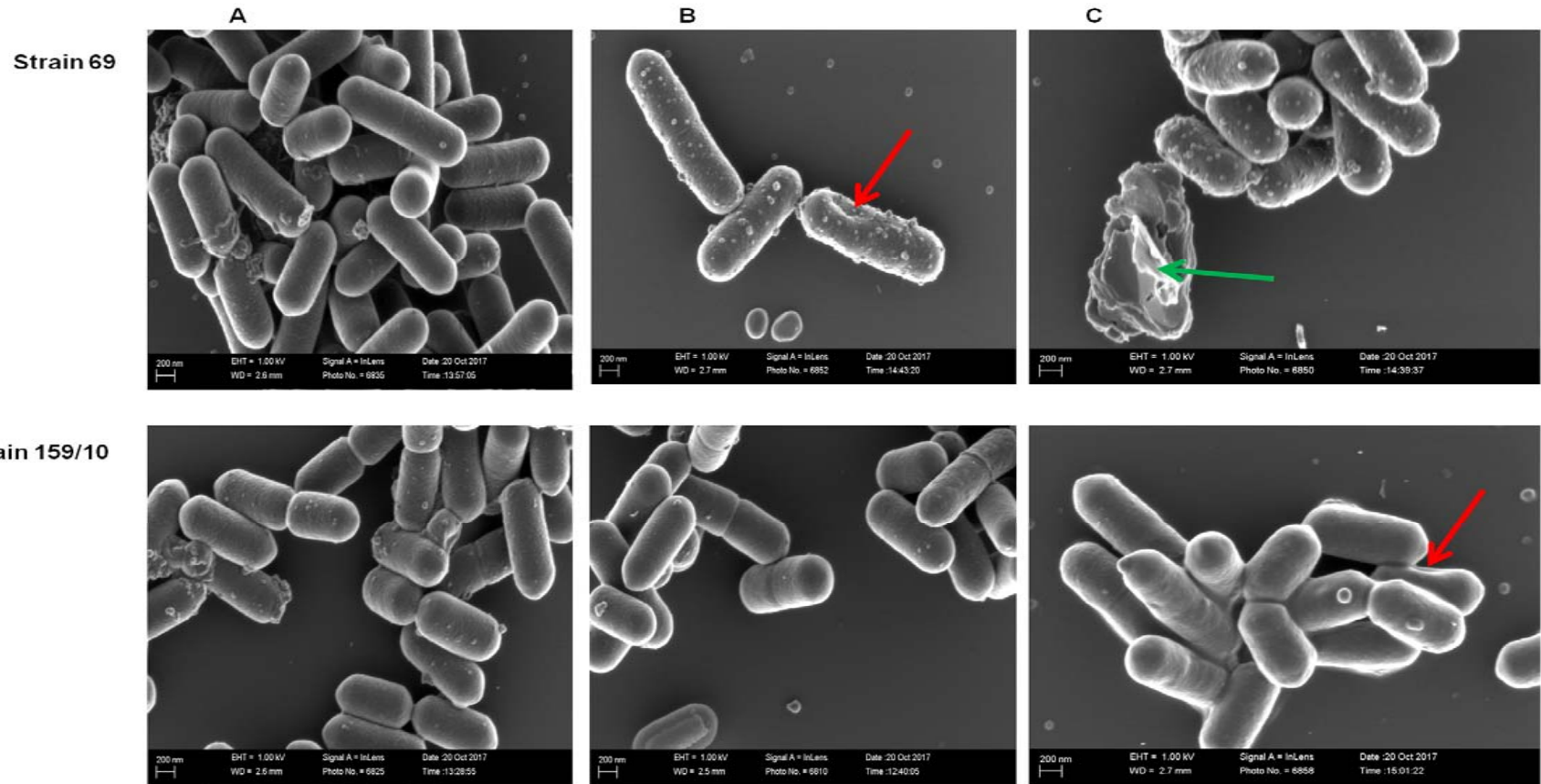
Figure 6.3: Scanning electron micrographs of acid treated cells of *L. monocytogenes* strain 69 and strain 159/10. A - Control cells with intact and smooth membranes. B and C – acid-treated cells. Evidence of membrane deformation is shown with a red arrow. The green arrow shows evidence of cell rupture.

6.5 Discussion

The analysis of stress-induced cellular changes in bacterial cells by FT-IR spectroscopy relies on the vibrational spectra of all biomolecules present in the cells (Rak et al., 2014). In addition, to established biochemical and molecular biology-based technologies, vibrational spectroscopy can, therefore, offer some useful insights on the study of microbial stress responses (Alvarez-Ordóñez et al., 2011). In the present chapter, FT-IR spectroscopy was utilized to study the effects of acid stress in two strains of *L. monocytogenes* which from Chapters 4 and 5, represented varying phenotypic responses. Of the two strains, *L. monocytogenes* 69 cells, exhibited a high sensitization to secondary stress agents once stressed while *L. monocytogenes* 159/10 strain exhibited tolerance to secondary agents once stressed.

Based on comparisons of spectral signatures of stressed and non-stressed control cells, *L. monocytogenes* 69 showed significant peak intensity alterations in C-H symmetric and asymmetric stretching vibrations of $-CH_2$ and $-CH_3$ groups in acyl chains of membrane lipids and PO_2^- asymmetric stretching vibrations associated with membrane phospholipids. In contrast, no significant alterations were observed in the same spectral region for *L. monocytogenes* 159/10. These variations reflect the differences in the acid stress response behavior of the two strains. Changes in vibration characteristics of membrane acyl chain have been linked to phase transition of bacterial cell membranes which influences membrane fluidity (Alvarez-Ordóñez and Prieto, 2010). Bacterial stress adaptation and survival depends a lot on the ability to adjust and maintain an appropriate fluidity of the cell membrane (Marielle and Sarrah, 2017). Using the technique of fluorescence anisotropy, Marielle and Sarrah (2017) showed that fluidity of bacterial membranes is related to cell viability and that under conditions of stress, the membrane changes from a fluid to a rigid state associated with

113

decreased viability and cell death. In their study, Alvarez-Ordóñez and Prieto (2010) deduced

membrane fluidity changes in stress-treated *Salmonella enterica* serovar Typhimurium cells

based on the analysis of the shift in peak of the –CH$_2$ stretching vibrations. In the present

study, no significant shifts in peaks between treated and control cells were observed.

Nevertheless, the observed reductions in peak intensities were indicative of changes in

bonding and conformation of the membrane lipid chains (Yoon et al., 2015). Thus the

findings of this study suggest that the differences in the stress susceptibilities of these strains

previously observed in Chapter 4 are related to the ability or lack thereof to maintain

membrane fluidity under environmental stress.

Spectral alterations in the Amide I band (wavenumbers 1655 – 1637 cm^{-1}) attributed to C=O

and C-N stretching vibrations of the protein backbone are a reflection of the changes in the

secondary structure of cellular proteins (α-helical structures and β-pleated sheets) (Hlaing et

al., 2017). The mechanism of acid-induced cell damage depends on the release of H$^+$ protons

into the cell cytoplasm which alters the internal pH of the cell (Brul and Coote, 1999). This

disruption of pH homeostasis affects protein structure through the disruption of intra- and

intermolecular hydrogen bonding and electrostatic interactions (Wu et al., 2013). In addition,

the acidification of the cytoplasm also causes denaturation of cytoplasmic proteins such as

enzymes (Lund et al., 2014). In both *L. monocytogenes* strains, it is clear that one of the

primary effects of acid-induced stress is the disruption of cellular proteins which

consequently affects other physiological and metabolic responses associated with stress

exposure (Lund et al., 2014).

The effects of acid treatments on the stability of nucleic acids were deduced from the

symmetric and asymmetric stretching vibrations of PO$_2^-$ groups (1084 and 1220 cm^{-1}), C=O

groups in base structures (1715 cm^{-1}), as well as C-O and C-C stretching vibrations of the DNA backbone (976 cm^{-1}). An increase in the intracellular concentration of H$^+$ protons and acidification of the cytoplasm induced by acid stress results in ionization and protonation of PO$_2$ groups and nitrogenous bases causing DNA denaturation and conformational changes (Tajmir-Riahi et al., 1995a). Studies on DNA exposed to low pH, have shown that the main alterations include base separation and stacking, destabilization of the helical structure and conversion from B to C and Z conformations (Tajmir-Riahi et al., 1995a; Tajmir-Riahi et al., 1995b). It would appear that while acid stress affected the phosphate groups, and the nucleic acid bases (which were prominent in both strains), it did not have any significant effect on the DNA backbone as deciphered from C-O and C-C stretching vibrations of the DNA backbone (976 cm^{-1}). This gives emphasis to observations from previous studies that stress-induced and adaptational changes in DNA mostly involve disruption of the helical structure and changes to non-B conformations (Kumar and Maiti, 1994; Wood, 2016).

The effects of stress on cellular polysaccharides as revealed spectral alteration in C-O-O-C stretching of polysaccharide ring structures at 1057 cm^{-1}. Given that the greatest amount of cellular carbohydrates is contained in the cell walls as peptidoglycan, and teichoic acid (Mistou et al., 2016), the alterations in the spectra of stress-treated cells are a reflection of the stress-induced loss of cell wall integrity. In addition to carbohydrates, the Gram-positive bacterial cell wall contains a lot of cross-linking peptides that regulate the physical properties of the peptidoglycan layer such as porosity and elasticity (Mistou et al., 2016; Vollmer and Seligman, 2010) as well as phosphate groups associated with teichoic acid (Schneewind and Missiakas, 2014). Given the effects of H$^+$ protons on the ionization of proteins and phosphate groups, it is likely that the structure of the peptidoglycan and teichoic acid would be affected

thereby compromising the integrity of the cell wall. Similar spectral changes were observed for *L. innocua* exposed to acetic acid at pH 4.13 (Wu et al., 2013).

Results from the SEM analysis provided evidence that physical damage to the cell wall and cell membrane was a possible manifestation of the acid-induced biomolecular changes providing an additional mechanism for acid-induced cell death in *L. monocytogenes*. The extensive formation of extracellular vesicles associated with *L. monocytogenes* 69 provided an additional feature of potential difference in acid stress response between the two strains. Extracellular vesicles have been identified as a secretory system that enables bacterial cells to secrete enzymes and other effector proteins enabling them to exploit their environment for survival (Kulp and Kuehn, 2010). As a stress response mechanism, these vesicles are important in cell to cell communication, biofilm formation, and nutrient acquisition that enables stressed cells to readily exploit their environment for survival (Kulp and Kuehn, 2010). In the context of this study, the formation of extracellular vesicles in *L. monocytogenes* 69 suggests that the less tolerant strains make-up for their low-stress resistance by investing in strategies that enhance survival potential through an improved exploitation of the environment to support growth.

In general, the effects of acid stress in both *L. monocytogenes* strains were reflected in alterations in the FT-IR spectra of proteins, lipids, nucleic acids, and polysaccharides. However, the outstanding feature of the response of the two strains was in the differences in the acyl chains of membrane lipids which suggested that the differences in the phenotypic responses of the two strains could be related to their ability or lack thereof in maintaining fluidity under environmental stress. This highlights membrane fluidity as a key determinant

of *L. monocytogenes* stress survival making it an important predictor of the pathogen's resistance to food preservation agents.

6.6   Conclusions

In general, exposure to acid stress in *L. monocytogenes* induces disruptions of protein secondary structure, nucleic acid conformations, and degradation of cellular polysaccharides. However, in stress-susceptible strains, this response is accompanied by changes in membrane lipid acyl chains that potentially cause reduced membrane fluidity. SEM analysis shows that physical damage to the cell wall and cell membrane is a possible manifestation of the acid-induced biomolecular changes providing an additional mechanism for acid-induced cell death. In stress-susceptible strains, the acid stress response also involves the formation of extracellular vesicles which possibly play a role in the survival potential through an improved exploitation of the environment to support growth. This study shows that membrane fluidity is a key determinant in *L. monocytogenes* survival which is vital in identifying predictors of the pathogen's resistance to food preservation agents.

7   CHAPTER 7

ACID STRESS-INDUCED PERSISTER CELL FORMATION IN *LISTERIA MONOCYTOGENES* AND THE DIFFERENTIAL EXPRESSION OF STRESS RESPONSE GENES IN REVIVED PERSISTERS

7.1 Abstract

Persistence of *Listeria monocytogenes* is a major challenge in the control of the pathogen in food processing environments. This study investigated the characteristics of persistent survivors following lethal acid stress treatment and the gene expression profiles of such cells when re-grown under mild stress conditions in two strains of *L. monocytogenes*. When overnight cultures of *L. monocytogenes* were treated with increasing doses of lactic acid (0.98 - 31.37 mM undissociated acid), a recalcitrant population of survivors remained regardless of increasing acid concentration. The surviving population was as susceptible to acid re-exposure as the parental population indicating a lack of acquired resistance. A reduced metabolic activity (based on carboxyfluorescein diacetate (CFDA) fluorescence) of survivors compared to control cells indicated a likely role of metabolic inactivity in the tolerance of survivors, a characteristic of persister cells. Microscopically, cells subjected to lethal acid stress showed the presence of extracellular vesicles which were lacking in control cells indicating the potential for other mechanisms accompanying survival of such stress in addition to metabolic inactivity. When persister survivors were re-grown under conditions of mild acid stress and cold stress, no significant differences ($p > 0.05$) were observed in the expression levels of stress response genes *sigB* (alternative sigma factor B (σ^B) *gadD2* (glutamate decarboxylase), *oppA* (oligopeptide permease), and *lmo1722* (DEAD-box RNA helicase) between re-grown survivors and control cells. However, significant differences were observed in the phosphotransferase system (PTS) coding gene *lmo1038*. The findings of this study suggest that the persistence of *L. monocytogenes* in the face severe stress as is the case in processing environments could be attributed to a dormant persister cell state. When such dormant cells are allowed to revive and re-grow, they do not exhibit any acquired stress resistance, an indication of an unlikely presence of heritable genetic differences from their parental generation. However, the lower transcript levels of the PTS system coding gene suggests that there could be a reduced affinity and uptake of sugars in re-grown survivors implying a potential for slow growth in cells re-grown from severe stress survivors.

Keywords: *Listeria monocytogenes*; persistence; gene expression.

7.2 Introduction

Bacterial persistence refers to the ability of particular types of microorganisms to survive for prolonged periods of time in certain habitats (Orgaz et al., 2013). In the case of *L. monocytogenes*, molecular profiling of strains has often identified the occurrence of specific molecular subtypes repeatedly associated with specific food processing plants resulting in recurrence of product contamination with the same strain over extended periods of time (Almeida et al., 2013; Rückerl et al., 2014). These processing plant persistent strains are often the cause of listeriosis outbreaks involving contaminated processed RTE foods (Jensen et al., 2008; Nowak et al., 2017; Wulff et al., 2006).

The underlying mechanisms of *L. monocytogenes* persistence still remain elusive (Orgaz et al., 2013). Several authors have suggested factors such as the ability of persistent strains to adhere to food-processing surfaces, and the ability to tolerate disinfectants as inherent differences influencing the ecology of persistent and non-persistent strains (Larsen et al., 2014; Møretrø and Langsrud, 2004). However as observed by Carpentier and Cerf (2011), there is no consensus in literature that disinfectant resistance or biofilm formation are the main factors responsible for *L. monocytogenes* persistence. Another proposition made by Fox et al. (2011) based on the phenotypic microarray analysis of the full metabolic profiles of persistent and non-persistent strains is that the latter possesses pathways for the metabolism of unique compounds not metabolized by other strains and co-existing microflora giving such strains a competitive advantage.

A third scenario also recognized as a potential explanation for bacterial persistence is the theory of persister cell formation. Based on this theory, persisters are regarded as highly tolerant phenotypic variants of normal cells that form stochastically in microbial populations

by entering a state of growth arrest (Brauner et al., 2016). While the stochastic formation of persisters is considered to be an evolutionary bet-hedging strategy, many environmental stresses have also been deemed to induce the transition of normal cells into this non-growing state making it an important survival state of microbial cells in the face of environmental pressures (Radzikowski et al., 2016). This phenomenon of persister cells has been known for a long time in clinical settings as a cause of persistent recurrent infections (Gefen and Balaban, 2009). Unlike antibiotic resistant infections, persister-mediated recurrent infections remain sensitive to antibiotic re-exposure. Similar to antibiotic-induced persister formation, it can also be argued that the routine use of disinfectants and sanitizers can potentially provide a driving force for the development of persister cells of *L. monocytogenes* in processing plants and thus consequently aid persistence of the pathogen.

Although the potential role of persisters as a factor facilitating *L. monocytogenes* survival and persistence in foods and food processing environments has previously been highlighted (Abee et al., 2016; Buchanan et al., 2017), no attention has been accorded to this cell state regarding the conditions under which such cells potentially form and the conditions under which such cells potentially revert to a growing state. Instead, most of the focus with regards to *L. monocytogenes* survival of food-related stresses has been on survivors with genetically mediated resistance (Metselaar et al., 2015; Van Boeijen et al., 2011, 2010). This is notwithstanding the observations from these studies that the majority of survivors comprised of non-resistant phenotypic variants.

As a long-term survival state, persister cells can be a food safety threat as such cells can potentially revive and resume of growth in contaminated foods. Although persister cells are genetically similar to their actively growing ancestral population, recent evidence suggests

that some the phenotypic variation that enables such cells to survive long-term exposure to stress, can potentially be passed on to offspring through a combination of molecular noise and epigenetic inheritance (Day, 2016). This is in addition to the established observation that the immediate environmental history of any cells influences the growth and adaptation of any population following a transition to a new environment (Ryall et al., 2012). It goes to reason therefore that, in the case of persister cells, the programmed gene regulation patterns enabling such cells to survive lethal stress could have an impact on the growth and adaptation of the cells when they resume growth. Therefore, the objectives of this study were to test the hypothesis that persister cell formation as a stress response mechanism is an underlying cause of *L. monocytogenes* persistence and to examine if persister *L. monocytogenes* survivors of lethal acid stress have any adaptational stress response gene expression profiles when resuming growth under conditions of mild acid and cold stress.

7.3 Materials and methods

7.3.1 Bacterial strains

The strains of *L. monocytogenes* used in this study and their growth conditions were as described in Section 4.3.1 (Chapter 4) of this thesis. Strains ATCC19115 and159/10, were specifically chosen because of their observed survival in a lactic soft cheese (Chapter 5).

7.3.2 Lactic acid solutions and determination of undissociated acid concentration

The experiments of bacterial exposure to lactic acid stress were done in stationary phase BHI broth cultures, acidified with L-Lactic acid (Sigma-Aldrich). As a reference for measuring the extent of acid stress treatment, the amount of undissociated acid was calculated for each acid incorporated broth using equation (7.1) derived from the Henderson-Hasselbalch equation (Wemmenhove et al., 2016).

$$[Undissociated\ Acid] = \frac{[Total\ Acid]}{1 + 10^{pH-pK_a}}$$

<div align="right">(7.1)</div>

where pK_a (lactic acid) = 3.86.

Lactic acid solutions in water with total acid concentrations ranging from 40 mM to 100 mM were incorporated into BHI broth on a ratio of 1:10 and used to pre-determine the undissociated acid concentrations before the bacterial exposure experiments. This achieved the following undissociated acid concentrations in broth; 0.98 mM (pH 5.46), 1.92 mM (pH 5.21), 5.25 mM (pH 4.79), 10.56 mM (pH 4.53), 14.57 mM (pH 4.44), 23.37 mM (pH 4.32) and 31.37 mM (pH 4.2). A non-acid (water) incorporated BHI broth was used as a negative control (0 mM (pH 7.46)).

7.3.3   *Lactic acid-induced* L. monocytogenes *persistence*

Persister cell formation was induced by a dose-dependent killing of planktonic cells in BHI broth following a method previously described by Wu et al. (2017). In line with the definition of persister cells, *L. monocytogenes* cells were subjected to treatment with lactic acid doses well above the minimum inhibitory concentration which was empirically determined to be 0.98 mM (pH 6.8) undissociated acid. A 900 µl volume of stationary phase culture (grown in 10 ml BHI broth at 37ºC for 18 h) was treated with 100 µl filter sterilized (0.2 µm filters, Pall Life Sciences) lactic acid solutions or blank (water) and the treatments were incubated at 37ºC for 24 h. Treatments were subsequently centrifuged at 5 000×g for 5 min and re-suspended in 1 ml PBS before viable counts were determined by plating on BHI agar. Each treatment was replicated three times.

For monitoring death kinetics, 9 ml volumes of stationary phase cultures were treated with 1 ml filter sterilized solution of lactic acid to achieve the highest concentration of 31.37 mM undissociated acid. Treatments were incubated at 37ºC and viable counts were monitored by plating on BHI agar at 0, 2, 4, 8 and 24 h.

7.3.4 Susceptibility of survivor isolates to severe acid stress re-exposure

Five colonies representing cell survivors at the highest concentration of lactic acid (31.37 mM) were re-grown in 10 ml fresh BHI broth at 37ºC for 18 h and 900 µl of the culture was re-subjected to acid treatment with 100 µl filter sterilized solution of lactic acid at the highest concentration of undissociated acid (31.37 mM). A culture of the parental cell population was used as a control. After 24 h at 37ºC, treatments were subsequently centrifuged at 5 000×g for 5 min and re-suspended in PBS before viable counts were determined by plating on BHI agar. Each treatment was replicated three times.

7.3.5 Metabolic activity of survivor cells

In order to evaluate the metabolic activity of survivor cells, the esterase activity of cells subjected to severe acid treatment was determined using flow cytometry (Kim et al., 2011). Treated cells (1 ml) were centrifuged at $5000 \times g$ for 10 min, washed twice and re-suspended in 1 ml PBS. A 100 µl of the cell suspension was then incubated with 1 µl of 10 µM 5-carboxyfluorescein diacetate (CFDA) (Molecular Probes) solution for 30 min in the dark at 37ºC. The CFDA stained cell suspensions were subsequently centrifuged at 5 000×g for 10 min to remove excess stain and the cells were re-suspended in fresh 100 µl PBS. The stained The intensity of the green fluorescence signal of the stained cells was measured using a flow cytometer (BD Accuri™ C6 Plus, BD Biosciences) equipped with a 20 mW argon laser emitting at 488 nm. Logarithmic signals for green fluorescence were recorded in the FL1

channel using a 533/30 bandpass filter. The obtained data were analyzed using FlowJo® v10.4 software (FlowJo LLC, USA). The fluorescence signal of the CFDA-stained surviving populations after lethal acid stress exposure was compared to a standardized population of CFDA-stained untreated control cells. Control cells were standardized by dilution to give a cell count equal to that of stress survivor cells (the average stress survivor cell population based on viable counts was $1.7Log_{10}$ CFU/ml and $2.2Log_{10}$ CFU/ml for *L. monocytogenes* ATCC19115 and 159/10 respectively). Also included in the experiment were unstained control cells. Data were presented as overlaid fluorescence histograms of treated and control cells.

7.3.6   *Effect of nutrients on* L. monocytogenes *persistence*

Duplicate 1 ml volumes of stationary phase culture (grown in 10 ml BHI broth at 37ºC for 18 h) were centrifuged at $5000 \times g$ for 10 min and washed twice with PBS. One cell pellet was re-suspended in 1 ml BHI broth and a second cell pellet was re-suspended in 1 ml dilute BHI broth (diluted 1:9 with water) (Wu et al., 2017). A 900 µl volume of the cell suspension was then treated with 100 µl filter sterilized lactic acid solution to achieve the highest concentration of 31.37 mM undissociated acid. After 24 h at 37ºC, treatments were subsequently centrifuged at 5 000×g for 5 min and re-suspended in PBS before viable counts were determined by plating on BHI agar. Also included in the experiment was a 900 µl volume of the stationary phase culture treated with 100 µl filter sterilized lactic acid solution representing spent broth. Each treatment was replicated three times.

7.3.7   *SEM examination of lethal acid stress-treated cells*

In order to understand some of the physical changes accompanying the survival of lethal acid stress, SEM observations of treated *L. monocytogenes* cells exposed to 31.37 mM acid stress

125

treatment was done. The method for the preparation of cells for SEM was as described in section 6.3.4 of this thesis (Chapter 6).

7.3.8 *Relative expression of stress response genes in persister survivors revived under conditions of mild acid and cold stress*

In order to test if persister cell survivors have any survival advantage over actively growing cells when they resume growth, the cells surviving lethal acid treatment were revived in BHI broth under conditions of mild acid and cold stress respectively. Surviving cells following lethal acid treatment were recovered by centrifugation of 1 ml suspension of treated cells at $5000 \times g$ for 5 min and the resultant pellet was washed twice with PBS. The washed cells (with approximately 10^2 viable cells as determined by plating) were then revived by addition of 2 ml BHI broth and incubated at 10ºC for 10 d for cold stress revival. For mild acid stress, washed cells were revived in BHI broth supplemented with lactic acid (0.08 mM undissociated acid) (pH 6.3) and incubated at 37ºC for 24 h. Together with severe acid stress survivors, control cells comprising of actively growing log phase cultures (10 – 12 hr) were standardized by dilution to give a cell count equal to that of inoculated persister cells in broth (the average stress survivor cell population inoculated into the broth was 1.7Log$_{10}$ CFU/ml and 2.2Log$_{10}$ CFU/ml for *L. monocytogenes* ATCC19115 and 159/10 respectively).

Given that the objective of the experiment was to evaluate if persistent survivors have any survival advantage over control cells when they resume growth under mild stress conditions relevant to foods, expression profiles of stress response genes were calculated for both persister survivors and control cells growing under mild stress conditions relative to transcript levels of the same genes under optimal growth conditions (Argueda-Villa et al., 2010). Therefore cultures of both persister survivors and control cells grown in BHI broth at 37ºC

were used as a reference for calculation of the relative expression of stress response genes. All cultures under all experimental conditions were grown to stationary phase (OD_{600nm} = 0.4 – 0.5) before RNA extraction.

7.3.8.1 RNA extraction

Cell suspensions were centrifuged at 5000×*g* for 5 min and total RNA was extracted from the resulting cell pellets using the ZR Fungal/Bacterial RNA MiniPrep™ kit (Zymo Research, Irvine, USA) following the manufacturer's instructions. An in-column DNase I digestion was performed using RNase free DNase (Zymo Research) for 15 min at room temperature to remove genomic DNA. Total RNA was eluted in 50 µl RNase free water and stored at -20ºC until use. RNA concentration was determined by measuring optical density (OD_{260nm}) using a nano spectrophotometer (Bibby Scientific, Staffordshire, UK).

7.3.8.2 Reverse transcription and quantitative real-time PCR (RT-qPCR)

Reverse transcription and qPCR was carried out in a one-step reaction using the QuantiNova® SYBR® Green RT-PCR Kit (Qiagen GmbH, Hilden, Germany). The reaction mixture comprised of; 5 µl of 2 × QuantiNova SYBR Green RT-PCR Master Mix (composed of Taq DNA Polymerase, SYBR Green RT-PCR Buffer and dNTP mix); 0.2 µl of 100 × QuantiNova RT Mix (composed of HotStaRT-Script Reverse Transcriptase and RNase inhibitor); 5 µM of each primer (Table 7.1); 1 µl of 5 – 10 ng of template RNA and nuclease-free water in a final volume of 10 µl. The reaction consisted of a reverse transcription step at 50ºC for 20 min followed by a PCR initial activation step at 95ºC for 2 min; denaturation at 95°C for 5 s; annealing at 57ºC for 30 s; extension at 72ºC for 30 s; a single fluorescence reading step after every cycle and a melting curve analysis at 65ºC – 95ºC in 0.5ºC/s increments in a Bio-Rad CFX96 Deep Well™ Real-Time System (Bio-Rad, Hercules, USA).

Primer sequences for the reference and target genes are shown in Table 1. Target genes, *sigB* (general stress response regulator, alternative sigma factor B (σ^B) (O'Byrne and Karatzas, 2008)), *gadD2* (a part of the glutamate decarboxylase (GAD) system that is expressed as part of the acid tolerance response (Lund et al., 2014)), *oppA* (coding for an oligopeptide permease responsible for the accumulation of peptides under cold growth conditions (Tasara and Stephan, 2006)), *lmo1722* (coding for a DEAD-box RNA helicase necessary for efficient translation by unwinding RNA secondary structures formed under low temperature growth (Netterling et al., 2012)) and *lmo1038* (coding for the PTS enzyme II responsible for the transport of sugars in bacteria (Liu et al., 2013)) were selected on the basis of their roles on the growth of *L. monocytogenes* in acid and low-temperature conditions. Negative controls consisting of a non-template control (NTC) a control with no reverse transcriptase (NoRT) were included in each reaction. A comparison of threshold cycle (C_T) values between the NoRT controls and their respective samples were used to evaluate the level of residual DNA contamination (Cabrita et al., 2015) and was considered negligible. The specificity of each reaction and purity of the amplification was confirmed by the presence of a single peak in melting curve analysis. Two reference genes *gap* and 16S were used based on available literature. Data (C_T values) was obtained from three biological replicates performed in duplicates.

7.3.8.3 PCR primer efficiency

Standard curves prepared from serial dilutions (10^0 to 10^{-4}) of template RNA extracted from *L. monocytogenes* ATCC19115 were used to determine the PCR efficiency (*E*) of each primer pair. The slope of the curve from a plot of C_T values versus the Log_{10} values of template concentration was used to calculate percentage efficiency for each primer pair (Table 7.1) using the Bio-Rad CFX manager 3.0 software (Bio-Rad).

7.3.8.4 **Normalization of transcript levels and calculation of relative gene expression**

C_T values of target and reference genes were subjected to efficiency correction and normalization for total RNA of each sample (Kubista et al., 2007). Corrected C_T values of target genes were normalized to the reference gene *gap*. Normalized transcript levels were used to calculate the relative expression of each target gene in using the $\Delta\Delta C_T$ method (Equation 1 and 2) (Livak and Schmittgen, 2001). Expression fold change was then expressed as Log_2 values.

$$\Delta\Delta C_T = (C_T Target - C_T Reference)_{Mild\ stress} - (C_T Target - C_T Reference)_{Optimal\ conditions}$$

(7.2)

$$Relative\ Expression\ of\ target\ gene = 2^{-\Delta\Delta C_T}$$

(7.3)

7.3.9 **Data analysis**

A one way ANOVA with Tukey test for multiple comparisons was used to compare surviving populations of each *L. monocytogenes* strain after exposure to increasing levels of lactic acid, the susceptibility of persister isolates to control cells following acid re-exposure and effect of nutrient availability on the level of persister survivors. The Student *t*-test was then used to analyze for any significant differences in gene expression levels between revived persisters and control cells. Principal component analysis (PCA) of the expression data was used to analyze for any correlation in the expression profiles of the target genes and experimental conditions as a multivariate tool. ANOVA and *t*-test were done in GraphPad Prism (GraphPad Software, Inc., USA). PCA analysis was done using Xlstat, 2016 (Addinsoft, New York, USA).

Table 7.1: Primer sequences of reference and target genes and the respective amplification efficiency values for each primer pairs

Housekeeping Genes	Primer Sequence (5′- 3′)	Reference	E	R^2
gap	F-ACCAGTGTAAGCGTGAA	Tasara and Stephan (2007)	99.3	0.97
	R-TCACAGCGCAAGACAAA			
16S	F-TGGGGAGCAAACAGGATTAG	Karatzas et al. (2010)	60.1	0.99
	R-TAAGGTTCTTCGCGTTGCTT			
Target Genes				
lmo1038	F-GGCTTAGAAACCGTATCCTT	Bae et al. (2012)	86.4	0.99
	R-CCTGCTTCTGCCTTAGTTAC			
oppA	F-GGATGATGTAGCTATTCAACCACTT	Cabrita et al. (2015)	88.5	0.98
	R-GTAAGTGTAATCTGGACCAAATGGA			
gadD2	F-AATACCTTGCCCATGCAGTC	Karatzas et al. (2010)	88.7	0.96
	R-GGCTTGGAAATCTTGGATGA			
lmo1722	F-CCACGAATGGAAATTTTGCT	Cabrita et al. (2015)	88.4	0.99
	R-CGTGAATTCCAGCTGCTTTTA			
sigB	F-TCATCGGTGTCACGGAAGAA	Mataragas et al. (2015c)	83.6	0.99
	R-TGACGTTGGATTCTAGACAC			

E - Efficiency of each primer pair estimated from standard curves using the equation, $E = (10^{(-1/slope)} - 1) \times 100$

F - Forward, R - Reverse, R^2 – Goodness of fit of the regression line for efficiency determination.

7.4   Results

7.4.1   Acid stress-induced persistence

Figure 7.1 shows the surviving populations of *L. monocytogenes* strains ATCC19115 and 159/10 exposed to increasing doses of lactic acid (0 – 31.37 mM of undissociated acid) including the MIC (empirically determined to be 0.98 mM (pH 6.8). A steady decline in surviving populations was observed at acid levels of 0.98 mM to 14.57 mM for strain ATCC19115 and acid levels of 0.98 mM to 23.37 mM for strain 159/10. There was no significant difference in surviving populations at acid levels in excess of 14.57 mM for both strains. A residual survivor population averaging 2.7Log$_{10}$ CFUml^{-1} for strain ATCC19115 and 3.4Log$_{10}$ CFUml^{-1} for strain 159/10 remained in the broth regardless of increasing acid concentrations.
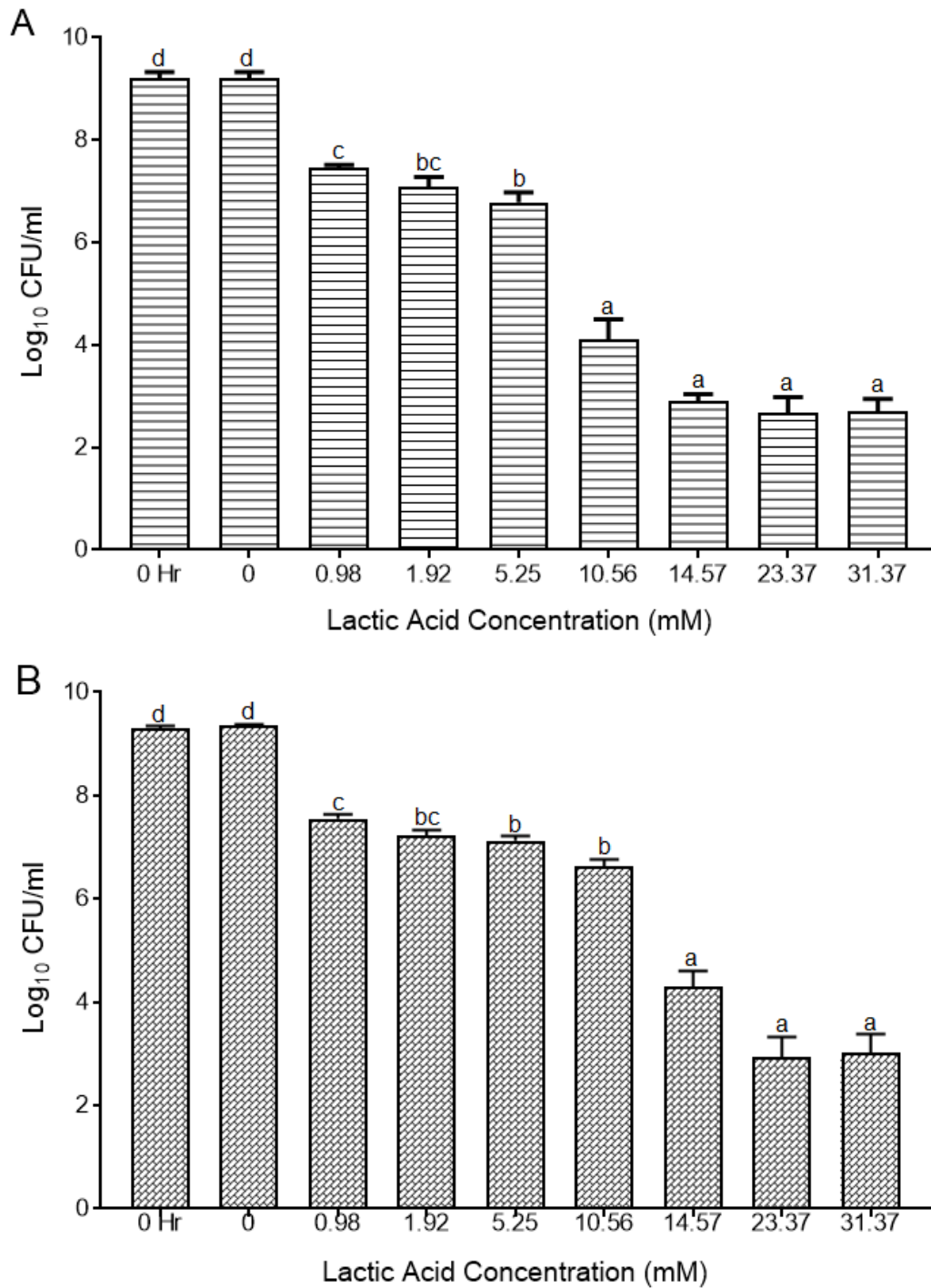
Figure 7.1: Surviving population of *L. monocytogenes* cells subjected to increasing lethal doses of lactic acid in BHI broth. A - *L. monocytogenes* ATCC19115, B - *L. monocytogenes* 159/10. Bars with different letters indicate significant differences (p < 0.05).

7.4.2 *Death kinetics of* L. monocytogenes *cells treated with lethal concentration of lactic acid*

The death kinetics of *L. monocytogenes* cells treated with the highest concentration of lactic acid (31.37 mM), is shown in Figure 7.2. A biphasic death curve typical of persister cell survivors was observed for strain ATCC19115. In the first phase of inactivation (first 8 h of exposure), a rapid decline from 9.1Log$_{10}$ CFUml^{-1} to 2.9Log$_{10}$ CFUml^{-1} was observed for this strain with the surviving tolerant population killed slowly over the remaining period. In the case of strain 159/10, a near log-linear decline in the surviving population was observed from 9.5Log$_{10}$ CFUml^{-1} to 3.0Log$_{10}$ CFUml^{-1} in 24 h.
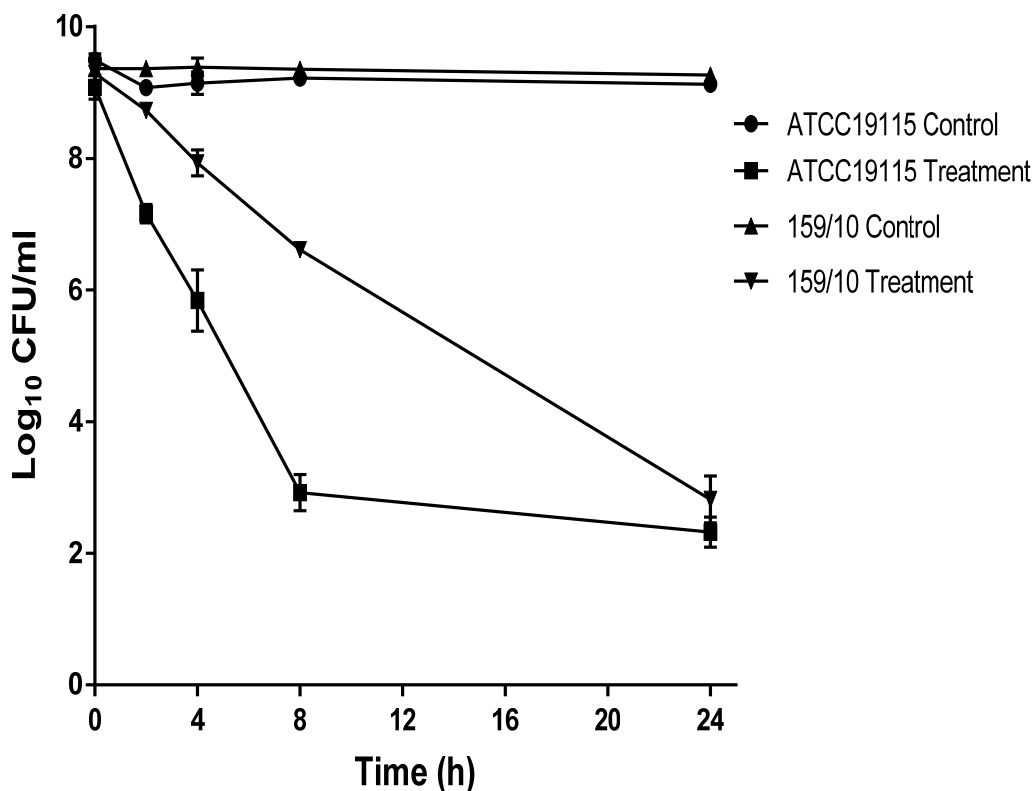
Figure 7.2: Death kinetics of *L. monocytogenes* cells subjected to lethal lactic acid treatment at 31.37 mM.

7.4.3 *Susceptibility of persister isolates upon re-exposure to lactic acid*

Figure 7.3 shows the susceptibility of persister isolates obtained from 24 h survivors of lactic acid treatment (31.37 mM). Survivors of acid treatment represented by colonies picked from BHI agar plates were re-grown in fresh BHI broth and their stationary phase cultures were subjected to re-treatment with 31.37 mM lactic acid. With the exception of one persister isolate of *L. monocytogenes* 159/10, no significant differences ($p > 0.05$) were observed in the susceptibility of persisters compared to the parental control cells an indication the lack of acquired resistance among the *L. monocytogenes* acid stress survivors.
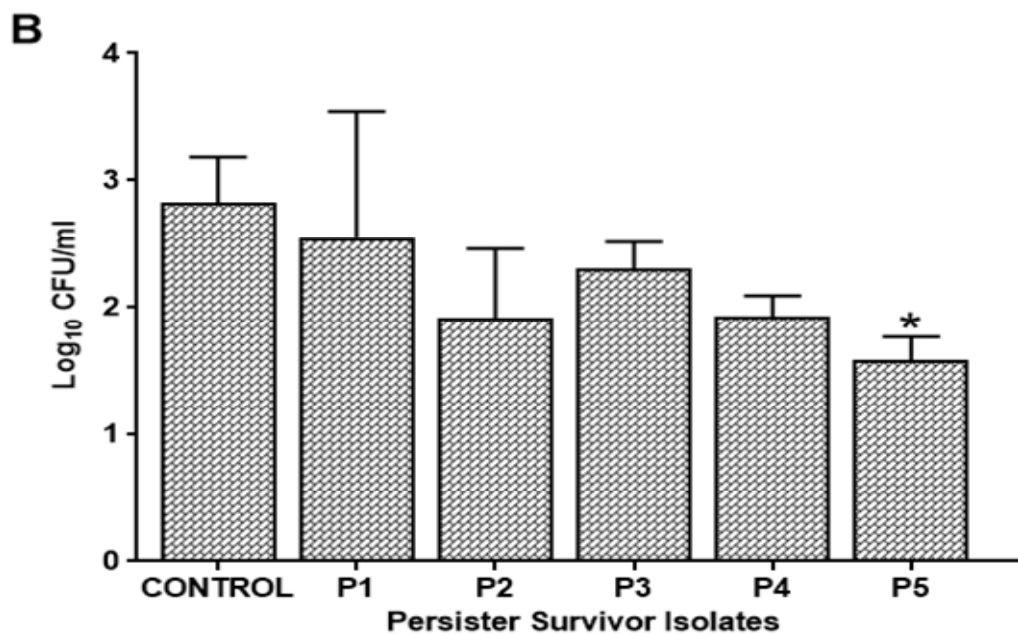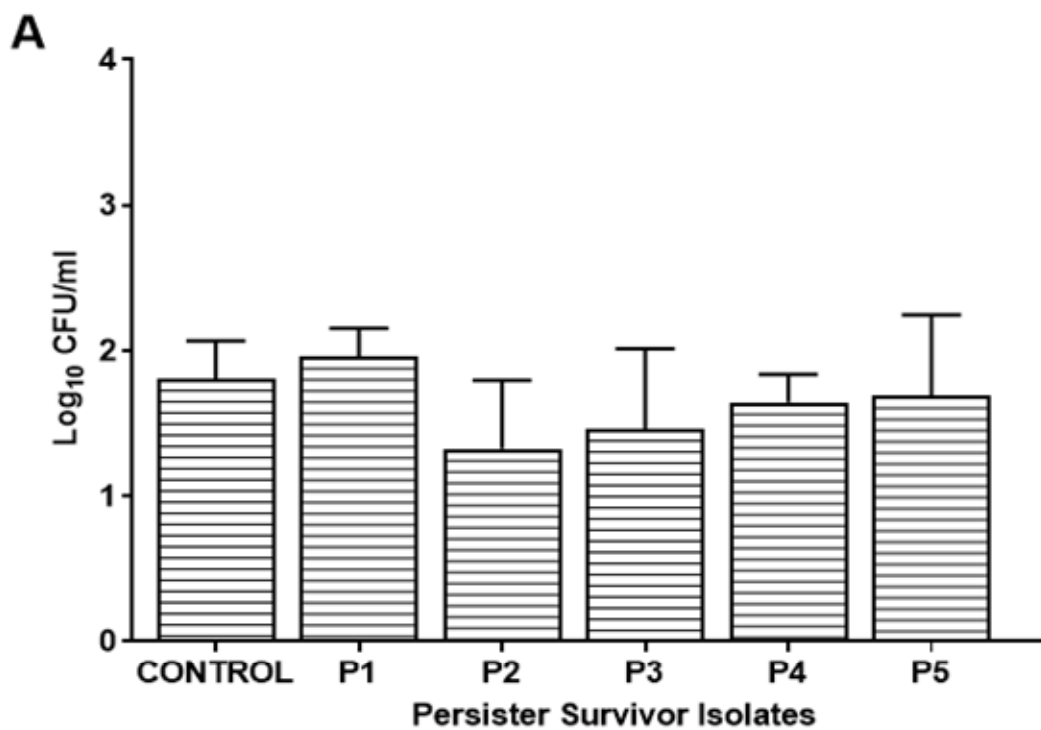
Figure 7.3: Survival of *L. monocytogenes* cells re-grown from persister survivor isolates upon re-exposure to lactic acid treatment at 31.37 mM. A - *L. monocytogenes* ATCC19115, B - *L. monocytogenes* 159/10. On each bar graph, P1 – P5 represent persister survivor isolates. Asterisk indicates a significant difference in survival compared to control cells ($p < 0.05$).

7.4.4 *Effect of nutrient availability on* **L. monocytogenes** *persistence*

The effect of nutrient availability on the susceptibility and persister cell formation of *L. monocytogenes* subjected to lactic acid treatment at 31.37 mM is shown in Figure 7.4. When *L. monocytogenes* overnight cultures were re-suspended in fresh and dilute BHI broth followed by acid treatment, persister cell survivors were significantly higher ($p < 0.05$) in fresh BHI broth than in nutrient deficient dilute and spent broth. However, there was no significant difference ($p > 0.05$) in the survivor population between treatments made in dilute and spent BHI broth in both strains.
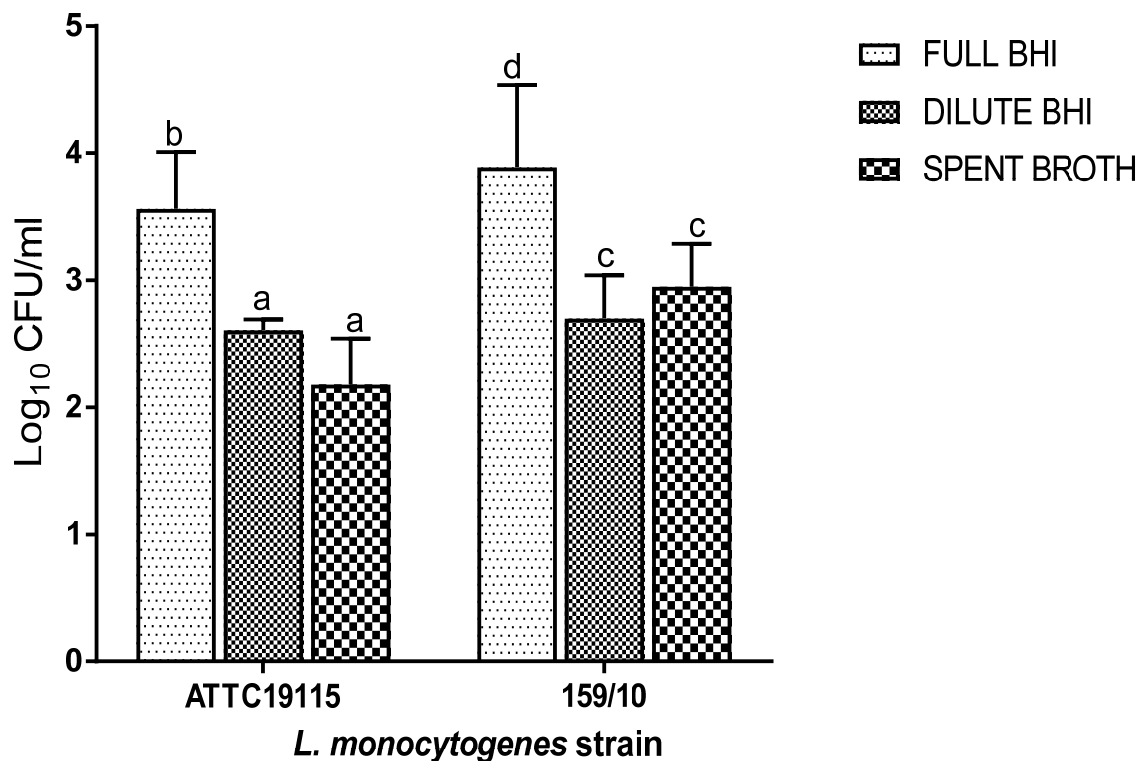
Figure 7.4: Effect of nutrient availability on persistence and tolerance of overnight cells of *L. monocytogenes* ATCC19115 and 159/10 re-suspended in fresh and dilute (1:9) BHI broth and treated with 31.37 mM lactic acid. For each strain, bars with the same letters are not significantly different ($p < 0.05$).

7.4.5 *Metabolic activity of persister cells*

Flow cytometry analysis (Figure 7.5) showed that persister survivors at a standardized cell count had a lower CFDA fluorescence signal compared to overnight control cells. The fluorescence signal was similar to that unstained control cells.
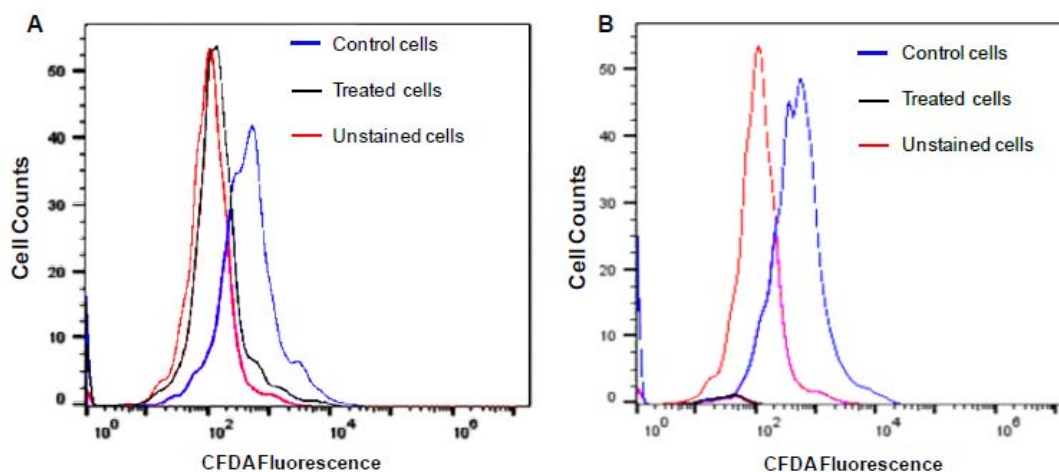
Figure 7.5: Flow cytometry histograms of normalized cell counts indicating reduced CFDA fluorescence of surviving *L. monocytogenes* cells after exposure to lethal acid stress. A - ATCC19115, B – 159/10.

In order to understand the physical changes in cell structure accompanying the shift from normal to persister cell state, scanning electron microscopy analysis was done on stationary phase control cells and persister cell survivors of lethal acid stress (Figure 7.6). Notably, non-treated control cells were mostly shorter rods with smooth outer surfaces while persister cells appeared as slightly elongated rods with a lot of granular surface structures that resembled extracellular vesicles. In addition, evidence of filamentation was observed in one image of strain ATCC19115 persister cells.
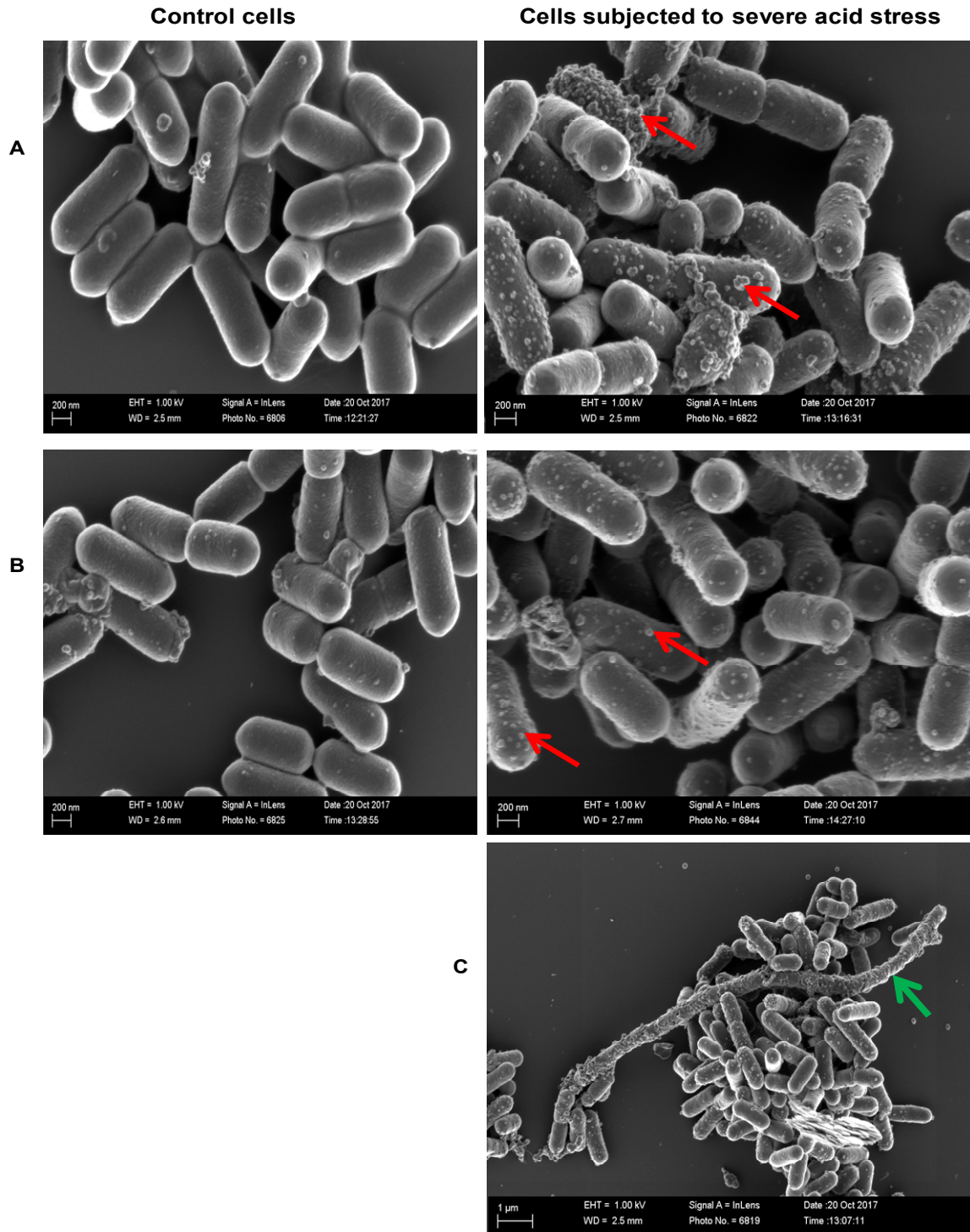
Figure 7.6: Scanning electron micrographs of *L. monocytogenes* ATCC19115 (A) and 159/10 (B) control cells and cells subjected to severe acid stress. Red arrows indicate the presence of extracellular vesicles in cells subjected to severe acid stress cells for both strains. The green arrow in image C indicates filamentation observed in ATCC19115 cells subjected to severe acid stress.

7.4.6 *Expression of stress response genes in cells re-grown from persister survivors*

The relative expression levels of the studied stress response genes in cells re-grown from persister survivors under mild acid growth are shown in Figure 7.7. The general stress response regulator gene *sigB* (> 1.5Log$_2$ fold) and the acid stress response gene *gadD2* (> 0.1 Log$_2$ fold) were generally up-regulated under conditions of mild acid stress, while the cold stress response genes *OppA* (< -0.5Log$_2$ fold) and *lmo1722* (-1.28Log$_2$ fold) were down-regulated in both control cells and revived persister survivors of both strains. However, no significant differences (*p* > 0.05) were observed in the expression levels of *sigB*, *gadD2*, *OppA*, *lmo1722* in cells re-grown from persister survivors compared to the control cells grown under similar conditions. Only the PTS encoding gene *lmo1038* showed a significant (*p* = 0.0012) down-regulation in revived persister cells of strain ATCC19115 (Figure 7.7 A) compared to control cells. A -3.97Log$_2$ fold down-regulation was observed in cells re-grown from persister survivors compared to control cells (0.77 Log$_2$ fold up-regulation). Interestingly, the same gene was up-regulated in both control cells and persister survivors of strain 159/10 growing under mild acid conditions (Figure 7.7 B), although no significant differences (p > 0.05) were observed.

Under conditions of cold stress, relative expression levels of the general stress response regulator, and the cold response genes were positive for both strains with only marginal changes in the acid stress response gene *gadD2* (Figure 7.8). Statistically, no significant differences were observed in the expression levels of *sigB*, *gadD2*, *OppA*, *lmo1722* in re-grown persister cells compared to the actively growing control cells grown under cold stress conditions. The only gene to show any significant differences (p < 0.00001) in levels of expression between persisters and control cells grown at 10ºC was the PTS encoding *lmo1038*. In *L. monocytogenes* ATCC19115, a 10.27Log$_2$ fold up-regulation was observed in

control cells compared to a -0.63 Log$_2$ fold down-regulation in persister survivors (Figure 7.8 A) while in strain 159/10, a 12.89Log$_2$ fold high up-regulation was observed in control cells compared to a -5.40 Log$_2$ fold down-regulation in persister survivors (Figure 7.8 B).
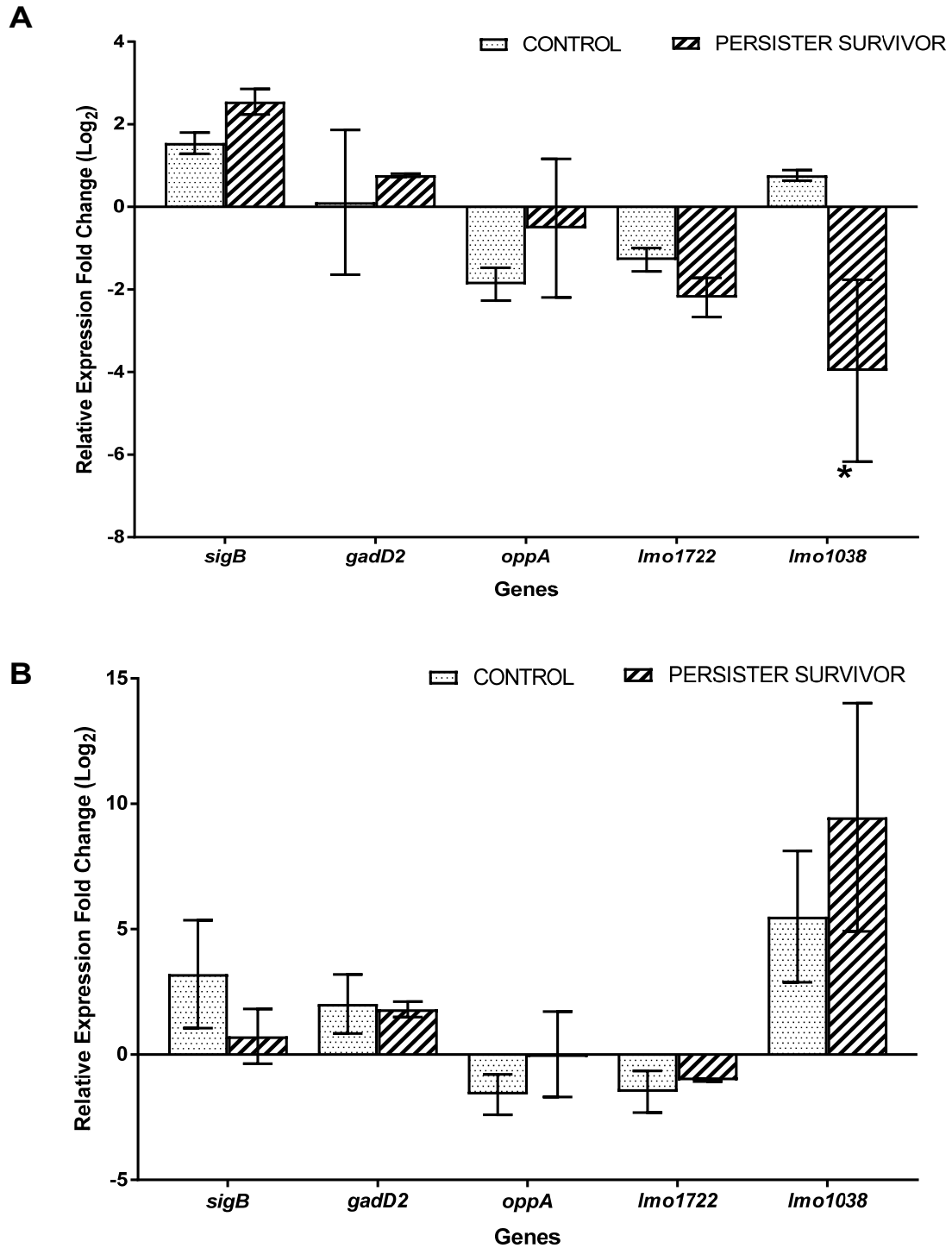
Figure 7.7: Relative gene expression profiles of *L. monocytogenes* cells re-grown from persister survivors and control cells grown to stationary phase in BHI broth supplemented with 0.08 mM undissociated lactic acid (pH6.3). Strain ATCC19115 (A) and strain 159/10 (B). Asterisks indicate significant differences in expression between the cells re-grown from persister survivors and control cells (p < 0.05).
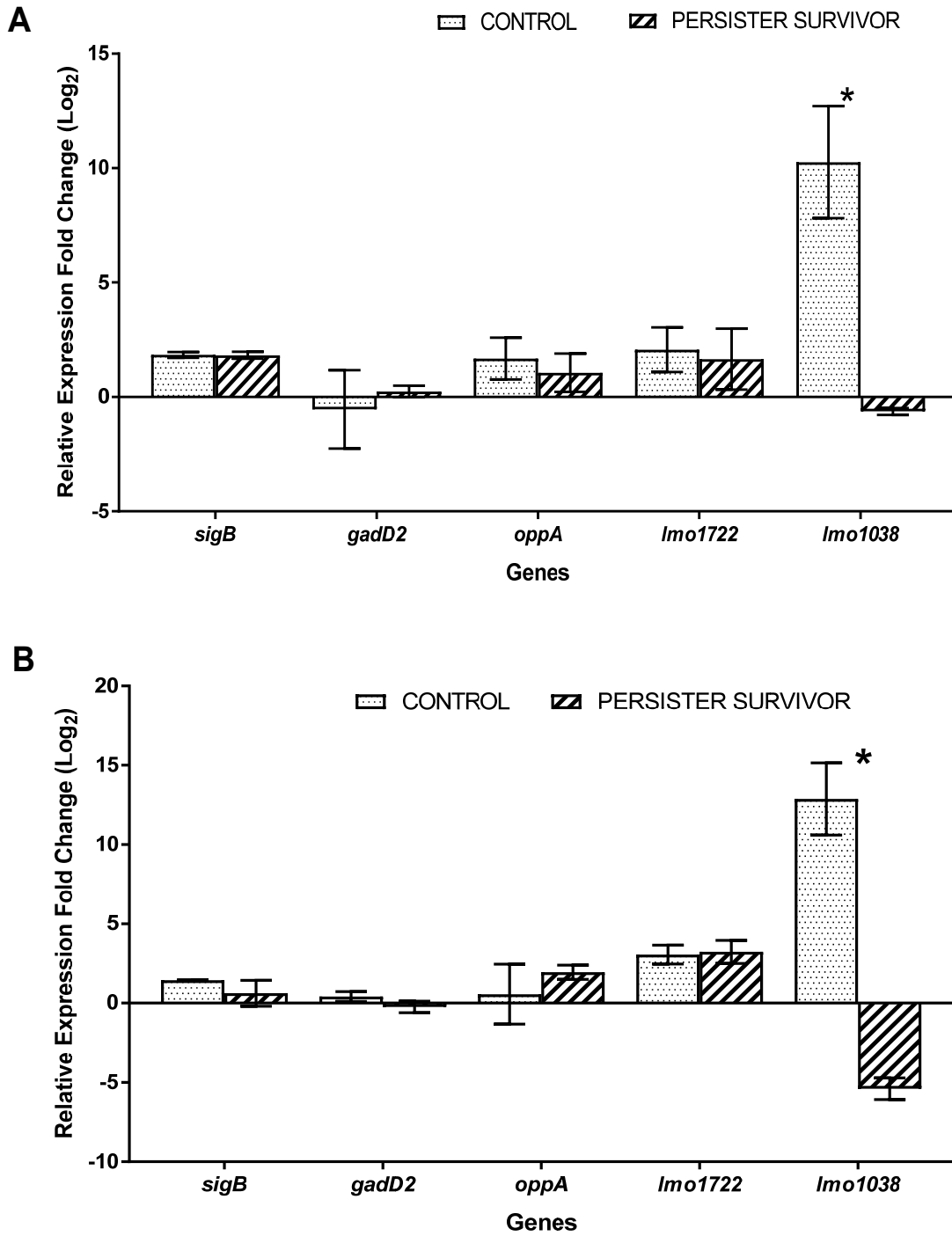
Figure 7.8: Relative gene expression profiles of *L. monocytogenes* cells re-grown from persister survivors and control cells grown in BHI broth at 10ºC for 10 d. Strain ATCC19115 (A) and strain 159/10 (B). Asterisks indicate significant differences in expression between the cells re-grown from persister survivors and control (p < 0.05).
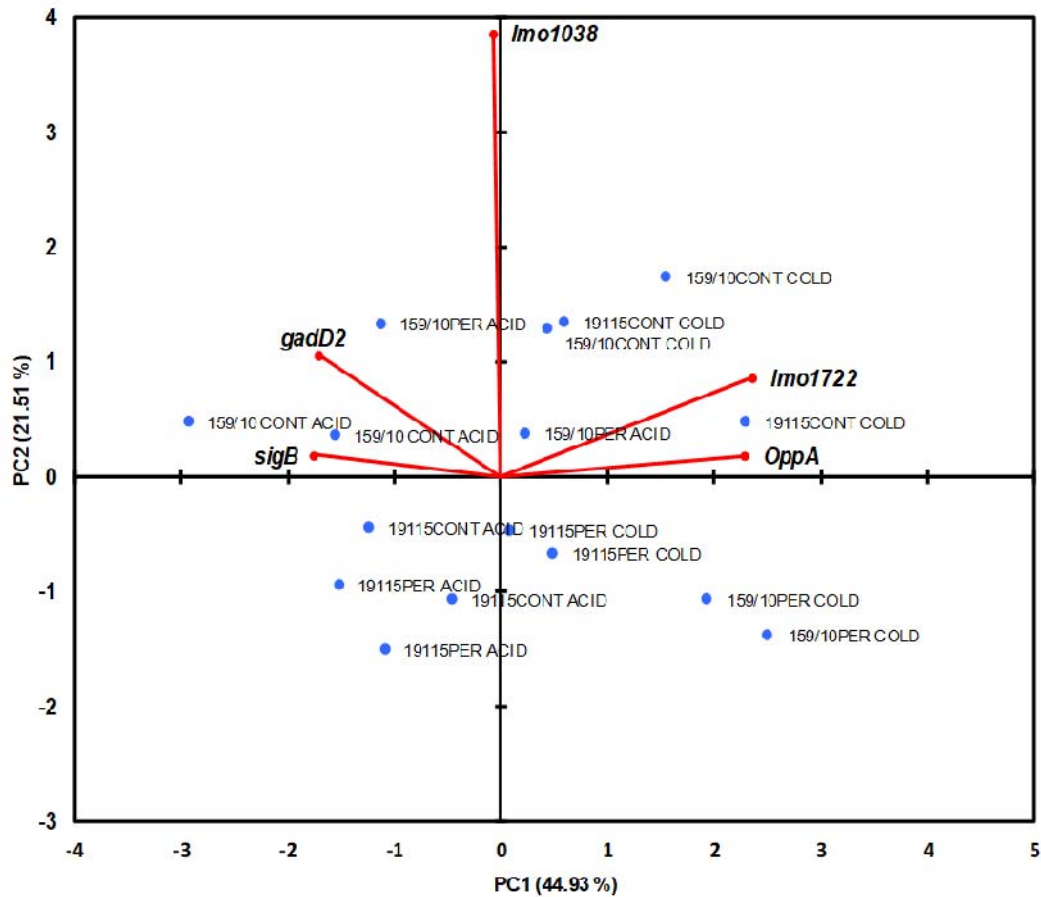
Figure 7.9: PCA classification of expression levels of *L. monocytogenes* stress response genes and re-growth conditions of persister and control cells projected on a PC1 and PC2 scale.

7.4.7 *Multivariate analysis of gene expression profiles*

The presence of any correlation in the expression profiles of the stress response genes and the re-growth conditions of *L. monocytogenes* persister and control cells was assessed using PCA (Figure 7.9). The first two principal components (PC1 and PC2) were used to show the relationship between the expression levels in the stress response genes and the re-growth conditions of the persister survivors as they accounted for 66.44% of the total variability. Generally, the expression of the genes *gadD2* and *sigB* were negatively correlated with that of

oppA and *lmo1722*. A Significant correlation was found only between the genes *oppA* and *lmo1722* (Pearson correlation coefficient = 0.60). Changes in the expression of the cold stress response genes *oppA* and *lmo1722* were related to growth of both cells re-grown from persister survivors and control cells under cold conditions in both strains. In contrast, increases in the expression of *sigB* and the acid stress response gene *gadD2* were strongly associated with mild acid re-growth of *L. monocytogenes* 159/10 control and cells re-grown from persister survivors. The expression of the PTS encoding *lmo1038* was associated mainly with control cells growing under both mild acid and cold stress conditions (positive values of PC2) except for revived acid grown persister survivors of strain 159/10.

7.5 Discussion

This study demonstrated that a residual population of highly tolerant *L. monocytogenes* cells survives lethal lactic acid stress treatment in broth. Given a MIC of 0.98 mM of undissociated acid, increasing acid concentrations of up to 31.37 mM, could not achieve a complete elimination of *L. monocytogenes* cells resulting in a surviving population averaging 2.7Log$_{10}$ CFUml^{-1} for strain ATCC19115 and 3.4Log$_{10}$ CFUml^{-1} for strain 159/10 from initial populations of ca. 10^9 CFUml^{-1}. In order to test if this surviving population owed its tolerance to development of resistance or a transient phenotypic switch, the susceptibility of survivor isolates to a severe acid re-exposure proved that the survivor population was phenotypically similar to the control population. These observations provided an indication that to large extent, *L. monocytogenes* severe acid stress survivors are phenotypic variants of the parental generation whose enhanced tolerance is not attributable to genetically mediated resistance. While both genetically mediated resistance and transient phenotypic switch result in enhanced tolerance and

therefore confer persistence and survival under conditions of lethal stress, the former arises from heritable mutations and therefore results in stable stress resistant mutants (Abee et al., 2016) while the latter arises from non-heritable phenotypic changes (Maisonneuve and Gerdes, 2014) conferring transient stress tolerance. Such a transiently tolerant cell state has previously been identified as the dominant survival state (80%) in *L. monocytogenes* cells exposed to heat and high hydrostatic pressure (Van Boeijen et al., 2011, 2010) and the findings of this study provide an indication that a similar cell state may also be responsible for the survival of severe acid stress.

The mechanisms responsible for the phenotypic switch that results in the formation of the persister cell state have not been elucidated for *L. monocytogenes*. However, based on studies with *E. coli,* persister cells enter into a state of reduced metabolic inactivity that results in growth cessation (Wood et al., 2013). In this study, the observed lower levels of green fluorescence emission of lethal acid stress survivors (indistinguishable from that of unstained control cells), provided an indication that lethal stress survivors of *L. monocytogenes* exhibit a reduced enzymatic activity suggesting that such cells could be in a state of growth arrest. This a proposition that has been made by many researchers supporting the theory of growth arrest as a means of severe stress survival (Amato et al., 2014; Pu et al., 2017, 2016).

The level of persistence observed in acid treatments done in nutrient-rich fresh BHI broth and dilute as well as spent BHI broth indicated that nutrient availability is an important factor in *L. monocytogenes* persistence. This observation has previously been made for *L. monocytogenes* exposed to lethal nisin treatments (Wu et al., 2017) and it implies that under conditions of lethal

stress, the presence of nutrients is necessary to provide resources needed to support the switch to the persister cell state, thus giving support to the theory of actively generated persister cells as a stress response mechanism (Nguyen et al., 2011). The implications of this nutrient enhanced persistence must be viewed in the context of *L. monocytogenes* survival in food systems which in spite of the presence of antimicrobials such as organic acid preservatives, the abundance of nutrients is likely to promote long-term survival and persistence of the pathogen.

An important aspect of the microscopic morphology of *L. monocytogenes* cells exposed to lethal acid stress observed in this study was the formation of extracellular membrane vesicles. The widespread extent of vesiculation observed in all treated cells (persister survivors and dead cells) suggests that this is part of a general response to lethal acid stress. In the only study to have profiled the composition of membrane vesicles from *L. monocytogenes*, the researchers identified transporters, stress response proteins, metabolism, and virulence agents as part of the protein constituents of the vesicles (Lee et al., 2013). The significance of this bacterial vesicular secretion system has been attributable to the need for bacteria to exploit a wider area of their environment under conditions of stress (Kulp and Kuehn, 2010). It could, therefore, be inferred that for *L. monocytogenes* persister survivors, enhanced vesiculation is an additional survival strategy under the conditions of lethal stress. In particular, the presence of effector proteins in the vesicles could play a role in sensing changes in the environment that help the dormant cells to arise and switch back to an active state.

In addition to vesiculation, filamentation was observed in only one case of *L. monocytogenes* ATCC19115 acid-treated cells. Several studies have reported such morphological changes in

bacterial cells induced by sub-lethal stress (Liu et al., 2017; Stackhouse et al., 2012). The current theory on the mechanism of filamentation in bacterial cells is that filaments arise from defects in cell division (Jones et al., 2013). Given the unlikely occurrence of cell growth under the lethal acid stress used in this study, it is possible that such filaments existed in the stationary phase cultures prior to acid treatment. While the filament formation is not likely to be related to persister cell state, as it is associated with dividing cells, it probably enhances survival under lethal stress and may consequently also enhance persistence. Because filament formation is a reversible process under optimum conditions, their formation has huge consequences for the enumeration of pathogens in foods resulting from an underestimation of bacterial numbers as many cells are enumerated as a single colony on solid media (Jones et al., 2013).

The lack of any significant differences in the expression levels of *sigB*, *gadD2*, *OppA*, *lmo1722* genes in control and cells re-grown from persister survivors under conditions of mild acid and cold stress gave emphasis to the fact that when *L. monocytogenes* survivors of lethal stress resume growth, they are not different from the parental generation from which they evolved in terms of tolerance to future stress exposures. Of these genes, *sigB* is a general stress response regulator controlling the transcription of over 200 genes involved in stress tolerance in *L. monocytogenes* and therefore plays a role under conditions of both acid and cold stress growth (O'Byrne and Karatzas, 2008). The lack of any significant difference particularly in the level of *sigB* transcripts between cells re-grown from persister survivors and control cells is perhaps an indication of the unlikely role of the gene in persister survival. In one study that investigated the potential role of *sigB* in persister cell formation in *L. monocytogenes*, Knudsen et al. (2013) concluded that persister survival in *L. monocytogenes* was independent of this general stress

response regulator. This is contrary to observations from other Gram-positive bacteria such as *Staphylococcus aureus* where TA systems responsible for the persister cell state are co-transcribed with *sigB* operons under stress conditions (Fu et al., 2007). It would appear therefore that the lack of any link between the general stress response and the persister survival state of *L. monocytogenes* could have implications on the expression levels of *sigB* and *sigB*-dependent stress response genes in cells re-grown from such survivors. Thus the transcription levels of the *sigB*-dependent *gadD2*, *OppA*, and *lmo1722* genes were not significantly affected in cells re-grown from persister survivors. Moreover, the lack of differential expression in these genes reinforces the theory of phenotypic switching that is the basis of persister cell definition (Brauner et al., 2016).

The only significantly altered expression levels in cells re-grown from persister survivors was that of the PTS enzyme II encoding *lmo1038*. The PTS system is responsible for the phosphorylation and transport of sugars in bacterial cells (Liu et al., 2013). Differences in the relative expression levels of *lmo1038* are likely related to the ability to utilize nutrients under conditions of stress. While the exact reason for the reduced expression in cells re-grown from persister survivors could not be established, it is likely that this is a feature emanating from the gene expression profiles of the severely stressed cells by epigenetic means. Bae et al. (2012) found that PTS coding genes were generally down-regulated in response to salt stress in *L. monocytogenes* with the expression levels of *lmo1038* specifically sensitive to salt concentration. Considering that a reduced metabolic activity is central to the persister cell state, it is possible that transcriptional signals triggering growth arrest such as ppGpp also result in reduced expression of genes for nutrient uptake. Once these cells resume growth, particularly under mild

stress conditions as was the case in this study, some of gene expression patterns associated with the state of reduced growth may still continue into subsequent generations of revived cells through epigenetic inheritance (Day, 2016). The reduced relative expression levels of PTS genes represented by that of *lmo1038* would imply a reduced ability to utilize nutrients by some survivors of lethal acid stress which would subsequently also imply a reduced growth potential. The potential phenotypic manifestations of this is a hypothesis that would need to be tested.

Overall, the patterns of gene expression revealed by PCA analysis indicated a link between mild acid growth and the positive expression of *sigB* and the acid stress response gene *gadD2* and a link between cold growth and the positive expression of cold stress response genes *lmo1722* and *oppA*. The positive correlation between *sigB* and *gadD2*, although not significant, was a reflection of the dependence of the acid tolerance response on *sigB* regulation (Wemekamp-Kamphuis et al., 2004) while the negative correlation with cold response genes is an indication that cold stress response genes could be subject to some other regulation in addition to *sigB* (Cabrita et al., 2015). Cold adaptation of *L. monocytogenes* has previously been identified to be subject to regulation by the alternative sigma factor 54 (σ^{54}) which may act redundantly with *sigB* (Tasara and Stephan, 2006). The association of *lmo1038* up-regulation with control cells of both strains and its down-regulation with cells re-grown from persister survivors provided the main difference between the two cell states and led to the inference that when *L. monocytogenes* persister survivors resume growth under conditions of mild stress, the cells adapt to the mild stress by reducing nutrient uptake.

7.6   Conclusions

When *L. monocytogenes* cells are challenged with lethal lactic acid treatment, a small fraction of highly tolerant cells survives the stress treatment. The surviving cells display a reduced metabolic activity indicating that survival of lethal stress in *L. monocytogenes* is due to the development of a dormant persister cell state. Moreover, the susceptibility of cultures re-grown from such survivors is not different from that of the parental generation indicating that such persister survivors are phenotypic variants with no acquired stress resistance. In addition, SEM morphology of the treated cells showed evidence of extracellular membrane vesicles inferring that survival of such persister cells could be aided by some other active means in addition to metabolic inactivity. Gene expression profiles of cells re-grown from persister survivors under conditions of mild stress show that for most of stress response genes, expression levels are not significantly different from parental control cells an indication of a lack of heritable genetic differences. However, revived cells have reduced transcript levels of the PTS system coding gene *lmo1038*, an indication that a down-regulation of PTS systems probably accompanies the persister cell state and that there is a potential transfer of such expression patterns in revived cells through epigenetic inheritance. This study shows that a persister cell state may be responsible for the long-term survival and persistence of *L. monocytogenes* under conditions related to foods and also that such persister cells may possess altered phenotypic characteristics when they re-grow in contaminated foods.

8   CHAPTER 8: GENERAL DISCUSSION

This general discussion focuses on a review of the methods applied in this study. The discussion also looks at the main findings of this study from the different research chapters and attempts to consolidate them with respect to the global objectives of the research.

8.1   REVIEW OF METHODOLOGY

Four strains of *L. monocytogenes* were utilized in this study, three of which were obtained from the Department of Food Science and Biotechnology, University of Free State, having been isolated from an avocado processing plant (Strydom et al., 2013) as well as strain ATCC19115. The justification for the choice of these strains was based on the fact that there were readily available and that the three strains represented the most recent isolates from a food processing environment and thus would possess some characteristics reflecting of *L. monocytogenes* survival in foods.

8.1.1   *Stress treatments and induction of cell injury*

In Chapter 4, the focus of the study was to examine the behavior of stress injured *L. monocytogenes* cells in terms of their resuscitation and growth kinetics under different temperature conditions in broth. In order to do that, the study had to induce cell injury in 4 strains of *L. monocytogenes* using three forms of stress that the organism would routinely encounter in the food value chain possibly as control or preservation hurdles. In the study, acid stress (pH 4.2 in saline solution), osmotic stress (10% NaCl solution) and heat stress (55ºC for 30

min in PBS) were used to induce injury. The considerations for using these levels of stress was to enhance sufficient level of cell injury given the known tolerance of the organism to these stresses (O'Driscoll et al., 1996; Skandamis et al., 2008). A previous study by Kennedy et al. (2011) had also shown that similar conditions could induce sufficient cell injury in *L. monocytogenes*. Secondly, the work intended to harvest and separate injured cells from non-injured cells by FACS cell sorting with the objective of studying the resuscitation and growth behavior of these cells without the interference of non-injured cells.

8.1.2 *Flow cytometry and FACS*

While it could be envisaged that the different forms of stress would affect different cellular targets, ranging from the cell membrane, the transmembrane potential, enzymes, and nucleic acids, the assessment of injury adopted for in this work was based on cell membrane damage. The considerations for this approach was that an intact cell membrane is almost a universal requirement for cell viability (Grégori et al., 2001; Stiefel et al., 2015), thus making the loss of membrane integrity to be a universal indicator of cell injury. However, the findings of the study seem to indicate that while membrane integrity could be well related to cell viability following acid and salt stress, there was no such relationship with heat stress. Therefore, the assessment of heat-induced cell injury and cell sorting could have been best done with alternative indicators of injury. The study of Kramer and Thielmann (2016), specifically noted that the transition from live to dead state in bacterial cells under thermal stress begins with the loss of respiratory activity, nutrient uptake and protein pump activity before the loss of membrane integrity, an indication that cells sorted on the basis of membrane integrity as injured cells could, in fact, be in a dead state. Hence, more representative indicators of heat-induced injury could have been the

loss of nutrient uptake ability. For instance, fluorescent indicators such as 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose) and 3,3′-dihexylocarbocyanine iodide have utilized for as indicators of loss of glucose uptake and respiratory activities respectively in heat-induced injury of bacterial cells (Arku et al., 2011; Kramer and Thielmann, 2016).

The technique of FACS cell sorting provides a powerful high throughput means of separation of cell subpopulations with special features from a mixture of cells (Amor et al., 2002). The ability to resolve injured cells from mixed populations of non-injured live and dead cells depends on a proper setting of gates to separate the subpopulations. For that purpose, this study utilized control cells consisting of live non-injured cells and dead cells to set up the gates. This approach has been used in previous cell sorting studies such as Stephens et al. (2000) and Kennedy et al. (2011). In this study, a confirmation of sort purity was done using a bead sort which confirmed a purity > 99%. However, the argued limitation of this approach is the lack of a more physical means of confirmation of the injury state of sorted cells. To that extent, the inclusion of fluorescence microscopy could have provided an extra assurance and a complete elimination of any potential spillover of live cells from the boundaries of the different gates.

As observed in this study and also in the study by Kennedy et al. (2011), not all sorted cells could re-grow on non-selective media. Although the potential of irreversible loss of viability due to the stress treatments could be an explanation, the toxicity of fluorescent stains on the already injured cells could be an additional possibility. The fluorescent label PI, used as the marker of cell integrity loss in this study was used at concentrations recommended in the BD™

Biosciences cell viability kit which has been optimized and validated for several bacterial species such as *E. coli*, *Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Bacillus globigii* (Alsharif and Godfrey, 2002, 2001). While this protocol is an established method for the quantification of cell injury, it has recommendations on cell sorting. With the additional step of cell sorting, the likelihood of PI and the sorting process affecting the downstream processes of cell recovery is a possibility. Notwithstanding these drawbacks of fluorescent probes, the technique of sorting cells based on their fluorescence characteristics remains the most convenient way of cell separation, with PI being the leading marker of cell damage (Sträuber and Müller, 2010; Yang et al., 2015). Despite the likely effects of fluorescent labels on the recovery ability of sorted cells, the stains do not have any effect on resuscitated cells. The restoration of the cell membrane and an energized state enables such cells to actively remove PI by efflux pumps (Joux and Lebaron, 2000).

8.1.3 *Resuscitation conditions and kinetic modeling*

In order to study the resuscitation and growth kinetics of sorted injured cells, the experimental design was meant to enable a comparison between injured cells and non-injured cells not exposed to stress. For an objective comparison, non-injured control cells (harvested from the late-log phase stage) were normalized to the same numbers as those of injured cells. The choice of the growth stage was governed by the need to use control cells in a balanced state of growth. With the onset of the stationary phase, a change in gene expression profile results in the development of stress tolerance responses (Llorens et al., 2010). The incubation conditions for the resuscitation of injured cells were at 37ºC, 25ºC, and 4ºC. The first two conditions fall within the growth temperature range for *L. monocytogenes* (Cole et al., 1990) and were selected to

represent the optimum, and ambient temperatures respectively. Because most RTE foods implicated in listeriosis outbreaks are often kept under refrigerated storage for a long time, the temperature of 4ºC was chosen to represent refrigeration storage conditions. In practice, however, *L. monocytogenes* ability to multiply in low-temperature stored foods is exacerbated by abuse especially with evidence that most domestic and retail refrigerator are well in excess of 4ºC and are in some instances > 10ºC (James et al., 2008). An inclusion of an additional temperature value at 10ºC would have added some valuable insight on the fate of injured cells in a context of temperature abuse. Because of the slow rate of resuscitation at 4ºC, the number of sampling intervals over a 15 d period were not enough to use for the estimation of growth parameters by the Baranyi and Roberts (1994) model. Although the growth kinetics could not be established at 4ºC, it was still pertinent that the ability of injured cells to resuscitate under such low-temperature conditions was examined as this relates in a practical way to the safety of low-temperature stored RTE foods contaminated with injured *L. monocytogenes.*

Given that the major challenge in *L. monocytogenes* is in RTE foods that support its growth. Hence, the choice of BHI broth as a resuscitation medium was premised on its general ability to support growth and permit repair of injured cells. However, the interpretation pathogen responses in broth must be treated with caution as it is not always easy to extrapolate the growth behavior of pathogens in broth to their behavior in foods. Despite these limitations, the use of BHI broth was considered satisfactory for the study of sorted injured cells.

Of the several deterministic growth models available for estimation of microbial growth parameters, the model of Baranyi and Roberts (1994), is considered to widely acceptable as it

incorporates an aspect of the physiological state of the cells and thus is ideal for the estimation of the lag phase duration (Baranyi and Roberts, 1994; Swinnen et al., 2004). Additionally, the model has been incorporated into user-friendly software (DMFit) (Institute of Food Research, Norwich, England) in which the growth parameters are reported in a biologically relevant way.

8.1.4 *Stress and population dynamics in soft cheese*

In Chapter 5, the focus was to understand the fate of *L. monocytogenes* cells surviving acid and osmotic stress exposures as both injured and non-injured tolerant cells in a model RTE food. Heat treated cells were not included in this part of the study as most of the heat-injured cells were from the earlier findings (Chapter 4), incapable of re-growth. A lactic soft cheese, produced by a lactic acid fermentation of pasteurized milk to an average pH of 4.2 and a total titrable acidity of 0.64%, was used as model RTE food. The final average pH of 4.2 meant that the soft cheese belonged to the category of foods incapable of supporting *L. monocytogenes* growth in term of Codex categorization (Codex Alimentarius Commission (CAC), 2009). As a no-growth food, an inoculation level of $10^4 – 10^5$ CFUml^{-1} was used in order to study the survival and inactivation kinetics of the organisms over a 15-day storage at 4ºC. In reality, however, it is the category of RTE that can support *L. monocytogenes* growth (pH > 4.4) that are the most high risk with regards to listeriosis (Codex Alimentarius Commission (CAC), 2009). It would have been desirable therefore if a typical growth supporting RTE food was included in the study as it would have given the study the added dimension of assessing the behavior of both injured and non-injured tolerant *L. monocytogenes* cells in an RTE food capable of supporting growth. Despite this limitation, this study was able to highlight some of the dangers of stress tolerance responses

in enhancing *L. monocytogenes* survival and persistence at levels that are at risk to the safety of non-growth supporting RTE foods.

The mathematical models applied in studying the population changes of stressed *L. monocytogenes* cells in the soft cheese were a collection of equations describing different shapes of inactivation curves (Peleg, 2000). The lack of linearity in microbial responses to non-thermal treatment processes, results in inactivation curves that are alterations of the first order log-linear kinetics such as; linear curves preceded by a shoulder; linear curves with a tail; and biphasic linear curves (Peleg, 2003) or curves with convex and concave shapes (Albert and Mafart, 2005). Of the four equations used in this work, two of them were variations of the log-linear inactivation response (log-linear with tail and biphasic) and the other two (weibull and weibull with tail) were variations of the weibull model, a model that can describe microbial responses showing concave and convex responses. Variations in microbial responses mean that essential parameters for predicting bacterial behavior in foods such as the inactivation rates must be based on the model giving the most accurate response. In this study, a LoF analysis followed by the AF and BF tests were utilized to test the adequacy of the four models in describing the bacterial responses. Although there are several methods of defining model accuracy, the LoF test is considered a first step in choosing suitable models as it measures the deviation of a model from experimental error (Drosinos et al., 2006). Because it was not possible to get one model passing the LoF test for all strains and treatments, two models (log-linear with tail and weibull) that passed the test for the most number of experimentals were used to obtain inactivation parameter and further subjected to AF and BF tests. The AF and BF tests are complementary measures proposed by Ross (1996). They are simple indices that provide a more practical interpretation of

model performance by providing model bias and accuracy in estimating the actual responses of microorganisms in foods. For practical purposes, it is important to avoid models that underestimate the risk (fail-dangerous) of microbial growth or survival in foods (Ross, 1996).

For the purposes of understanding how co-contamination of soft cheese with mixed strains influences the diversity of surviving populations, GTG_5 PCR fingerprinting was chosen to profile the genetic relatedness of surviving populations to the parental strains used. While the strains were previously typed with pulsed-field gel electrophoresis (PFGE) (Strydom et al., 2013), there was a need to find a more economical and convenient means of separating the strains and repetitive elements PCR (REP-PCR) was considered ideal under the conditions of the study. The resolution power of REP-PCR is much lower than PFGE in the subtyping of *L. monocytogenes* as seen in the study of Zunabovic et al. (2012). However, considering that the objective and design of the study, the application of the technique was merely to match the fingerprint patterns of the surviving population to that of the inoculated strain. Hence the GTG_5 PCR fingerprint patterns of the four strains were done before the cocktail was made. When it was evident that two strains 159/10 and 243 could not be distinguished using this technique, strain 243 was then excluded from the analysis in all experiments of the cheese challenge so that only strains with distinctly identifiable GTG_5 patterns were used. The inability of GTG_5 fingerprinting to distinguish the two strains which were hitherto subtyped by PFGE indicated the inferiority of this technique alone in subtyping of *L. monocytogenes*. A more improved resolution ability of REP-PCR can be achieved by including more than one primer in the analysis as in studies by Chou and Wang, (2006) and Zunabovic et al. (2012).

8.1.5 *FT-IR spectroscopy*

In Chapter 6, the biochemical changes accompanying stress responses in *L. monocytogenes* were elucidated using FT-IR microspectroscopy. This technique is based on the vibration behavior of biomolecules exposed to infrared radiation and therefore it reflects the changes in the main constituents of the cell such as lipids, carbohydrates, proteins, and nucleic acids (Alvarez-Ordóñez et al., 2011), thus it reflects the changes in the structural components such as the cell membrane and other physiological changes such as alterations in protein structure as well as DNA damage (Alvarez-Ordóñez et al., 2011). The strength of this technique is that unlike other methods for analyzing cellular changes such as fluorescence-based and chromatographic methods, it is label-free and non-invasive, requiring no cell lysis (Le Roux et al., 2015a). Because of the complexity of the bacterial spectra, due to numerous overlapping absorption peaks, differences between studied cells are not easily discernible unless the spectra are subjected to processing and chemometric analysis. When combined with chemometric analysis, the technique has been used to distinguish between injured, and live as well the effects of different stress exposures on microbial cells (Alvarez-Ordóñez et al., 2010; Zoumpopoulou et al., 2010). The limitation of this technique is that it is expensive and requires a huge capital outlay to set up. This is especially the case with the microspectroscope which incorporates a specialized IR microscope in addition to the normal FT-IR equipment. However, once established, routine analysis is less expensive compared to fluorescence-based and chromatographic methods. Because of technical challenges of long equipment breakdown, only the acid stress effect was analyzed using this technique. For that analysis, two strains (69 and 159/10) that showed different susceptibilities to acid stress in Chapter 4 and 5 were utilized. SEM was included to provide some information on the ultrastructural changes in the cells resulting from acid

treatment. While the power of SEM provided information on the morphological and cell surface changes on the acid-treated cells, it does not give information on the effects of stress within the cell. The inclusion of transmission electron microscopy (TEM) would, therefore, have provided additional information on the internal cellular changes.

8.1.6   *Lactic acid-induced persistence*

A lactic acid treatment in BHI broth was used to study the acid-induced persistence of *L. monocytogenes*. Persistence is routinely observed as the ability of an organism to survive high concentrations of an antimicrobial agent without the development of resistance. In most cases, where such studies involve antibiotics, microbial exposures are done in antibiotic-supplemented broth (Jõers et al., 2010; Knudsen et al., 2013). A similar approach was also used to study the nisin induced persistence of *L. monocytogenes* (Wu et al., 2017). The concentrations of the lactic acid in the treatments were expressed in terms of the undissociated form of the acid in order to account for the buffering effect of the broth which lowers the effective concentration of the lactic acid in the broth. Graded doses of the undissociated acid in excess of the MIC (0.98 mM to 31.37 mM) were used to check for the existence of persister survivors that occur regardless of increasing concentration in line with the established criteria of identifying such cells (Knudsen et al., 2013). The justification for using lactic acid in the experiment was that it is the main antimicrobial agent in lactic soft cheese from which the persistence of *L. monocytogenes* strains used in this study was initially observed (Chapter 5). As the main inhibitory agent, it was hypothesized to be the main inducer of persistence. In addition, it is a commonly used acid as a preservative and acidulant in foods.

8.1.7   Metabolic status of persister survivors

Flow cytometry coupled with CFDA labeling was used to assess the metabolic activity of persister cells recovered from a 24 h treatment with 31.17 mM lactic acid. CFDA is a non-fluorescent ester that is converted to a green fluorescent product by cellular esterases of metabolically active cells. The method provides a way of confirming the metabolic status of bacterial cells. A potential weakness of this method is a sometimes weak fluorescence signal (Hoefel et al., 2003). However, the method was able to distinguish the fluorescence signal of acid treated cells which was the same as that of unstained cells.

8.1.8   Considerations for RT-qPCR in gene expression

RT-qPCR was used to monitor the gene expression profile of revived persister cells. This technique relies on the synthesis of cDNA from the available mRNA transcripts of bacterial cells. The technique requires an immediate stabilization of all mRNA in the sample to avoid loss and an efficient conversion of the mRNA to cDNA. A commercial RNA extraction kit, ZR Fungal/Bacterial RNA MiniPrep™ kit (Zymo Research) was utilized. Most commercial RNA extraction kits incorporate an RNA stabilizing agent such as Trizol reagent in the lysis buffer. A commercial RT-PCR kit, QuantiNova® SYBR® Green RT-PCR Kit (Qiagen) with an optimized master mix of reverse transcriptase and Taq DNA polymerase. Relative expression using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) was utilized to quantify the expression levels of stress response genes in revived persister cells compared to control cells. This method requires reliable stably expressed internal reference genes for normalization and two house-keeping genes (*gap* and *16S*) were chosen reference genes. However, after analyzing the amplification efficiency, the *16S* gene was excluded from the analysis because of low amplification efficiency.

The RT-qPCR technique is useful in cases where the expression profiles of a selected small number of target genes are required. It is hence difficult to extract the complete transcriptomic profile of the cells using this method. RNA microarrays can overcome this challenge by providing a global analysis of the complete transcriptome of the organism thus showing the whole set of up-regulated and down-regulated genes (Cordero et al., 2016). Notwithstanding these challenges, the RT-qPCR is a very sensitive method that is amenable to analyses involving a small set of genes as was the case in this study.

8.1.9 *Growth conditions for the revival of persister survivors and choice of stress response genes*

Growth conditions for the revival of persister survivors were chosen to represent the conditions that the pathogen likely encounters in contaminated foods. In many RTE foods, low pH and a_w often combined with refrigeration are common hurdles for the control of *L. monocytogenes* growth. Hence persister survivors were revived under mildly acidic conditions and under also conditions of slightly abusive temperature (10ºC). The choice of stress response genes was based on literature evidence that the expression levels of such genes in *L. monocytogenes* can serve as stress resistance biomarkers (Mataragas et al., 2015c).

8.2 RESEARCH FINDINGS

In this study, the influences of *L. monocytogenes* stress responses on three main aspects that relate to food safety were examined. These three aspects were the influence on; cell injury and resuscitation; persistence and persister cell formation; and population dynamics in an RTE food product. These aspects are discussed separately in the following parts of this discussion.

8.2.1 *Influence of strain heterogeneity and stress variability on cell injury response and resuscitation kinetics of injured cells*

This study (in Chapter 4), found varying responses among *L. monocytogenes* strains in terms of the extent of cell injury upon exposure to acid, osmotic and heat stresses and also in terms of the resuscitation kinetics of sorted injured cells. Variations among strains are attributable to genetic heterogeneity within the species, which is a driving force in the adaptive evolution of the organism (Lianou and Koutsoumanis, 2013). Several studies have cited strain variability as a key factor influencing the differences in inactivation responses of *L. monocytogenes* to stresses such as acid and heat (Arguedas-Villa et al., 2014; Lianou et al., 2006; Rodríguez et al., 2016). The implication of such variability is that the response behavior of one strain cannot be extended to other strains (Lianou and Koutsoumanis, 2013). Another important finding was that the extent of cell injury varied significantly among the three forms of stress, with acid stress causing the highest loss of cell integrity and osmotic stress causing the least. Different forms of stress affect different targets on the bacterial cell, hence the extent of cell injury would be expected to differ. Acid stress, in particular, is known to affect the trans-membrane electrochemical gradient that leads to a loss of membrane potential (Alakomi et al., 2000), while osmotic stress impacts on the osmotic potential and cell turgor (Wood, 1999). Considering that the method of assessing cell injury in this study was the uptake of the membrane-impermeant dye PI which reflects a loss of membrane integrity, stress treatments that impact membrane permeability would result in a higher degree of cell injury than treatments that affect other cellular targets. In addition to its direct effects on the trans-membrane potential, acid stress has been proven to cause permeabilization of bacterial cells (Alakomi et al., 2000) thus it would result in higher uptake of

PI and higher percentage injury. On the other hand, the cellular targets for heat stress include enzymes and nucleic acids with membrane integrity loss often occurring after (Kramer and Thielmann, 2016). This implies that the extent of cell injury arising from heat stress was potentially underestimated in this study as most of the treated cells were observed as membrane intact. This underestimation of cell injury to heat was also manifested as a complete lack of growth for sorted cells suggesting that although these cells were considered viable in terms of membrane integrity indicators, they had in fact lost viability.

When injured cells were FACS sorted and resuscitated in BHI broth, differences (related to strain and stress variabilities) were observed only in the lag phase duration. The lag phase duration is the time it takes for an organism to commence exponential growth following an environmental transition (Swinnen et al., 2004). In the context of injured cells, it is an indicator of the period of repair. What was discernible from these findings was that variability in the extent of cell injury attributable to strain and stress factors influence the period of injury repair, but once the cell damage has been repaired, the growth rates of the strains are the same. Thus the key factor with regards to the food safety implications of injured cells is their ability to repair. However, it has to be noted that the resuscitation of the injured cells in this study was done in BHI broth which makes it difficult to make extrapolations about the fate of such cells in a food system. Although injured cells are known to be sensitive to many inhibitory agents that non-injured cells are able to tolerate (Wesche et al., 2009), and are expected to be sensitive to the sub-lethal stress hurdles used in preservation of RTE foods, it is still necessary to be able to predict the fate of such cells in foods especially for RTE foods that are at risk of *L. monocytogenes* growth.

8.2.2 Trade-offs between stress tolerance and growth ability possibly explain the differences in stress susceptibility and growth ability of L. monocytogenes strains

A striking finding of this study was the consistent lack of culturability on selective agar for strain 69 after all stress treatments, an observation that was inconsistent with the high level of membrane intact (TO-positive/PI-negative) cells. Consequently, this resulted in a big gap in the extent of cell injury as measured by flow cytometry and differential plating. What was discerned from this, and therefore was used throughout this thesis was that this strain is intrinsically susceptible to stress. Although most of the cells were deemed membrane intact on the basis of impermeability to PI, the susceptibility to selective agents used in PALCAM agar suggested that these cells become more sensitive to inhibitory agents once they are stressed. Moreover, the same strain exhibited the shortest period of repair for its injured cells following cell sorting and was the only strain capable of resuscitation after heat injury. What was deduced from the response of this strain was that, while stressed cells of this organism have an increased sensitivity to inhibitory agents the same cells have a high growth ability when growth conditions permit. The possible explanation for this behavior lies in the theory of self-preservation and nutritional competence (SPANC) (Ferenci, 2005). This theory, based on the trade-offs between stress resistance and growth ability is an adaptational tool in living organisms that underpins intra-species diversity (Ferenci, 2016). This balance means that the more stress tolerant an organism is, the less efficient it is in nutrient utilization. In *E. coli*, the control of this balance is achieved through the regulation of levels of the general stress response, RpoS, and the stringent response regulator ppGpp (Ferenci, 2005). Strain-specific variations in the levels of RpoS and ppGpp have been established in *E. coli* (Ferenci et al., 2011). In *L. monocytogenes*, no available information is yet available on the regulation of stress resistance and growth ability trade-offs. However,

based on evidence from studies on *E. coli*, it is likely that the low-stress tolerance of *L. monocytogenes* 69 and its quick resuscitation in BHI broth could be related to low levels of the general stress response regulator (sigB).

The differences in stress susceptibility and growth ability of *L. monocytogenes* strains have immense implications on food safety. On exposure to sublethal stresses hurdles, strains that possess better stress susceptibilities, survive preservation hurdles as process survivors (Yousef and Courtney, 2003) whose potential deleterious effects include subsequent contamination of finished products. On the other hand, while susceptible strains might not survive food processing hurdles, the ability of low numbers of such cells contaminating foods will also impose serious consequences especially for RTE foods that are capable of supporting *L. monocytogenes* growth. The differences in stress susceptibilities of strains also have implications with respect to survival and strain competition in contaminated foods. Although the lineage and serotype of these strains were unknown, the differences draw parallels with the known fact that some *L. monocytogenes* strains belonging to lineage II are better competitors in the food environment than other lineages (Orsi and Wiedmann, 2016).

8.2.3 *Individual strain susceptibility and stress tolerance responses influence population dynamics in lactic soft cheese*

Apart from the potential food safety impacts of injured cells (the focus of Chapter 4), a lot of cells were observed to survive the stress exposures as non-injured cells (Figure 4.2). Because of the stress exposure, the non-injured cell fraction potentially consists of stress hardened tolerant cells (Yousef and Courtney, 2003). Hence the interest in Chapter 5 was on how such cells behave when they potentially contaminated a model RTE food. What was established from this study

was that while stress exposure reduces the inactivation rate for some strains, the opposite occurs in other strains. This is potentially attributed to variability in the intrinsic stress resistance of individual strains. The quick inactivation of strain 69 with upward concave inactivation curves (Figures 5.1 - 5.3) was consistent with its intrinsic susceptibility to stress discussed earlier. The already stressed cells are sensitized to the subsequent exposure to the food stress hence accelerating their death (Barker and Park, 2001). The physiological basis of this sensitization stems from the extra homeostatic burden that such already stressed cells have to cope with when subjected to the subsequent food stress (Skandamis et al., 2008). For the other two strains, however, the development of stress tolerance responses appear to be the cause of the enhanced survival in the soft cheese. Stress tolerance responses are adaptive responses that protect an organism against future exposures of the same or different stress factors (Brauner et al., 2016). The development of stress tolerance response imposes the biggest threat to food safety as it renders food preservation hurdles ineffective (Yousef and Courtney, 2003). Also of concern was the observation of osmotic stress-treated cells were also tolerant to the food stress which is predominantly based on an acid stress hurdle implying a cross-tolerance between osmotic and acid stress (Alvarez-Ordóñez et al., 2015). The practical implication of these findings is that, for a pathogen like *L. monocytogenes*, whose prominence in RTE foods is a result of the lack of lethal hurdles post-processing, stress tolerance responses have a potential for enhanced survival in foods and for an RTE food that does not support *L. monocytogenes* growth like a lactic soft cheese, contamination of the product with higher than the safety threshold (10^2 CFUg^{-1}) levels of the pathogen would result in sustained survival of the pathogen for the duration of the product shelf-life.

As explained earlier that the implications of strain heterogeneity means that responses of *L. monocytogenes* cannot be extrapolated from one strain (Lianou and Koutsoumanis, 2013). It is often argued that the challenge of strain heterogeneity in predicting pathogen responses in foods can be overcome by using a cocktail of mixed strains in challenge tests (Beaufort, 2011). However, because of the potential for strain competition, the measured data essentially derives from the most dominant strain thus underestimating the behavior of individual strains whose survival potential in the absence of strain competition could be much higher (Lianou and Koutsoumanis, 2013). What was established from the mixed strain challenge tests was that strain 159/10 out-lived all strains and was the only survivor after 15 days of cheese storage. It has already been established that competition among *L. monocytogenes* in co-cultures can cause the suppression of less competitive strains by the dominant strain (Zilelidou et al., 2016b, 2016a). The underlying mechanisms for these observations, however, have yet to be explained. What makes it even difficult to explain the survival of strain 159/10 over the other strains is that most strain competition studies so far have been done in growth experiments, while the conditions of this study involved inactivating conditions. According to a hypothesis, posited by Cornforth and Foster (2013), there is a relationship between microbial competition and stress response. In their arguments, the authors suggest that several of the major bacterial stress responses detect ecological competition by sensing cell damage caused by interfering microflora. The support for this hypothesis is that microorganisms secrete inhibitory substances such as antibiotics and bacteriocins only in response to biotic stress.

8.2.4 *Acid stress results in disruption of protein structure, nucleic acid conformations, and cellular polysaccharides*

Based on findings from FT-IR analysis, it can be concluded that the effects of acid stress on *L. monocytogenes* cells involve disruptions of protein secondary structure, nucleic acid conformations, and cellular polysaccharides. The mechanism of antimicrobial action of acids probably explains these biomolecular changes. The disruption of the internal pH of the cells due to the influx of H^+ protons causes the protonation of essential functional groups in biomolecules (Krulwich et al., 2011). The change in the ionic states affects the stability of many biomolecules with potential effects on proteins, for example, causing loss of normal conformation and loss of biological activity. Based on the comparison of the spectral changes of *L. monocytogenes* 69 and 159/10 whose phenotypic susceptibilities to stress were contrastingly different, the main distinguishing feature in their responses to acid stress were changes in acyl chains of membrane lipids. Changes in acyl chains of membrane lipids influence membrane fluidity (Yoon et al., 2015). It is therefore suggested that membrane composition in strain 69 that likely influence an increase in fluidity when stressed is key to its intrinsic susceptibility to stress. Some stresses such as low temperature and alkaline stress have been associated with increased fluidity in *L. monocytogenes* (Alonso-Hernando et al., 2010; Annous et al., 1997). It was, however, interesting that while stress treatment seemed to cause a change in membrane fluidity in this strain, results from flow cytometry analysis (Chapter 4) seemed to show that this did not result in an increased uptake of PI by stressed cells.

8.2.5 *Persister cell formation facilitates* L. monocytogenes *persistence and survival of lethal acid stress*

In Chapter 7 of this thesis, the focus was to understand some of the underlying facts behind *L. monocytogenes* persistence in foods and food processing environments. The ability of *L. monocytogenes* to survive for prolonged periods of time in foods and habitats associated with food processing is well acknowledged as key food safety factor with regards to listeriosis outbreaks (Larsen et al., 2014). Remarkably, the factors commonly used to explain *L. monocytogenes* persistence in the context of food processing environments such as resistance to disinfectants and formation of biofilms have not fully accounted for this phenomenon (Carpentier and Cerf, 2011). The findings of this study indicated that a subpopulation of persistent *L. monocytogenes* cells survives high levels of lethal acid stress. Importantly, the susceptibility of representatives of such stress survivors when re-subjected to the acid treatment indicated a lack of acquired resistance. What could be deciphered from this was that most of the stress survivors of *L. monocytogenes* are phenotypic variants of the original population. Unlike the development of resistance which is genetically mediated and therefore heritable, phenotypic variants result from a transient state of tolerance to lethal stress (Brauner et al., 2016).

Based on studies with *E. coli*, the theory accounting for the development of persister cells is that these are cells that avoid the effect of stress by transition into a dormant, metabolically inactive state (Wood et al., 2013). Consistent with this theory, the acid stress survivors in this study showed a lower enzymatic activity compared to actively growing control cells. In terms of the dormancy theory, the switch from an actively growing to a dormant state arises from variations in gene expression at individual cell level with the attendant effect of producing phenotypically

different subpopulations in a genetically identical cell population (Maisonneuve and Gerdes, 2014). At the core of the phenotypic switch is variations in the expression levels of the toxin-antitoxin (TA) operon of individual cells (Balaban, 2011). The TA operon codes for a protein toxin that inhibits cell growth and an RNA or protein antitoxin that regulates the activity of the toxin (Radzikowski et al., 2017). Cells with higher than threshold expression of the toxin are associated with cessation of growth which underlies the persister cell state (Radzikowski et al., 2017). In steady state log phase cultures, a very small subpopulation of cells may present a dormant state, an indication that the regulation of expression of TA operons is stochastic (Radzikowski et al., 2016). However, under conditions of stress, regulatory genes with pleiotropic effects such as the general stress response regulator *sigB* and the stringent response regulator guanosine tetraphosphate (ppGpp) can mediate a switch on of the TA operon leading to enhanced persister cell formation (Amato et al., 2013; Radzikowski et al., 2017). It has been argued that the ecological significance of bacterial cells entering a dormant state is a bet-hedging strategy in which dormant cells formed under conditions of environmental stress act as a seed bank for future revival of the population when conditions become favorable (Lennon and Jones, 2011). This theory of phenotypic heterogeneity that leads to persister cell formation appears suitable for explaining the persistence of *L. monocytogenes* in foods and processing environments given that persistent *L. monocytogenes* strains survive in these environments despite the routine use of inhibitory and lethal concentrations of disinfectants, without any evidence of acquired resistance (Carpentier and Cerf, 2011).

Although surviving cells could not be separated from dead cells the findings of this study indicated at least that lethal acid stress treatment is accompanied by the formation of

extracellular vesicles. Extracellular membrane vesicles have been identified and extensively studied as a secretory system in Gram-negative bacteria emanating from the outer membrane (Kulp and Kuehn, 2010). In Gram-positive bacteria, the formation of such vesicles has not received much attention as the absence of the outer membrane and the presence of a thick cell wall has been thought to preclude their formation (Brown et al., 2015). However recent studies have since identified the occurrence of such vesicles in some Gram-positive bacteria (Lee et al., 2009; Rivera et al., 2010). Based on profiling of their composition, it is now established that as a secretory system, extracellular membrane vesicles contain a variety of effector proteins that enable bacterial cells to exploit environments beyond their immediate vicinity without having to move around (Kulp and Kuehn, 2010). In the context of *L. monocytogenes* stress survival, it could be reasoned that the formation of these vesicles is a potential response mechanism accompanying stress survival. It is possible that these vesicles could play a part when surviving persisters emerge from their state of growth arrest.

8.2.6 *Gene expression profiles of persister survivors upon growth resumption*

The fact that cells re-grown from persister survivors of acid stress were not different from the parental generation in terms the relative expression profiles of most of the stress response genes studied (*sigB*, *gadD2*, *oppA*, and *lmo1722*) is a reflection of a lack of heritable stress resistance. This conclusion is based on the fact that stress resistance results from heritable mutations that confer the resistance characteristic to future generations (Brauner et al., 2016). Given that the persister survivors were revived under conditions of mild acid and cold stress, a development of resistance would have resulted in increased expression levels of the acid stress response gene *gadD2* as well as the cold stress response genes *oppA* and *lmo1722* in addition to the general

stress response gene *sigB*. One previous study has shown that acid stress resistant variants of *L. monocytogenes* carrying mutations in the *rpsU* gene, encoding ribosomal protein S21 have elevated levels of glutamate decarboxylase, a product of the *gadDT* operon (Metselaar et al., 2015). The implication of these findings is to suggest that the persister cells as a long-term survival states of the pathogen do not possess any growth advantage compared to the parental population when they resume growth.

Notwithstanding the fact that most of the genes analyzed showed a lack of heritable resistance, this study also found that cells re-grown from persister survivors had reduced relative expression levels of PTS enzyme II encoding *lmo1038* gene. It is difficult to discern how survivors that showed a lack of heritable resistance could have a different expression profile from their parental cells. The potential explanation for this lies in the possibility that the switch that results in growth arrest and formation of persister cells is accompanied by a down-regulation of PTS genes. This reasoning is supported by previous observations that expression of PTS genes is down-regulated in response to stress (Bae et al., 2012) and also by the fact that persister cell development is itself based on a reduction of metabolic activity and growth arrest (Amato et al., 2013). It has been argued that some phenotypic traits such as gene expression levels can be transmitted from parent to offspring through epigenetic means (Day, 2016; Gefen and Balaban, 2009). Based on this theory, it would imply that the down-regulation of *lmo1038* in cells re-grown from persister survivors is a characteristic inherited from the dormant state. For instance, regulators such as ppGpp that are associated with the dormant state of persisters can be passed on to generations of revived cells at higher than normal levels (Korch et al., 2003). can reasonably result in variations in expression of genes for nutrient uptake. The potential for such regulators

being transferred to revived cell generations could result in variations in the transcription of genes for nutrient uptake.

It should be emphasized, however, that the expression profiles of bacterial cells are complex and subject to control by many regulatory networks. More comprehensive approaches based on single-cell analysis techniques are necessary to decipher the changes in persister cells as they transition back to actively dividing cells as well as their subsequent generations. Notwithstanding these limitations, the findings of this study make it possible to conclude in a broad sense, if the persister cell state contributes to *L. monocytogenes* persistence and if such survivors of extreme environmental stress possess any growth advantage when they resume growth under conditions pertinent to RTE foods.

The food safety implications of *L. monocytogenes* persister cell formation are potentially significant. It is not surprising that one of the questions recently posed by Buchanan et al. (2017) in a review of the outcomes of a workshop of experts hosted by the Joint Institute of Food Safety and Applied Nutrition (JIFSAN) in Maryland, USA in 2015, whose purpose was to evaluate the latest information on risk factors of *L. monocytogenes* was the extent to which persister cells are a factor in the pathogen's survival in foods. This study provides some attempt to decipher the basis of *L. monocytogenes* persistence under food relevant conditions and what the findings showed was that, although persister cells have not been given much attention, as a cause of *L. monocytogenes* persistence, such cells are likely the main long-term survival states of the pathogen in food processing environments. What is also concerning from the findings of the study is the extent to which such cells can survive lethal stress implying that their eradication

from food processing environments could be potentially difficult. The positive aspect though is that such cells did not show any acquired resistance to mild stress factors used as preservation hurdles implying that RTE foods in the no-growth category should be protected. However, RTE foods that support *L. monocytogenes* growth could be at risk if contaminated.

9 CHAPTER 9: CONCLUSIONS AND RECOMMENDATIONS

This study revealed that flow cytometry and differential plating show differences in assessment of stress-induced cell injury. Based on flow cytometry and fluorescent staining using membrane integrity indicators, this study showed that acid (pH 4.2), heat (55°C for 30 min) and osmotic (10% NaCl solution) stresses result in varying levels of cell injury that is also influenced by *L. monocytogenes* strain heterogeneity. In general, acid stress causes higher levels of cell membrane damage than heat and osmotic stresses an indication that the antimicrobial mechanism of acid stress involves membrane damage more than the other stress factors. When injured cells are FACS sorted into BHI broth, only acid and osmotic stress injured are capable of re-growth an indication that while membrane integrity is a good indicator of cell injury and viability loss due to acid and osmotic stress, it is not a sufficient indicator of heat stress-induced injury. Based on analysis of resuscitation kinetics of sorted cells at 37 and 25°C, the duration of the lag phase, is the main difference in the resuscitation behavior of injured cells indicating that repair is dependent on the extent of injury determined by the form of stress and strain variabilities. Once injured cells repair the cellular damage, their growth rate is not different from non-injured cells regardless of the form of stress and strain differences and resuscitation conditions. This study also found that low temperature (representative of refrigerated storage conditions) cannot prevent resuscitation of injured *L. monocytogenes* cells. Also revealed by this study was that stress-susceptible strains (that lack growth on selective media after stress exposure) have the shortest repair period after cell sorting indicating a potential trade-off between stress tolerance and growth ability in *L. monocytogenes*.

This study also revealed that the inactivation kinetics of *L. monocytogenes* strains in lactic soft cheese (a low pH based RTE food) are influenced by individual strain susceptibilities to acid and osmotic stress pre-exposures. In stress-susceptible strains (based on the response of strain 69), acid and osmotic stress pre-exposures result in enhanced inactivation of the pathogen in lactic soft cheese an indication that stress exposures induce cell injury that sensitizes the susceptible cells to the acidity of the food. In contrast, the same exposures result in enhanced survival for tolerant strains an indication that stress exposures also result in the induction of tolerance responses that protect the tolerant cells against the acidity of the soft cheese. While both acid and osmotic stress exposures cause the development of tolerance responses, the acid tolerance response imposes a better protection of *L. monocytogenes* against the acidity of the soft cheese. Based on genetic profiling of surviving populations from a mixed strain challenge, this study shows that one persistent *L. monocytogenes* strain out-lives and dominates the microflora after 15 days of cheese storage an indication that the measured inactivation kinetics is potentially attributable to one longest surviving and persistent strain. This may have implications with regards the quantification of the responses of the pathogen in RTE foods. As could be established from this study, more cautious estimates for prediction of *L. monocytogenes* responses in foods can be obtained from the inactivation kinetics data of hardened cells pre-exposed to stress and also from persistent strains that survive the longest in contaminated foods.

With regards to cellular targets of acid stress, this study shows that in both stress-susceptible and tolerant strains, disruptions of protein secondary structure, nucleic acid conformations, and cellular polysaccharides occur as shown by FT-IR spectral analysis. The distinguishing feature of

the acid stress response of the susceptible, strain 69 and tolerant strains appears to be in the cell membrane lipids with disruptions of lipid acyl chains showing the most significant differences. These stress-induced changes in membrane lipids are probably related to the phenotypic susceptibility this strain. Along with the biomolecular effects, the formation of extracellular membrane vesicles is part of the acid stress response of susceptible strains.

When *L. monocytogenes* cells are challenged with lethal lactic acid treatment, a small fraction of highly tolerant persistent cells survives the treatment. The surviving population has no acquired acid resistance and owes its tolerance to a metabolically inactive state characteristic of persister cells. Moreover, the transition into the persister cell appears to be accompanied by the formation of extracellular membrane vesicles probably as an active response in aid of metabolic inactivity. When the persister survivors are revived under conditions of mild acid and cold conditions, their relative expression levels of some stress response genes are not different from their control parental generation an indication of a possible lack of heritable genetic differences. Notwithstanding the general lack of heritable genetic differences, revived persister survivors show a significantly lower level of expression of the PTS system coding gene *lmo1038*. With reduced nutrient uptake being a feature of persistence, it is probable that a down-regulation of PTS systems is part of the persister cell state and that there is a potential carry-over of such expression patterns in revived cells through epigenetic means.

Even though this study provided some valuable information on the possible behavior of stress-injured and persister cells, there is also a need for a more expanded focus on the potential for resuscitation and growth of such cells in different RTE foods especially those that have the

potential to support *L. monocytogenes* growth. More detailed studies on the physiology of these stress-induced cell states can help to decipher and close some of the knowledge gaps with regards to the contributions of such cells in food safety. To that extent, the application of high throughput techniques such as whole genome sequencing, pan-genome analysis, RNA microarrays, proteomic and cell sorting approaches should be valuable in understanding the physiology and genetic basis of *L. monocytogenes* survival and persistence. Over and above the physiology and genetics of stress injured and persister cells, there is a need to carry out detailed phenotypic characterizations with respect to growth behavior in RTE foods under different conditions. This is particularly recommended because the conclusions on the behavior of both injured and persister cells in this study were based on growth in broth. It is further recommended that studies on these cells should be extended to include the virulence and dose-response characteristics. and Such studies should ultimately lead to the development of new models for use quantitative risk assessment.

10 CHAPTER 10: REFERENCES

Abee, T., Koomen, J., Metselaar, K.I., Zwietering, M.H., den Besten, H.M.W., 2016. Impact of pathogen population heterogeneity and stress-resistant variants on food safety. Annu. Rev. Food Sci. Technol. 7, 439–456.

Abram, F., Starr, E., Karatzas, K.A.G., Matlawska-Wasowska, K., Boyd, A., Wiedmann, M., Boor, K.J., Connally, D., O'Byrne, C.P., 2008. Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. Appl. Environ. Microbiol. 74, 6848–6858.

Adrião, A., Vieira, M., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R.P., Chambel, L., Barata, B., Zilhao, I., Shama, G., Perni, S., Jordan, S.J., Andrew, P.W., Faleiro, M.L., 2008. Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. Int. J. Food Microbiol. 123, 142–150.

Akanuma, G., Nanamiya, H., Natori, Y., Yano, K., Suzuki, S., Omata, S., Ishizuka, M., Sekine, Y., Kawamura, F., 2012. Inactivation of ribosomal protein genes in *Bacillus subtilis* reveals importance of each ribosomal protein for cell proliferation and cell differentiation. J. Bacteriol. 194, 6282–6291.

Al-Qadiri, H.M., Lin, M., Al-Holy, M.A., Cavinato, A.G., Rasco, B.A., 2008. Detection of sublethal thermal injury in *Salmonella enterica* serotype Typhimurium and *Listeria monocytogenes* using Fourier Transform Infrared (FT-IR) spectroscopy (4000 to 600 cm-1). J. Food Sci. 73, M54–M61.

Alakomi, H.L., Skyttä, E., Saarela, M., Mattila-Sandhlm, T., Latva-Kala, K., Helander, I.M., 2000. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. 66, 2001–2005.

Albert, I., Mafart, P., 2005. A modified Weibull model for bacterial inactivation. Int. J. Food Microbiol. 100, 197–211.

Almeida, G., Magalhães, R., Carneiro, L., Santos, I., Silva, J., Ferreira, V., Hogg, T., Teixeira, P., 2013. Foci of contamination of *Listeria monocytogenes* in different cheese processing

plants. Int. J. Food Microbiol. 167, 303–309.

Alonso-Hernando, Alicia, Alonso-Calleja, C., Capita, R., 2010. Effects of exposure to poultry chemical decontaminants on the membrane fluidity of *Listeria monocytogenes* and *Salmonella* enterica strains. Int. J. Food Microbiol. 137, 130–136.

Alsharif, R., Godfrey, W., 2002. Bacterial detection and Live/Dead discrimination by flow cytometry, Microbial Cytometry Application Note. BD Biosciences, Immunocytometry Systems; San Jose, CA.

Alsharif, R., Godfrey, W., 2001. Bacterial disinfectant efficacy using flow cytometry. Microb. Cytom. Appl. Note. BD Biosci. Immunocytometry Syst. San Jose, CA.

Alvarez-Ordóñez, A., Broussolle, V., Colin, P., Nguyen-The, C., Prieto, M., 2015. The adaptive response of bacterial food-borne pathogens in the environment, host and food: Implications for food safety. Int. J. Food Microbiol. 213, 99–109.

Alvarez-Ordóñez, A., Halisch, J., Prieto, M., 2010. Changes in Fourier transform infrared spectra of *Salmonella enterica* serovars Typhimurium and Enteritidis after adaptation to stressful growth conditions. Int. J. Food Microbiol. 142, 97–105.

Alvarez-Ordóñez, A., Mouwen, D.J.M., López, M., Prieto, M., 2011. Fourier transform infrared spectroscopy as a tool to characterize molecular composition and stress response in foodborne pathogenic bacteria. J. Microbiol. Methods 84, 369–378.

Alvarez-Ordóñez, A., Prieto, M., 2010. Changes in ultrastructure and Fourier Transform Infrared spectrum of *Salmonella enterica* serovar Typhimurium cells after exposure to stress conditions. Appl. Environ. Microbiol. 76, 7598–7607.

Amato, S.M., Fazen, C.H., Henry, T.C., Mok, W.W.K., Orman, M.A., Sandvik, E.L., Volzing, K.G., Brynildsen, M.P., 2014. The role of metabolism in bacterial persistence. Front. Microbiol. 5.

Amato, S.M., Orman, M. a, Brynildsen, M.P., 2013. Metabolic control of persister formation in *Escherichia coli*. Mol. Cell 50, 475–487.

Amor, K. Ben, Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D.L., De Vos, W.M., Abee, T., 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. Appl. Environ. Microbiol. 68, 5209–5216.

Angelidis, A.S., Papageorgiou, D.K., Tyrovouzis, N.A., Stoforos, N.G., 2013. Kinetics of *Listeria monocytogenes* cell reduction in processed cheese during storage. Food Control 29, 18–21.

Angelidis, A.S., Smith, G.M., 2003. Three transporters mediate uptake of glycine betaine and carnitine by *Listeria monocytogenes* in response to hyperosmotic stress. Appl. Environ. Microbiol. 69, 1013–1022.

Angelo, K.M., Conrad, A.R., Saupe, A., Dragoo, H., West, N., Sorenson, A., Barnes, A., Doyle, M., Beal, J., Jackson, K.A., Stroika, S., Tarr, C., Kucerova, Z., Lance, S., Hould, L.H., Wise, M., Jackson, B.R., 2017. Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014-2015. Epidemiol. Infect. 145, 848–856.

Annous, B.A., Becker, L.A., Bayles, D.O., Labeda, D.P., Wilkinson, B.J., 1997. Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. Appl. Environ. Microbiol. 63, 3887–3894.

Argueda-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. Food Microbiol. 27, 653–660.

Arguedas-Villa, C., Kovacevic, J., Allen, K.J., Stephan, R., Tasara, T., 2014. Cold growth behaviour and genetic comparison of Canadian and Swiss *Listeria monocytogenes* strains associated with the food supply chain and human listeriosis cases. Food Microbiol. 40, 81–87.

Arku, B., Fanning, S., Jordan, K., 2011. Flow cytometry to assess biochemical pathways in heat-stressed *Cronobacter* spp. (formerly *Enterobacter sakazakii*). J. Appl. Microbiol. 111, 616–624.

Aryani, D.C., de Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015. Quantifying variability on thermal resistance of *Listeria monocytogenes*. Int. J. Food Microbiol. 193, 130–138.

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015. Quantifying strain variability in modeling growth of Listeria monocytogenes. Int. J. Food Microbiol. 208, 19–29. doi:10.1016/j.ijfoodmicro.2015.05.006

Aspridou, Z., Koutsoumanis, K.P., 2015. Individual cell heterogeneity as variability source in population dynamics of microbial inactivation. Food Microbiol. 45, 216–221.

Augustin, J.C., Rosso, L., Carlier, V., 2000. A model describing the effect of temperature history on lag time for *Listeria monocytogenes*. Int. J. Food Microbiol. 57, 169–181.

Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, D., Marchiabo, G., Novara, O., Leone, L., Salmaso, S., 2000. An outbreak of febrile gastroenteritis associated with corn contaminated with *Listeria monocytogenes*. N. Engl. J. Med. 342, 1236–1241.

Bae, D., Liu, C., Zhang, T., Jones, M., Peterson, S.N., Wang, C., 2012. Global gene expression of *Listeria monocytogenes* to salt stress. J. Food Prot. 75, 906–912. doi:10.4315/0362-028X.JFP-11-282

Balaban, N.Q., 2011. Persistence: Mechanisms for triggering and enhancing phenotypic variability. Curr. Opin. Genet. Dev. 21, 768–775.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., Leibler, S., 2004. Bacterial persistence as a phenotypic switch. Science (80-.). 305, 1622–1625.

Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277–294.

Bäreclev, C., Vaitkevicius, K., Netterling, S., Johansson, J., 2014. DExD-box RNA-helicases in *Listeria monocytogenes* are important for growth, ribosomal maturation, rRNA processing and virulence factor expression. RNA Biol. 11, 1457–1466.

Barker, C., Park, S.F., 2001. Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. Appl. Environ. Microbiol. 67, 1594–1600.

Bayles, D.O., Annous, B.A., Wilkinson, B.J., 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. Environ. Microbiol. 62, 1116–1119.

Bayles, D.O., Wilkinson, B.J., 2000. Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. Lett. Appl. Microbiol. 30, 23–27.

Beaufort, A., 2011. The determination of ready-to-eat foods into *Listeria monocytogenes* growth and no growth categories by challenge tests. Food Control 22, 1498–1502.

Bergholz, T.M., de Bakker, H.C., Fortes, E.D., Boor, K.J., Wiedmann, M., 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. Foodborne

Pathog. Dis. 7, 1537–1549.

Beumer, R.R., Te Giffel, M.C., Cox, L.J., Rombouts, F.M., Abee, T., 1994. Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal growth medium. Appl. Environ. Microbiol. 60, 1359–1363.

Bhagat, A.A.S., Bow, H., Hou, H.W., Tan, S.J., Han, J., Lim, C.T., 2010. Microfluidics for cell separation. Med. Biol. Eng. Comput. 48, 999–1014.

Bishop, A.L., Rab, F.A., Sumner, E.R., Avery, S. V, 2007. Phenotypic heterogeneity can enhance rare-cell survival in "stress-sensitive" yeast populations. Mol. Microbiol. 63, 507–520.

Bolocan, A.S., Oniciuc, E.A., Alvarez-Ordóñez, A., Wagner, M., Rychli, K., Jordan, K., Nicolau, A.I., 2015. Putative cross-contamination routes of *Listeria monocytogenes* in a meat processing facility in Romania. J. Food Prot. 78, 1664–1674.

Bonsaglia, E.C.R., Silva, N.C.C., Júnior, A.F., Júnior, J.P.A., Tsunemi, M.H., Rall, V.L.M., 2014. Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. Food Control 35, 386–391.

Booth, I.R., 2002. Stress and the single cell: Intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. Int. J. Food Microbiol. 78, 19–30.

Brauner, A., Fridman, O., Gefen, O., Balaban, N.Q., 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat. Rev. Microbiol. 14, 320–330.

Bridier, A., Hammes, F., Canette, A., Bouchez, T., Briandet, R., 2015. Fluorescence-based tools for single-cell approaches in food microbiology. Int. J. Food Microbiol. 213, 2–16.

Brown, L., Wolf, J.M., Prados-Rosales, R., Casadevall, A., 2015. Through the wall: Extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat. Rev. Microbiol. 13, 620–630.

Brul, S., Coote, P., 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. Int. J. Food Microbiol. 50, 1–17.

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., Whiting, R.C., 2017. A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Control 75, 1–13.

Burall, L.S., Grim, C.J., Datta, A.R., 2017. A clade of Listeria monocytogenes</i> serotype 4b

variant strains linked to recent listeriosis outbreaks associated with produce from a defined geographic region in the US. PLoS One 12, e0176912.

Burgess, C.M., Gianotti, A., Gruzdev, N., Holah, J., Knøchel, S., Lehner, A., Margas, E., Esser, S.S., Sela Saldinger, S., Tresse, O., 2016. The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. Int. J. Food Microbiol. 221, 37–53.

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

Cabrita, P., Ferreira, R.B., Brito, L., 2015. Differences in the expression of cold stress-related genes and in the swarming motility among persistent and sporadic strains of *Listeria monocytogenes*. Foodborne Pathog. Dis. 12, 576–584.

Caggia, C., Scifo, G.O., Restuccia, C., Randazzo, C.L., 2009. Growth of acid-adapted *Listeria monocytogenes* in orange juice and in minimally processed orange slices. Food Control 20, 59–66.

Carpentier, B., Cerf, O., 2011. Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int. J. Food Microbiol. 145, 1–8.

Casadesús, J., Low, D.A., 2013. Programmed heterogeneity: Epigenetic mechanisms in bacteria. J. Biol. Chem. 288, 13929–13935.

Castro, H., Jaakkonen, A., Hakkinen, M., Korkeala, H., Lindström, M., 2018. Longitudinal study of the occurrence, persistence and contamination routes of *Listeria monocytogenes* genotypes on three Finnish dairy cattle farms. Appl. Environ. Microbiol. 84, e02000-17.

Cataldo, G., Conte, M.P., Chiarini, F., Seganti, L., Ammendolia, M.G., Superti, F., 2007. Acid adaptation and survival of Listeria monocytogenes in Italian-style soft cheeses. J. Appl. Microbiol. 103, 185–193.

Centre for Disease Control and Prevention (CDC), 2015a. Multistate utbreak of listeriosis linked to Blue Bell Creameries products (Final Update). https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html (accessed 12.15.16).

Centre for Disease Control and Prevention (CDC), 2015b. Wholesome Soy Products, Inc. Sprouts and Investigation of Human Listeriosis Cases (Final Update) http://www.cdc.gov/listeria/outbreaks/bean-sprouts-11-14/index.html (accessed 12.15.16).

Centre for Disease Control and Prevention (CDC), 2015c. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from Bidart Bros.Apples (Final Update) https://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html (accessed 12.10.16).

Cerf, 0, 1977. Tailing of survival curves of bacterial spores. J. Appl. Bacteriol. 42, 1–19.

Cerutti, F., Mallet, L., Painset, A., Hoede, C., Moisan, A., Bécavin, C., Duval, M., Dussurget, O., Cossart, P., Gaspin, C., Chiapello, H., 2017. Unraveling the evolution and coevolution of small regulatory RNAs and coding genes in *Listeria*. BMC Genomics 18, 882.

Cetin, M.S., Zhang, C., Hutkins, R.W., Benson, A.K., 2004. Regulation of transcription of compatible solute transporters by the general stress sigma factor, σB, in *Listeria monocytogenes*. J. Bacteriol. 186, 794–802.

Chan, Y.C., Wiedmann, M., 2008. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. Crit. Rev. Food Sci. Nutr. 49, 237–253.

Chaturongakul, S., Boor, K.J., 2006. σB activation under environmental and energy stress conditions in *Listeria monocytogenes*. Appl. Environ. Microbiol. 72, 5197–5203.

Chaturongakul, S., Boor, K.J., 2004. RsbT and RsbV contribute to σB-dependent survival under environmental, energy, and intracellular stress conditions in *Listeria monocytogenes*. Appl. Environ. Microbiol. 70, 5349–5356.

Cheng, Y., Siletzky, R.M., Kathariou, S., 2008. Genomic Divisions/Lineages, Epidemic Clones, and Population Structure, in: Liu, D. (Ed.), Handbook of Listeria Monocytogenes. CRC Press, Boca Raton, pp. 337–358.

Chiara, M., Caruso, M., D'Erchia, A.M., Manzari, C., Fraccalvieri, R., Goffredo, E., Latorre, L., Miccolupo, A., Padalino, I., Santagada, G., Chiocco, D., Pesole, G., Horner, D.S., Parisi, A., 2015. Comparative genomics of *Listeria* Sensu Lato: Genus-wide differences in evolutionary dynamics and the progressive gain of complex, potentially pathogenicity-related traits through lateral gene transfer. Genome Biol. Evol. 7, 2154–2172.

Chilton, P., Isaacs, N.S., Manas, P., Mackey, B.M., 2001. Biosynthetic requirements for the repair of membrane damage in pressure-treated *Escherichia coli*. Int. J. Food Microbiol. 71, 101–104.

Choi, K.-H., Heath, R.J., Rock, C.O., 2000. β-ketoacyl-acyl carrier protein synthase III (FabH) is

a determining factor in branched-chain fatty acid biosynthesis. J. Bacteriol. 182, 365–370.

Chorianopoulos, N., Giaouris, E., Grigoraki, I., Skandamis, P., Nychas, G.J., 2011. Effect of acid tolerance response (ATR) on attachment of *Listeria monocytogenes* Scott A to stainless steel under extended exposure to acid or/and salt stress and resistance of sessile cells to subsequent strong acid challenge. Int. J. Food Microbiol. 145, 400–406.

Chou, C., Wang, C., 2006. Genetic relatedness between *Listeria monocytogenes* isolates from seafood and humans using PFGE and REP-PCR. Int. J. Food Microbiol. 110, 135–148.

Codex Alimentarius Commission (CAC), 2009. Guidelines on the application of general principles of food hygiene to the control of *Listeria monocytogenes* in foods (CAC/GL 61 - 2007). Codex Aliment. Comm. 1–28.

Cole, M.B., Jones, M. V., Holyoak, C., 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J. Appl. Microbiol. 69, 63–72.

Commission, E., 2013. Guidance Document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuff [WWW Document]. URL https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_mc_guidance_document_lysteria.pdf (accessed 11.10.17).

Cordero, N., Maza, F., Navea-Perez, H., Aravena, A., Marquez-Fontt, B., Navarrete, P., Figueroa, G., González, M., Latorre, M., Reyes-Jara, A., 2016. Different transcriptional responses from slow and fast growth rate strains of *Listeria monocytogenes* adapted to low temperature. Front. Microbiol. 7, 229.

Cornforth, D.M., Foster, K.R., 2013. Competition sensing: the social side of bacterial stress responses. Nat. Rev. Microbiol. 11, 285–293.

Costa, A., Bertolotti, L., Brito, L., Civera, T., 2016. Biofilm formation and disinfectant susceptibility of persistent and nonpersistent *Listeria monocytogenes* isolates from Gorgonzola cheese processing plants. Foodborne Pathog. Dis. 13, 602–609.

Cotter, P.D., Gahan, C.G.M., Hill, C., 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol. Microbiol. 40, 465–475.

Cotter, P.D., O'Reilly, K., Hill, C., 2001. Role of the glutamate decarboxylase acid resistance

system in the survival of *Listeria monocytogenes* LO28 in low pH foods. J. Food Prot. 64, 1362–1368.

Cotter, P.D., Ryan, S., Gahan, C.G.M., Hill, C., 2005. Presence of GadD1 glutamate decarboxylase in selected *Listeria monocytogenes* strains is associated with an ability to grow at low pH. Appl. Environ. Microbiol. 71, 2832–2839.

Csonka, L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53, 121–147.

Currie, A., Farber, J.M., Nadon, C., Sharma, D., Whitfield, Y., Gaulin, C., Galanis, E., Bekal, S., Flint, J., Tschetter, L., Pagotto, F., Lee, B., Jamieson, F., Badiani, T., MacDonald, D., Ellis, A., May-Hadford, J., McCormick, R., Savelli, C., Middleton, D., Allen, V., Tremblay, F.-W., MacDougall, L., Hoang, L., Shyng, S., Everett, D., Chui, L., Louie, M., Bangura, H., Levett, P.N., Wilkinson, K., Wylie, J., Reid, J., Major, B., Engel, D., Douey, D., Huszczynski, G., Di Lecci, J., Strazds, J., Rousseau, J., Ma, K., Isaac, L., Sierpinska, U., 2015. Multi-Province Listeriosis outbreak linked to contaminated deli meat consumed primarily in institutional settings, Canada, 2008. Foodborne Pathog. Dis. 12, 645–652.

da Silva, M.F., Ferreira, V., Magalhães, R., Almeida, G., Alves, A., Teixeira, P., 2017. Detection of premature stop codons leading to truncated internalin A among food and clinical strains of *Listeria monocytogenes*. Food Microbiol. 63, 6–11.

da Silva, T.L., Piekova, L., Mileu, J., Roseiro, J.C., 2009. A comparative study using the dual staining flow cytometric protocol applied to *Lactobacillus rhamnosus* and *Bacillus licheniformis* batch cultures. Enzyme Microb. Technol. 45, 134–138.

Dalzini, E., Bernini, V., Bertasi, B., Daminelli, P., Losio, M.-N., Varisco, G., 2016. Survey of prevalence and seasonal variability of *Listeria monocytogenes* in raw cow milk from Northern Italy. Food Control 60, 466–470.

Dalzini, E., Cosciani-Cunico, E., Bernini, V., Bertasi, B., Losio, M.-N., Daminelli, P., Varisco, G., 2015. Behaviour of *Escherichia coli* O157 (VTEC), *Salmonella* Typhimurium and *Listeria monocytogenes* during the manufacture, ripening and shelf life of low fat salami. Food Control 47, 306–311.

Danielsson-Tham, M.-L., Bille, J., Brosch, R., Buchrieser, C., Persson, K., Rocourt, J., Schwarzkopf, A., Tham, W., Ursing, J., 1993. Characterization of *Listeria* strains isolated

from soft cheese. Int. J. Food Microbiol. 18, 161–166.

Davis, M.J., Coote, P.J., O'Byrne, C.P., 1996. Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology 142, 2975–2982.

Davis, R., Mauer, L.J., 2010. Fourier transform infrared (FT-IR) spectroscopy: a rapid tool for detection and analysis of foodborne pathogenic bacteria, in: Méndez-Vilas, A. (Ed.), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. pp. 1582–1594.

Day, T., 2016. Interpreting phenotypic antibiotic tolerance and persister cells as evolution via epigenetic inheritance. Mol. Ecol. 25, 1869–1882.

de las Heras, A., Cain, R.J., Bielecka, M.K., Vázquez-Boland, J.A., 2011. Regulation of *Listeria* virulence: PrfA master and commander. Curr. Opin. Microbiol. 14, 118–127.

den Bakker, H.C., Manuel, C.S., Fortes, E.D., Wiedmann, M., Nightingale, K.K., 2013. Genome sequencing identifies *Listeria fleischmannii* subsp. *coloradonensis* subsp. nov., isolated from a ranch. Int. J. Syst. Evol. Microbiol. 63, 3257–3268.

den Bakker, H.C., Warchocki, S., Wright, E.M., Allred, A.F., Ahlstrom, C., Manuel, C.S., Stasiewicz, M.J., Burrell, A., Roof, S., Strawn, L.K., Fortes, E., Nightingale, K.K., Kephart, D., Wiedmann, M., 2014. *Listeria floridensis* sp. nov., *Listeria aquatica* sp. nov., *Listeria cornellensis* sp. nov., *Listeria riparia* sp. nov. and *Listeria grandensis* sp. nov., from agricultural and natural environments. Int. J. Syst. Evol. Microbiol. 64, 1882–1889.

Deng, X., Phillippy, A.M., Li, Z., Salzberg, S.L., Zhang, W., 2010. Probing the pan-genome of *Listeria monocytogenes*: new insights into intraspecific niche expansion and genomic diversification. BMC Genomics 11, 500.

Department of Health of the Republic of South Africa (DoH), 2018. Listeriosis outbreak situation Report - 26/07/2018. Johannesburg.

Díaz, M., Herrero, M., García, L.A., Quirós, C., 2010. Application of flow cytometry to industrial microbial bioprocesses. Biochem. Eng. J. 48, 385–407.

Dikici, A., Calicioglu, M., 2013. Survival of *Listeria monocytogenes* during production and ripening of traditional Turkish Savak Tulum cheese and in synthetic gastric fluid. J. Food Prot. 76, 1801–1805.

Doijad, S.P., Barbuddhe, S.B., Garg, S., Poharkar, K. V., Kalorey, D.R., Kurkure, N. V., Rawool, D.B., Chakraborty, T., 2015. Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. PLoS One 10, 9.

Doijad, S.P., Poharkar, K. V., Kale, S.B., Kerkar, S., Kalorey, D.R., Kurkure, N. V., Rawool, D.B., Malik, S.V.S., Ahmad, R.Y., Hudel, M., Chaudhari, S.P., Abt, B., Overmann, J., Weigel, M., Hain, T., Barbuddhe, S.B., Chakraborty, T., 2018. *Listeria goaensis* sp. nov. Int. J. Syst. Evol. Microbiol. 68, 3285–3291.

Donovan, S., 2015. Listeriosis: A rare but deadly disease. Clin. Microbiol. Newsl. 37, 135–140.

Drevets, D.A., Bronze, M.S., 2008. *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. FEMS Immunoogy Med. Microbiol. 53, 151–165.

Drosinos, E.H., Mataragas, M., Vesković-Moracanin, S., Gasparik-Reichardt, J., Hadziosmanović, M., Alagić, D., 2006. Quantifying non-thermal inactivation of *Listeria monocytogenes* in European fermented sausages using bacteriocinogenic lactic acid bacteria or their bacteriocins: a case study for risk assessment. J. Food Prot. 69, 2648–2663.

Duché, O., Trémoulet, F., Glaser, P., Labadie, J., 2002. Salt stress proteins induced in *Listeria monocytogenes*. Appl. Environ. Microbiol. 68, 1491–1498.

Dupont, C., Augustin, J.-C., 2009. Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half fraser broth. Appl. Environ. Microbiol. 75, 3069–3076.

European Commission, 2013. Guidance Document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuff https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_mc_guidance_document_lysteria.pdf (accessed 11.10.17).

Faleiro, M.L., Andrew, P.W., Power, D., 2003. Stress response of *Listeria monocytogenes* isolated from cheese and other foods. Int. J. Food Microbiol. 84, 207–216.

Feehily, C., Karatzas, K.A.G., 2012. Role of glutamate metabolism in bacterial responses towards acid and other stresses. J. Appl. Microbiol. 114, 11–24.

Ferenci, T., 2016. Trade-off mechanisms shaping the diversity of bacteria. Trends Microbiol. 24, 209–223.

Ferenci, T., 2005. Maintaining a healthy SPANC balance through regulatory and mutational adaptation. Mol. Microbiol. 57, 1–8.

Ferenci, T., Galbiati, H.F., Betteridge, T., Phan, K., Spira, B., 2011. The constancy of global regulation across a species: The concentrations of ppGpp and RpoS are strain-specific in Escherichia coli. BMC Microbiol. 11, 62.

Ferreira, A., Gray, M., Wiedmann, M., Boor, K.J., 2004. Comparative genomic analysis of the sigB operon in *Listeria monocytogenes* and in other Gram-positive bacteria. Curr. Microbiol. 48, 39–46.

Ferreira, A., O'Byrne, C.P., Boor, K.J., 2001. Role of σB in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. Appl. Environ. Microbiol. 67, 4454–4457.

Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J. Food Prot. 77, 150–170.

Fox, E.M., Leonard, N., Jordan, K., 2011. Physiological and transcriptional characterization of persistent and nonpersistent *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 77, 6559–6569.

Francis, G.A., O'Beirne, D., 2005. Variation among strains of *Listeria monocytogenes*: differences in survival on packaged vegetables and in response to heat and acid conditions. Food Control 16, 687–694.

Freitag, N.E., Port, G.C., Miner, M.D., 2009. *Listeria monocytogenes*: from saprophyte to intracellular pathogen. Nat. Rev. Microbiol. 7, 623.

Fretz, R., Sagel, U., Ruppitsch, W., Pietzka, A.T., Stöger, A., Huhulescu, S., Heuberger, S., Pichler, J., Much, P., Pfaff, G., Stark, K., Prager, R., Flieger, A., Feenstra, O., Allerberger, F., 2010. Listeriosis outbreak caused by acid curd cheese "Quargel", Austria and Germany 2009. Eurosurveillance 15, 1–2.

Fu, Z., Donegan, N.P., Memmi, G., Cheung, A.L., 2007. Characterization of MazFSa, an endoribonuclease from *Staphylococcus aureus*. J. Bacteriol. 189, 8871–8879.

Gahan, C.G.M., O'Mahony, J., Hill, C., 2001. Characterization of the *groESL* operon in *Listeria monocytogenes*: Utilization of two reporter systems (*gfp* and *hly*) for evaluating in vivo

expression. Infect. Immun. 69, 3924–3932.

Gandhi, M., Chikindas, M.L., 2007. *Listeria*: A foodborne pathogen that knows how to survive. Int. J. Food Microbiol. 113, 1–15.

Gao, W., Zhang, W., Meldrum, D.R., 2011. RT-qPCR based quantitative analysis of gene expression in single bacterial cells. J. Microbiol. Methods 85, 221–227.

García, D., Hassani, M., Mañas, P., Condón, S., Pagán, R., 2005. Inactivation of *Escherichia coli* O157:H7 during the storage under refrigeration of apple juice treated by pulsed electric fields. J. Food Saf. 25, 30–42.

García, D., Mañas, P., Gómez, N., Raso, J., Pagán, R., 2006. Biosynthetic requirements for the repair of sublethal membrane damage in *Escherichia coli* cells after pulsed electric fields. J. Appl. Microbiol. 100, 428–435.

Geeraerd, A.H., Herremans, C.H., Van Impe, J.F., 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int. J. Food Microbiol. 59, 185–209.

Geeraerd, A.H., Valdramidis, V.P., Van Impe, J.F., 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102, 95–105.

Gefen, O., Balaban, N.Q., 2009. The importance of being persistent: Heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol. Rev. 33, 704–717.

Gerhardt, P.N.M., Smith, L.T., Smith, G.M., 2000. Osmotic and chill activation of glycine betaine porter II in *Listeria monocytogenes* membrane vesicles. J. Bacteriol. 182, 2544–2550.

Giaouris, E., Chorianopoulos, N., Nychas, G.J., 2014. Acquired acid adaptation of *Listeria monocytogenes* during its planktonic growth enhances subsequent survival of its sessile population to disinfection with natural organic compounds. Food Res. Int. 64, 896–900.

Gillesberg Lassen, S., Ethelberg, S., Björkman, J.T., Jensen, T., Sørensen, G., Jensen, A.K., Müller, L., Nielsen, E.M., Mølbak, K., 2016. Two listeria outbreaks caused by smoked fish consumption - using whole-genome sequencing for outbreak investigations. Clin. Microbiol. Infect. 22, 620–624.

Gorski, L., Flaherty, D., Mandrell, R.E., 2006. Competitive fitness of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed cultures with and without Food in the U.S. Food and

Drug Administration enrichment protocol. Appl. Environ. Microbiol. 72, 776–783.

Gossett, D.R., Weaver, W.M., Mach, A.J., Hur, S.C., Tse, H.T.K., Lee, W., Amini, H., Di Carlo, D., 2010. Label-free cell separation and sorting in microfluidic systems. Anal. Bioanal. Chem. 397, 3249–3267.

Graves, L.M., Helsel, L.O., Steigerwalt, A.G., Morey, R.E., Daneshvar, M.I., Roof, S.E., Orsi, R.H., Fortes, E.D., Milillo, S.R., den Bakker, H.C., Wiedmann, M., Swaminathan, B., Sauders, B.D., 2010. *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. Int. J. Syst. Evol. Microbiol. 60, 1280–1288.

Gray, M.J., Freitag, N.E., Boor, K.J., 2006. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infect. Immun. 74, 2505–2512.

Grégori, G., Citterio, S., Ghiani, A., Labra, M., Sgorbati, S., Brown, S., Denis, M., 2001. Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. Appl. Environ. Microbiol. 67, 4662–4670.

Guldimann, C., Boor, K.J., Wiedmann, M., Guariglia-Oropeza, V., 2016. Resilience in the face of uncertainty: Sigma factor B fine-tunes gene expression to support homeostasis in Gram-positive bacteria. Appl. Environ. Microbiol. 82, 4456–4469.

Hanawa, T., Kai, M., Kamiya, S., Yamamoto, T., 2000. Cloning, sequencing, and transcriptional analysis of the dnaK heat shock operon of *Listeria monocytogenes*. Cell Stress Chaperones 5, 21–9.

Health Canada Food Directorate, 2010. Policy on *Listeria monocytogenes* in ready-to-eat foods. http://members.wto.org/crnattachments/2010/sps/CAN/10_4322_00_e.pdf (accessed 3.10.17).

Hecker, M., Schumann, W., Völker, U., 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. 19, 417–28.

Heiman, K.E., Garalde, V.B., Gronostaj, M., Jackson, K.A., Beam, S., Joseph, L., Saupe, A., Ricotta, E., Waechter, H., Wellman, A., Adams-Cameron, M., Ray, G., Fields, A., Chen, Y., Datta, A., Burall, L., Sabol, A., Kucerova, Z., Trees, E., Metz, M., Leblanc, P., Lance, S., Griffin, P.M., Tauxe, R.V., Silk, B.J., 2016. Multistate outbreak of listeriosis caused by

imported cheese and evidence of cross-contamination of other cheeses, USA, 2012. Epidemiol. Infect. 144, 2698–2708.

Hill, C., Cotter, P.D., Sleator, R.D., Gahan, C.G.M., 2002. Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. Int. Dairy J. 12, 273–283.

Hlaing, M.M., Wood, B.R., Mcnaughton, D., Ying, D., Dumsday, G., Augustin, M.A., 2017. Effect of drying methods on protein and DNA conformation changes in *Lactobacillus rhamnosus* GG cells by Fourier Transform Infrared Spectroscopy. J. Agric. Food Chem. 65, 1724–1731.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., Saint, C.P., 2003. A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. J. Microbiol. Methods 52, 379–388.

Horlbog, J.A., Kent, D., Stephan, R., Guldimann, C., 2018. Surviving host- and food relevant stresses: Phenotype of *L. monocytogenes* strains isolated from food and clinical sources. Nat. Sci. Reports 8, 12931.

Huang, L., Hwang, A., Phillips, J., 2011. Effect of temperature on microbial growth rate - Mathematical analysis : The Arrhenius and Eyring – Polanyi connections. J. Food Sci. 76, 553–560.

Jamali, H., Radmehr, B., Thong, K.L., 2013. Prevalence, characterisation, and antimicrobial resistance of *Listeria* species and *Listeria monocytogenes* isolates from raw milk in farm bulk tanks. Food Control 34, 121–125.

James, S.J., Evans, J., James, C., 2008. A review of the performance of domestic refrigerators. J. Food Eng. 87, 2–10.

Jeffers, G.T., Bruce, J.L., McDonough, P.L., Scarlett, J., Boor, K.J., Wiedmann, M., 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiology 147, 1095–1104.

Jensen, A., Williams, D., Irvin, E.A., Gram, L., Smith, M.A., 2008. A processing plant persistent strain of *Listeria monocytogenes* crosses the fetoplacental barrier in a pregnant guinea pig model. J. Food Prot. 71, 1028–1034.

Jõers, A., Kaldalu, N., Tenson, T., 2010. The frequency of persisters in Escherichia coli reflects

the kinetics of awakening from dormancy. J. Bacteriol. 192, 3379–3384.

Jones, T.H., Vail, K.M., McMullen, L.M., 2013. Filament formation by foodborne bacteria under sublethal stress. Int. J. Food Microbiol. 165, 97–110.

Joux, F., Lebaron, P., 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes Infect. 2, 1523–1535.

Karatzas, K.-A.G., Brennan, O., Heavin, S., Morrissey, J., O'Byrne, C.P., 2010. Intracellular accumulation of high levels of γ-aminobutyrate by *Listeria monocytogenes* 10403S in response to low pH: Uncoupling of γ-aminobutyrate synthesis from efflux in a chemically defined medium. Appl. Environ. Microbiol. 76, 3529–3537.

Kastbjerg, V.G., Larsen, M.H., Gram, L., Ingmer, H., 2010. Influence of sublethal concentrations of common disinfectants on expression of virulence genes in *Listeria monocytogenes*. Appl. Environ. Microbiol. 76, 303–309.

Kataoka, A.I., Wang, H., Elliott, P.H., Whiting, R.C., Hayman, M.M., 2017. Growth of *Listeria monocytogenes* in thawed frozen foods. J. Food Prot. 80, 447–453.

Kazmierczak, M.J., Mithoe, S.C., Boor, K.J., Wiedmann, M., 2003. *Listeria monocytogenes* σB regulates stress response and virulence functions. J. Bacteriol. 185, 5722–5734.

Kazmierczak, M.J., Wiedmann, M., Boor, K.J., 2006. Contributions of *Listeria monocytogenes* σB and PrfA to expression of virulence and stress response genes during extra- and intracellular growth. Microbiology 152, 1827–1838.

Kennedy, D., Cronin, U.P., Wilkinson, M.G., 2011. Responses of *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* to simulated food processing treatments, determined using fluorescence-activated cell sorting and plate counting. Appl. Environ. Microbiol. 77, 4657–4668.

Kim, J.S., Heo, P., Yang, T.J., Lee, K.S., Jin, Y.S., Kim, S.K., Shin, D., Kweon, D.H., 2011. Bacterial persisters tolerate antibiotics by not producing hydroxyl radicals. Biochem. Biophys. Res. Commun. 413, 105–110.

Klinkert, B., Narberhaus, F., 2009. Microbial thermosensors. Cell. Mol. Life Sci. 66, 2661–2676.

Knudsen, G.M., Ng, Y., Gram, L., 2013. Survival of bactericidal antibiotic treatment by a persister subpopulation of *Listeria monocytogenes*. Appl. Environ. Microbiol. 79, 7390–7397.

Ko, R., Smith, L.T., 1999. Identification of an ATP-driven, osmoregulated glycine betaine transport system in *Listeria monocytogenes*. Appl. Environ. Microbiol. 65, 4040–4048.

Koch, J., Dworak, R., Prager, R., Becker, B., Brockmann, S., Wicke, A., Wichmann-Schauer, H., Hof, H., Werber, D., Stark, K., 2010. Large listeriosis outbreak linked to cheese made from pasteurized milk, Germany, 2006-2007. Foodborne Pathog. Dis. 7, 1581–1584.

Kocot, A.M., Olszewska, M.A., 2017. Biofilm formation and microscopic analysis of biofilms formed by *Listeria monocytogenes* in a food processing context. LWT - Food Sci. Technol. 84, 47–57.

Komora, N., Bruschi, C., Magalhães, R., Ferreira, V., Teixeira, P., 2017. Survival of *Listeria monocytogenes* with different antibiotic resistance patterns to food-associated stresses. Int. J. Food Microbiol. 245, 79–87.

Korch, S.B., Henderson, T.A., Hill, T.M., 2003. Characterization of the *hipA7* allele of Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. Mol. Microbiol. 50, 1199–1213.

Koutsoumanis, K., 2008. A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations. Int. J. Food Microbiol. 128, 116–121.

Koutsoumanis, K.P., Aspridou, Z., 2017. Individual cell heterogeneity in Predictive Food Microbiology: Challenges in predicting a "noisy" world. Int. J. Food Microbiol. 240, 3–10.

Koutsoumanis, K.P., Lianou, A., 2013. Stochasticity in colonial growth dynamics of individual bacterial cells. Appl. Environ. Microbiol. 79, 2294–2301.

Kramer, B., Thielmann, J., 2016. Monitoring the live to dead transition of bacteria during thermal stress by a multi-method approach. J. Microbiol. Methods 123, 24–30.

Krämer, C.E.M., Wiechert, W., Kohlheyer, D., 2016. Time-resolved, single-cell analysis of induced and programmed cell death via non-invasive propidium iodide and counterstain perfusion. Sci. Rep. 6, 32104.

Krulwich, T.A., Sachs, G., Padan, E., 2011. Molecular aspects of bacterial pH sensing and homeostasis. Nat. Rev. Microbiol. 9, 330–343.

Kubista, M., Sindelka, R., Tichopad, A., Bergkvist, A., Lindh, D., Forootan, A., 2007. The Prime Technique: Real-Time PCR Data Analysis. G.I.T. Lab. J. 9–10, 33–35.

Kuhn, M., Goebel, W., 2007. Pathogenesis of Listeria monocytogenes, in: Ryser, E.T., Marth,

E.H. (Eds.), Listeria, Listeriosis and Food Safety. CRC Press, pp. 97–130.

Kulp, A., Kuehn, M.J., 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu. Rev. Microbiol. 64, 163–184.

Kumar, S.G., Maiti, M., 1994. DNA polymorphism under the influence of low pH and low temperature. J. Biomol. Struct. Dyn. 12, 183–201.

Laksanalamai, P., Joseph, L.A., Silk, B.J., Burall, L.S., Tarr, C.L., Gerner-Smidt, P., Datta, A.R., 2012. Genomic characterization of *Listeria monocytogenes* strains involved in a multistate listeriosis outbreak associated with Cantaloupe in US. PLoS One 7, 1–11.

Larsen, H.E., Seeliger, H.P.R., 1966. A mannitol fermenting *Listeria*: *Listeria grayi* sp. n. Proc. Third Int. Symp. List. 1994, 35–39.

Larsen, M.H., Dalmasso, M., Ingmer, H., Langsrud, S., Malakauskas, M., Mader, A., Møretrø, T., Možina, S.S., Rychli, K., Wagner, M., Wallace, R.J., Zentek, J., Jordan, K., 2014. Persistence of foodborne pathogens and their control in primary and secondary food production chains. Food Control 44, 92–109.

Le Roux, K., Prinsloo, L., Meyer, D., 2015a. Fourier Transform Infrared spectroscopy discloses different types of cell death in flow cytometrically sorted cells. Toxicol. Vitr. 29, 1932–1940.

Le Roux, K., Prinsloo, L.C., Meyer, D., 2015b. Cellular injury evidenced by impedance technology and infrared microspectroscopy. Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 138, 321–330.

Lee, E.-Y., Choi, D.-Y., Kim, D.-K., Kim, J.-W., Park, J.O., Kim, S., Kim, S.-H., Desiderio, D.M., Kim, Y.-K., Kim, K.-P., Gho, Y.S., 2009. Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. Proteomics 9, 5425–5436.

Lee, J.H., Choi, C.-W., Lee, T., Kim, S.I., Lee, J.-C., Shin, J.-H., 2013. Transcription factor σB plays an important role in the production of extracellular membrane-derived vesicles in *Listeria monocytogenes*. PLoS One 8, e73196.

Lee, J.Y., Jung, B.S., Yoon, H.J., Kim, K.T., Paik, H.D., Lee, J.Y., 2014. Predictive model for the growth kinetics of *Listeria monocytogenes* in raw pork meat as a function of temperature. Food Control 44, 16–21.

Lee, S., Chen, Y., Gorski, L., Ward, T.J., Osborne, J., Kathariou, S., 2018. *Listeria monocytogenes* source distribution analysis indicates regional heterogeneity and ecological niche preference among serotype 4b clones. MBio 9, e00396-18.

Lennon, J.T., Jones, S.E., 2011. Microbial seed banks: The ecological and evolutionary implications of dormancy. Nat. Rev. Microbiol. 9, 119–130.

Li, Z., Pérez-Osorio, A., Wang, Y., Eckmann, K., Glover, W.A., Allard, M.W., Brown, E.W., Chen, Y., 2017. Whole genome sequencing analyses of *Listeria monocytogenes* that persisted in a milkshake machine for a year and caused illnesses in Washington State. BMC Microbiol. 17, 134.

Liamkaew, R., Thipayarat, A., Saranak, J., 2014. *Listeria* detection in microscale solid state inoculation with minimal selective agents. Food Control 43, 183–192.

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: A review. Int. J. Food Microbiol. 167, 310–321.

Lianou, A., Stopforth, J.D., Yoon, Y., Wiedmann, M., Sofos, J.N., 2006. Growth and stress resistance variation in culture broth among *Listeria monocytogenes* strains of various serotypes and origins. J. Food Prot. 69, 2640–2647.

Lim, B., Gross, C.A., 2011. Cellular response to heat shock and cold shock, in: Storz, G., Hengge, R. (Eds.), Bacterial Stress Responses. ASM Press, Washington, DC, pp. 93–114.

Linke, K., Rückerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., Tichy, A., Wagner, M., Stessl, B., 2014. Reservoirs of *Listeria* species in three environmental ecosystems. Appl. Environ. Microbiol. 80, 5583–5592.

Liu, S., Graham, J.E., Bigelow, L., Morse II, P.D., Wilkinson, B.J., 2002. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. Appl. Environ. Microbiol. 68, 1697–1705.

Liu, X., Basu, U., Miller, P., McMullen, L.M., 2017. Differential gene expression and filamentation of *Listeria monocytogenes* 08-5923 exposed to sodium lactate and sodium diacetate. Food Microbiol. 63, 153–158.

Liu, Y., Ceruso, M., Jiang, Y., Datta, A.R., Carter, L., Strain, E., Pepe, T., Anastasi, A., Fratamico, P., 2013. Construction of *Listeria monocytogenes* mutants with in-frame deletions in the phosphotransferase transport system (PTS) and analysis of their growth

under stress conditions. J. Food Sci. 78, M1392–M1398.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-Delta Delta CT Method. Methods 25, 402–408.

Llorens, J.M.N., Tormo, A., Martínez-García, E., 2010. Stationary phase in gram-negative bacteria. FEMS Microbiol. Rev. 34, 476–495.

Low, J.C., Donachie, W., 1997. A review of *Listeria monocytogenes* and listeriosis. Vet. J. 153, 9–29.

Luber, P., Crerar, S., Dufour, C., Farber, J., Datta, A., Todd, E.C.D., 2011. Controlling *Listeria monocytogenes* in ready-to-eat foods: Working towards global scientific consensus and harmonization - Recommendations for improved prevention and control. Food Control 22, 1535–1549.

Lund, P., Tramonti, A., De Biase, D., 2014. Coping with low pH: molecular strategies in neutralophilic bacteria. FEMS Microbiol. Rev. 38, 1091–1125.

Mafart, P., Couvert, O., Gaillard, S., Leguerinel, I., 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. Int. J. Food Microbiol. 72, 107–114.

Maisonneuve, E., Gerdes, K., 2014. Molecular mechanisms underlying bacterial persisters. Cell 157, 539–548.

Makino, S.-I., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, M., Yamamoto, S., Igimi, S., 2005. An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. Int. J. Food Microbiol. 104, 189–196.

Marielle, B., Sarrah, G., 2017. Assessment of bacterial membrane fluidity by flow cytometry. J. Microbiol. Methods 143, 50–57.

Markkula, A., Lindstrom, M., Johansson, P., Bjorkroth, J., Korkeala, H., 2012. Roles of four putative DEAD-box RNA helicase genes in growth of *Listeria monocytogenes* EGD-e under heat, pH, osmotic, ethanol, and oxidative stress conditions. Appl. Environ. Microbiol. 78, 6875–6882.

Martín, B., Perich, A., Gómez, D., Yangüela, J., Rodríguez, A., Garriga, M., Aymerich, T., 2014. Diversity and distribution of *Listeria monocytogenes* in meat processing plants. Food Microbiol. 44, 119–127.

Martin, R.M., Leonhardt, H., Cardoso, M.C., 2005. DNA labeling in living cells. Cytom. Part A 67, 45–52.

Massicotte, R., Mafu, A.A., Ahmad, D., Deshaies, F., Pichette, G., Belhumeur, P., 2017. Comparison between flow cytometry and traditional culture methods for efficacy assessment of six disinfectant agents against nosocomial bacterial species. Front. Microbiol. 8, 112.

Mataragas, M., Alessandria, V., Rantsiou, K., Cocolin, L., 2015a. Evaluation of the *Listeria monocytogenes* inactivation during post-process storage of fermented sausages: A basis for the development of a decision support tool. Food Control 50, 568–573.

Mataragas, M., Rantsiou, K., Alessandria, V., Cocolin, L., 2015b. Estimating the non-thermal inactivation of *Listeria monocytogenes* in fermented sausages relative to temperature, pH and water activity. Meat Sci. 100, 171–178.

Mataragas, M., Rovetto, F., Bellio, A., Alessandria, V., Rantsiou, K., Decastelli, L., 2015c. Differential gene expression profi ling of *Listeria monocytogenes* in Cacciatore and Felino salami to reveal potential stress resistance biomarkers. Food Microbiol. 46, 408–417.

Mataragas, M., Stergiou, V., Nychas, G.-J.E., 2008. Modeling survival of *Listeria monocytogenes* in the traditional Greek soft cheese Katiki. J. Food Prot. 71, 1835–1845.

Maury, M.M., Tsai, Y.-H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., Criscuolo, A., Gaultier, C., Roussel, S., Brisabois, A., Disson, O., Rocha, E.P.C., Brisse, S., Lecuit, M., 2016. Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. Nat. Genet. 48, 308.

McCollum, J.T., Cronquist, A.B., Silk, B.J., Jackson, K.A., O'Connor, K.A., Cosgrove, S., Gossack, J.P., Parachini, S.S., Jain, N.S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N.W., Gorny, J.R., Mogen, K.M., Spires, C., Teitell, P., Joseph, L.A., Tarr, C.L., Imanishi, M., Neil, K.P., Tauxe, R. V., Mahon, B.E., 2013. Multistate outbreak of listeriosis associated with cantaloupe. N. Engl. J. Med. 369, 944–953.

Mclauchlin, J., Mitchell, R.T., Smerdon, W.J., Jewell, K., 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 92, 15–33.

Mellefont, L.A., Ross, T., 2003. The effect of abrupt shifts in temperature on the lag phase

duration of *Escherichia coli* and *Klebsiella oxytoca*. Int. J. Food Microbiol. 83, 295–305.

Melo, J., Andrew, P.W., Faleiro, M.L., 2015. *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. Food Res. Int. 67, 75–90.

Mendum, M.L., Smith, L.T., 2002. Characterization of glycine betaine porter I from *Listeria monocytogenes* and its roles in salt and chill tolerance. Appl. Environ. Microbiol. 68, 813–819.

Metselaar, K.I., Abee, T., Zwietering, M.H., den Besten, M.W., 2016. Modeling and validation of the ecological behavior of wild-type Listeria monocytogenes and stress-resistant variants. Appl. Environ. Mcrobiology 82, 5389–5401.

Metselaar, K.I., den Besten, H.M.W., Abee, T., Moezelaar, R., Zwietering, M.H., 2013. Isolation and quantification of highly acid resistant variants of *Listeria monocytogenes*. Int. J. Food Microbiol. 166, 508–514.

Metselaar, K.I., den Besten, H.M.W., Boekhorst, J., van Hijum, S.A.F.T., Zwietering, M.H., Abee, T., 2015. Diversity of acid stress resistant variants of *Listeria monocytogenes* and the potential role of ribosomal protein S21 encoded by rpsU. Front. Microbiol. 6, 1–12.

Miller, F.A., Brandao, T.R.S., Teixeira, P., Silva, C.L.M., 2006. Recovery of heat-injured *Listeria innocua*. Int. J. Food Microbiol. 112, 261–265.

Miner, M.D., Port, G.C., Freitag, N.E., 2007. Regulation of *Listeria monocytogenes* virulence genes, in: Goldfine, H., Shen, H. (Eds.), Listeria Monocytogenes: Pathogenesis and Host Response. Springer Science+Business Media, LLC, pp. 139–158.

Mir, S.A., Shah, M.A., Mir, M.M., Dar, B.N., Greiner, R., Roohinejad, S., 2018. Microbiological contamination of ready-to-eat vegetable salads in developing countries and potential solutions in the supply chain to control microbial pathogens. Food Control 85, 235–244.

Mistou, M.Y., Sutcliffe, I.C., van Sorge, N.M., 2016. Bacterial glycobiology: Rhamnose-containing cell wall polysaccharides in gram-positive bacteria. FEMS Microbiol. Rev. 40, 464–479.

Montero, D., Bodero, M., Riveros, G., Lapierre, L., Gaggero, A., Vidal, R.M., Vidal, M., 2015. Molecular epidemiology and genetic diversity of *Listeria monocytogenes* isolates from a wide variety of ready-to-eat foods and their relationship to clinical strains from listeriosis

outbreaks in Chile. Front. Microbiol. 6, 384.

Moorhead, S.M., Dykes, G.A., Cursons, R.T., 2003. An SNP-based PCR assay to differentiate between *Listeria monocytogenes* lineages derived from phylogenetic analysis of the *sigB* gene. J. Microbiol. Methods 55, 425–432.

Møretrø, T., Langsrud, S., 2004. *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. Biofilms 1, 107–121.

Mortimer, F.C., Mason, D.J., Gant, V.A., 2000. Flow cytometric monitoring of antibiotic-induced injury in *Escherichia coli* using cell-impermeant fluorescent probes. Antimicrob. Agents Chemother. 44, 676–681.

Muhterem-Uyar, M., Dalmasso, M., Bolocan, A.S., Hernandez, M., Kapetanakou, A.E., Kuchta, T., Manios, S.G., Melero, B., Minarovičová, J., Nicolau, A.I., Rovira, J., Skandamis, P.N., Jordan, K., Rodríguez-Lázaro, D., Stessl, B., Wagner, M., 2015. Environmental sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. Food Control 51, 94–107.

Murray, E.G.D., Webb, R.A., Swann, M.B.R., 1926. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp). J. Pathol. 29, 407–439.

Nair, S., Derré, I., Msadek, T., Gaillot, O., Berche, P., 2000. CtsR controls class III heat shock gene expression in the human pathogen *Listeria monocytogenes*. Mol. Microbiol. 35, 800–811.

Nedwell, D.B., 1999. Effect of low temperature on microbial growth : lowered affinity for substrates limits growth at low temperature. FEMS Microbiol. Ecol. 30, 101–111.

Nelson, K.E., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., Rasko, D.A., Angiuoli, S. V., Gill, S.R., Paulsen, I.T., Peterson, J., White, O., Nelson, W.C., Nierman, W., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Haft, D.H., Selengut, J., Van Aken, S., Khouri, H., Fe-Dorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L.D., Uhlich, G.A., Bayles, D.O., Luchansky, J.B., Fraser, C.M., 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. 32, 2386–2395.

Netterling, S., Vaitkevicius, K., Nord, S., Johansson, J., 2012. A *Listeria monocytogenes* RNA helicase essential for growth and ribosomal maturation at low temperatures uses its C Terminus for appropriate interaction with the ribosome. J. Bacteriol. 194, 4377–4385.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B.E., K, S.P., 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science. 334, 982–986.

Nightingale, K.K., Ivy, R.A., Ho, A.J., Fortes, E.D., Njaa, B.L., Peters, R.M., Wiedmann, M., 2008. *inlA* premature stop codons are common among *Listeria monocytogenes* isolates from foods and yield virulence-attenuated strains that confer protection against fully virulent strains. Appl. Environ. Microbiol. 74, 6570–6583.

Nightingale, K.K., Windham, K., Martin, K.E., Yeung, M., Wiedmann, M., 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry disctinct nonsense mutations in *inlA*. Appl. Environ. Microbiol. 71, 8764–8772.

Norman, T.M., Lord, N.D., Paulsson, J., Losick, R., 2015. Stochastic switching of cell fate in microbes. Annu. Rev. Microbiol. 69, 381–403.

Novo, D.J., Perlmutter, N.G., Hunt, R.H., Shapiro, H.M., 2000. Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. Antimicrob. Agents Chemother. 44, 827–834.

Nowak, J., Cruz, C.D., Tempelaars, M., Abee, T., van Vliet, A.H.M., Fletcher, G.C., Hedderley, D., Palmer, J., Flint, S., 2017. Persistent *Listeria monocytogenes* strains isolated from mussel production facilities form more biofilm but are not linked to specific genetic markers. Int. J. Food Microbiol. 256, 45–53.

Núñez-Montero, K., Leclercq, A., Moura, A., Vales, G., Peraza, J., Pizarro-Cerdá, J., Lecuit, M., 2018. *Listeria costaricensis* sp. nov. Int. J. Syst. Evol. Microbiol. 68, 844–850.

Nyarko, E., Donnelly, C., 2015. Differentiation of different mixed *Listeria* strains and also acid-injured, heat-injured, and repaired cells of *Listeria monocytogenes* using Fourier Transform Infrared spectroscopy. J. Food Prot. 78, 540–548.

O'Byrne, C.P., Karatzas, K.A.G., 2008. The role of Sigma B (σB) in the stress adaptations of

Listeria monocytogenes: Overlaps between stress adaptation and virulence, in: Advances in Applied Microbiology. Elsevier Masson SAS, pp. 115–140.

O'Driscoll, B., Gahan, C.G.M., Hill, C., 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl. Environ. Microbiol. 62, 1693–1698.

Olsen, S.J., Patrick, M., Hunter, S.B., Reddy, V., Kornstein, L., MacKenzie, W.R., Lane, K., Bidol, S., Stoltman, G.A., Frye, D.M., Lee, I., Hurd, S., Jones, T.F., LaPorte, T.N., Dewitt, W., Graves, L., Wiedmann, M., Schoonmaker-Bopp, D.J., Huang, A.J., Vincent, C., Bugenhagen, A., Corby, J., Carloni, E.R., Holcomb, M.E., Woron, R.F., Zansky, S.M., Dowdle, G., Smith, F., Ahrabi-Fard, S., Ong, A.R., Tucker, N., Hynes, N.A., Mead, P., 2005. Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. Clin. Infect. Dis. 40, 962–967.

Orgaz, B., Puga, C.H., Martínez-Suárez, J. V, Sanjose, C., 2013. Biofilm recovery from chitosan action: A possible clue to understand *Listeria monocytogenes* persistence in food plants. Food Control 32, 484–489.

Orsi, R.H., den Bakker, H.C., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301, 79–96.

Orsi, R.H., Wiedmann, M., 2016. Characteristics and distribution of *Listeria* spp., including *Listeria* species newly described since 2009. Appl. Microbiol. Biotechnol. 100, 5273–5287.

Pagadala, S., Parveen, S., Rippen, T., Luchansky, J.B., Call, J.E., Tamplin, M.L., Porto-Fett, A.C.S., 2012. Prevalence, characterization and sources of *Listeria monocytogenes* in blue crab (*Callinectus sapidus*) meat and blue crab processing plants. Food Microbiol. 31, 263–270.

Peleg, M., 2003. Calculation of the non-isothermal inactivation patterns of microbes having sigmoid isothermal and semi-logarithmic survival curves. Crit. Rev. Food Sci. Nutr. 43, 645–658.

Peleg, M., 2000. Microbial survival curves - the reality of flat "shoulders'''' and absolute thermal death times." Food Res. Int. 33, 531–538.

Peleg, M., Penchina, C.M., 2000. Modeling microbial survival during exposure to a lethal agent with varying intensity. Crit. Rev. Food Sci. Nutr. 40, 159–172.

Perfetto, S.P., Chattopadhyay, P.K., Lamoreaux, L., Nguyen, R., Ambrozak, D., Koup, R.A., Roederer, M., 2006. Amine reactive dyes: An effective tool to discriminate live and dead cells in polychromatic flow cytometry. J. Immunol. Methods 313, 199–208.

Phadtare, S., 2004. Recent developments in bacterial cold-shock response. Curr. Issues Mol. Biol. 6, 125–136.

Phadtare, S., Alsina, J., Inouye, M., 1999. Cold-shock response and cold-shock proteins. Curr. Opin. Microbiol. 2, 175–180.

Piffaretti, J.-C., Kressebuch, H., Aeschbacher, M., Bille, J., Bannermant, E., Musser, J.M., Selander, R.K., Rocourt, J., 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. 86, 3818–3822.

Pirie, H.J., 1940. *Listeria*: change of name for a genus bacteria. Nature 145, 264.

Pirie, H.J., 1927. A new disease of veld rodents 'tiger river disease.' Publ S Afr Inst Med Res 3, 163–187.

Pizarro-Cerdá, J., Cossart, P., 2007. Invasion of host cells by *Listeria monocytogenes*, in: Goldfine, H., Shen, H. (Eds.), Listeria Monocytogenes: Pathogenesis and Host Response. Springer Science+Business Media, LLC, pp. 159–176.

Pouillot, R., Lubran, M.B., 2011. Predictive microbiology models vs. modeling microbial growth within *Listeria monocytogenes* risk assessment: What parameters matter and why. Food Microbiol. 28, 720–726.

Pu, Y., Ke, Y., Bai, F., 2017. Active efflux in dormant bacterial cells – New insights into antibiotic persistence. Drug Resist. Updat. 30, 7–14.

Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., Ke, Y., Zhu, Y., Chen, H., Baker, M.A.B., Ge, H., Sun, Y., Xie, X.S., Bai, F., 2016. Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Mol. Cell 62, 284–294.

Radzikowski, J.L., Schramke, H., Heinemann, M., 2017. Bacterial persistence from a system-level perspective. Curr. Opin. Biotechnol. 46, 98–105.

Radzikowski, J.L., Vedelaar, S., Siegel, D., Ortega, Á.D., Schmidt, A., Heinemann, M., 2016. Bacterial persistence is an active σS stress response to metabolic flux limitation. Mol. Syst. Biol. 12, 882.

Rak, S., De Zan, T., Stefulj, J., Kosoví, M., Gamulin, O., Osmak, M., 2014. FTIR spectroscopy

reveals lipid droplets in drug resistant laryngeal carcinoma cells through detection of increased ester vibrational bands intensity. Analyst 139, 3407–3415.

Renier, S., Hébraud, M., Desvaux, M., 2011. Molecular biology of surface colonization by *Listeria monocytogenes*: An additional facet of an opportunistic Gram-positive foodborne pathogen. Environ. Microbiol. 13, 835–850.

Rivera, J., Cordero, R.J.B., Nakouzi, A.S., Frases, S., Nicola, A., Casadevall, A., 2010. *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins. Proc. Natl. Acad. Sci. 107, 19002–19007.

Rocourt, J., Buchrieser, C., 2007. The genus Listeria and Listeria monocytogenes: Phylogenetic position, taxonomy, and identification, in: Ryser, E.T., Marth, E.H. (Eds.), Listeria, Listeriosis and Food Safety. CRC Press, Boca Raton, pp. 1–20.

Rodríguez, M.R., Aguirre, J.S., Lianou, A., Parra-flores, J., de Fernando, G.D.G., 2016. Analysis of the variability in microbial inactivation by acid treatments. LWT - Food Sci. Technol. 66, 369–377.

Rogga, K.J., Samelis, J., Kakouri, A., Katsiari, M.C., Savvaidis, I.N., Kontominas, M.G., 2005. Survival of *Listeria monocytogenes* in Galotyri, a traditional Greek soft acid-curd cheese, stored aerobically at 4°C and 12°C. Int. Dairy J. 15, 59–67.

Roncarati, D., Scarlato, V., 2017. Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. FEMS Microbiol. Rev. 41, 549–574.

Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiology. J. Appl. Bacteriol. 81, 501–508.

Rowan, N.J., Anderson, J.G., 1998. Effects of above-optimum growth temperature and cell morphology on thermotolerance of *Listeria monocytogenes* cells suspended in bovine milk. Appl. Environ. Microbiol. 64, 2065–2071.

Rückerl, I., Muhterem-Uyar, M., Muri-Klinger, S., Wagner, K.-H., Wagner, M., Stessl, B., 2014. *L. monocytogenes* in a cheese processing facility: Learning from contamination scenarios over three years of sampling. Int. J. Food Microbiol. 189, 98–105.

Russell, N.J., 2002. Bacterial membranes: the effects of chill storage and food processing. An overview. Int. J. Food Microbiol. 79, 27–34.

Ryall, B., Eydallin, G., Ferenci, T., 2012. Culture history and population heterogeneity as

determinants of bacterial adaptation: the adaptomics of a single environmental transition. Microbiol. Mol. Biol. Rev. 76, 597–625.

Ryan, S., Begley, M., Gahan, C.G.M., Hill, C., 2009. Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. Environ. Microbiol. 11, 432–445.

Ryan, S., Hill, C., Gahan, C.G.M., 2008. Acid stress responses in *Listeria monocytogenes*, in: Advances in Applied Microbiology. Elsevier Masson SAS, pp. 67–91.

Rychli, K., Müller, A., Zaiser, A., Schoder, D., Allerberger, F., Wagner, M., Schmitz-Esser, S., 2014. Genome sequencing of *Listeria monocytogenes* '"Quargel"' listeriosis outbreak strains reveals two different strains with distinct in vitro virulence potential. PLoS One 9, 1–11.

Sadeghi-Mehr, A., Lautenschlaeger, R., Drusch, S., 2016. Behavior of *Salmonella* spp. and *Listeria monocytogenes* throughout the manufacture and shelf-life of dry-cured formed ham. Food Control 64, 22–28.

Schmid, B., Klumpp, J., Raimann, E., Loessner, M.J., Stephan, R., Tasara, T., 2009a. Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl. Environ. Microbiol. 75, 1621–1627.

Schmid, B., Klumpp, J., Raimann, E., Loessner, M.J., Stephan, R., Tasara, T., 2009b. Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl. Environ. Microbiol. 75, 1621–1627.

Schneewind, O., Missiakas, D., 2014. Lipoteichoic acids, phosphate-containing polymers in the envelope of Gram-positive bacteria. J. Bacteriol. 6, 1133–1142.

Schoder, D., Rossmanith, P., Glaser, K., Wagner, M., 2012. Fluctuation in contamination dynamics of *L. monocytogenes* in quargel (acid curd cheese) lots recalled during the multinational listeriosis outbreak 2009/2010. Int. J. Food Microbiol. 157, 326–331.

Schvartzman, M.S., Belessi, X., Butler, F., Skandamis, P., Jordan, K., 2010. Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. J. Appl. Microbiol. 109, 1790–1799.

Seeliger, H.P.R., Höhne, K., 1979. Serotyping of *Listeria monocytogenes* and related species. Methods Microbiol. 13, 31–49.

Shabala, L., Lee, S.H., Cannesson, P., Ross, T., 2008. Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels on inactivation kinetics. J. Food Prot. 71, 1169–1177.

Shapiro, H.M., 2001. Multiparameter flow cytometry of bacteria: Implications for diagnostics and therapeutics. Cytometry 43, 223–226.

Shen, Q., Jangam, P.M., Soni, K.A., Nannapaneni, R., Schilling, W., Silva, J.L., 2014. Low, medium, and high heat tolerant strains of *Listeria monocytogenes* and increased heat stress resistance after exposure to sublethal heat. J. Food Prot. 77, 1298–1307.

Silva-Angulo, A.B., Zanini, S.F., Rodrigo, D., Rosenthal, A., Martinez, A., 2014. Growth kinetics of *Listeria innocua* and *Listeria monocytogenes* under exposure to carvacrol and the occurrence of sublethal damage. Food Control 37, 336–342.

Silva, A., Genovés, S., Martorell, P., Zanini, S.F., Rodrigo, D., Martinez, A., 2015. Sublethal injury and virulence changes in *Listeria monocytogenes* and *Listeria innocua* treated with antimicrobials carvacrol and citral. Food Microbiol. 50, 5–11.

Singh, A.K., Zhang, Y.M., Zhu, K., Subramanian, C., Li, Z., Jayaswal, R.K., Gatto, C., Rock, C.O., Wilkinson, B.J., 2009. FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol. Lett. 301, 188–192.

Singh, S., Shalini, R., 2016. Effect of hurdle technology in food preservation: A Review. Crit. Rev. Food Sci. Nutr. 56, 641–649.

Skalina, L., Nikolajeva, V., 2010. Growth potential of *Listeria monocytogenes* strains in mixed ready-to-eat salads. Int. J. Food Microbiol. 144, 317–321.

Skandamis, P.N., Yoon, Y., Stopforth, J.D., Kendall, P.A., Sofos, J.N., 2008. Heat and acid tolerance of *Listeria monocytogenes* after exposure to single and multiple sublethal stresses. Food Microbiol. 25, 294–303.

Sleator, R.D., Gahan, C.G.M., Abee, T., Hill, C., 1999. Identification and disruption of betL, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28. Appl. Environ. Microbiol. 65, 2078–2083.

Sleator, R.D., Wouters, J., Gahan, C.G.M., Abee, T., Hill, C., 2002. Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of *Listeria*

monocytogenes. Appl. Environ. Microbiol. 67, 2692–2698.

Spano, G., Chieppa, G., Beneduce, L., Massa, S., 2004. Expression analysis of putative *arcA, arcB* and *arcC* genes partially cloned from *Lactobacillus plantarum* isolated from wine. J. Appl. Microbiol. 96, 185–193.

Stackhouse, R.R., Faith, N.G., Kaspar, C.W., Czuprynski, C.J., Wong, A.C.L., 2012. Survival and virulence of *Salmonella enterica* Serovar Enteritidis filaments induced by reduced water activity. Appl. Environ. Microbiol. 78, 2213–2220.

Stepanyan, K., Wenseleers, T., Duèňez-Guzmán, E.A., Muratori, F., Van Den Bergh, B., Verstraeten, N., De Meester, L., Verstrepen, K.J., Fauvart, M., Michiels, J., 2015. Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen *Pseudomonas aeruginosa*. Mol. Ecol. 24, 1572–1583.

Stephan, R., Althaus, D., Kiefer, S., Lehner, A., Hatz, C., Schmutz, C., Jost, M., Gerber, N., Baumgartner, A., Hächler, H., Mäusezahl-Feuz, M., 2015. Foodborne transmission of *Listeria monocytogenes* via ready-to-eat salad: A nationwide outbreak in Switzerland 2013-2014. Food Control 57, 14–17.

Stephens, P.J., Druggan, P., Nebe-von Caron, G., 2000. Stressed *Salmonella* are exposed to reactive oxygen species from two independent sources during recovery in conventional culture media. Int. J. Food Microbiol. 60, 269–285.

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K., Ren, Q., 2015. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. BMC Microbiol. 15, 36.

Sträuber, H., Müller, S., 2010. Viability states of bacteria -Specific mechanisms of selected probes. Cytom. Part A 77, 623–634.

Strydom, A., Bester, I.M., Cameron, M., Franz, C.M.A.P., Witthuhn, C.C., 2013. Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE. Food Control 31, 274–279.

Sue, D., Fink, D., Wiedmann, M., Boor, K.J., 2004. σB-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. Microbiology 150, 3843–3855.

Swinnen, I.A.M., Bernaerts, K., Dens, E.J.J., Geeraerd, A.H., Van Impe, J.F., 2004. Predictive

modelling of the microbial lag phase: a review. Int. J. Food Microbiol. 94, 137–159.

Tajmir-Riahi, H.A., Ahmad, R., Naoui, M., Diamantoglou, S., 1995. The effect of HCl on the solution structure of calf thymus DNA: A comparative study of DNA denaturation by proton and metal cations using Fourier Transform IR difference spectroscopy. Biopolymers 35, 493–501.

Tajmir-Riahi, H.A., Neault, J.F., Naoui, M., 1995. Does DNA acid fixation produce left-handed Z structure? FEBS Lett. 370, 105–108.

Takada, H., Morita, M., Shiwa, Y., Sugimoto, R., Suzuki, S., Kawamura, F., Yoshikawa, H., 2014. Cell motility and biofilm formation in *Bacillus subtilis* are affected by the ribosomal proteins, S11 and S21. Biosci. Biotechnol. Biochem. 78, 898–907.

Tamburini, S., Ballarini, A., Ferrentino, G., Moro, A., Foladori, P., Spilimbergo, S., Jousson, O., 2013. Comparison of quantitative PCR and flow cytometry as cellular viability methods to study bacterial membrane permeabilization following supercritical CO2 treatment. Microbiology 159, 1056–1066.

Tasara, T., Stephan, R., 2007. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. FEMS Microbiol. Lett. 269, 265–272.

Tasara, T., Stephan, R., 2006. Cold stress tolerance of *Listeria monocytogenes*: A review of molecular adaptive mechanisms and food safety implications. J. Food Prot. 69, 1473–1484.

Tiganitas, A., Zeaki, N., Gounadaki, A.S., Drosinos, E.H., Skandamis, P.N., 2009. Study of the effect of lethal and sublethal pH and aw stresses on the inactivation or growth of Listeria monocytogenes and *Salmonella* Typhimurium. Int. J. Food Microbiol. 134, 104–112.

Tiwari, U., Walsh, D., Rivas, L., Jordan, K., Duffy, G., 2014. Modelling the interaction of storage temperature, pH, and water activity on the growth behaviour of *Listeria monocytogenes* in raw and pasteurised semi-soft rind washed milk cheese during storage following ripening. Food Control 42, 248–256.

United States Food and Drug Administration (USFDA). 2017. Control of *Listeria monocytogenes* in ready-to-eat foods: Guidance for industry. https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinfo rmation/ucm535981.pdf (accessed 3.10.17).

Uyttendaele, M., Rajkovic, A., Van Houteghem, N., Boon, N., Thas, O., Debevere, J., Devlieghere, F., 2008. Multi-method approach indicates no presence of sub-lethally injured *Listeria monocytogenes* cells after mild heat treatment. Int. J. Food Microbiol. 123, 262–268.

Valderrama, W.B., Cutter, C.N., 2013. An ecological perspective of *Listeria monocytogenes* biofilms in food processing facilities. Crit. Rev. Food Sci. Nutr. 53, 801–817.

Van Boeijen, I.K.H., Casey, P.G., Hill, C., Moezelaar, R., Zwietering, M.H., Gahan, C.G.M., Abee, T., 2013. Virulence aspects of *Listeria monocytogenes* LO28 high pressure-resistant variants. Microb. Pathog. 59–60, 48–51.

Van Boeijen, I.K.H., Chavaroche, A.A.E., Valderrama, W.B., Moezelaar, R., Zwietering, M.H., Abee, T., 2010. Population diversity of *Listeria monocytogenes* LO28: Phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. Appl. Environ. Mcrobiology 76, 2225–2233.

Van Boeijen, I.K.H., Francke, C., Moezelaar, R., Abee, T., Zwietering, M.H., 2011. Isolation of highly heat-resistant *Listeria monocytogenes* variants by use of a kinetic modeling-based sampling scheme. Appl. Environ. Microbiol. 77, 2617–2624.

Van Nevel, S., Koetzsch, S., Proctor, C.R., Besmer, M.D., Prest, E.I., Vrouwenvelder, J.S., Knezev, A., Boon, N., Hammes, F., 2017. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Res. 113, 191–206.

Van Stelten, A., Simpson, J.M., Ward, T.J., Nightingale, K.K., 2010. Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in *inlA* are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. Appl. Environ. Microbiol. 76, 2783–2790.

Vázquez-Boland, J.A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., Goebel, W., 2001. Pathogenicity islands and virulence evolution in *Listeria*. Microbes Infect. 3, 571–584.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. J. Immunol. Methods 243, 191–210.

Vollmer, W., Seligman, S.J., 2010. Architecture of peptidoglycan: more data and more models. Trends Microbiol. 18, 59–66.

Vorob'eva, L.I., 2004. Stressors, stress reactions, and survival of bacteria: A review. Appl. Biochem. Microbiol. 40.

Waage, A.S., Vardund, T., Lund, V., Kapperud, G., 1999. Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. Appl. Environ. Microbiol. 65, 1636–1643.

Wagner, M., McLauchlin, J., 2008. Biology of *Listreria monocytogenes*, in: Dongyou, L. (Ed.), Handbook of Listeria Monocytogenes. CRC Press, Boca Raton, pp. 3–26.

Wang, J., Ray, A.J., Hammons, S.R., Oliver, H.F., 2015. Persistent and transient *Listeria monocytogenes* strains from retail deli environments vary in their ability to adhere and form biofilms and rarely have *inlA* premature stop codons. Foodborne Pathog. Dis. 12, 151–158.

Wang, X.M., Lü, X.F., Yin, L., Liu, H.F., Zhang, W.J., Si, W., Yu, S.Y., Shao, M.L., Liu, S.G., 2013. Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods. Food Control 32, 153–158.

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., Bielawski, J.P., 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 74, 7629–7642.

Ward, T.J., Usgaard, T., Evans, P., 2010. A targeted multilocus genotyping assay for lineage, serogroup, and epidemic clone typing of *Listeria monocytogenes*. Appl. Environ. Microbiol. 76, 6680–6684.

Warriner, K., Namvar, A., 2009. What is the hysteria with *Listeria*? Trends Food Sci. Technol. 20, 245–254.

Weller, D., Andrus, A., Wiedmann, M., den Bakker, H.C., 2015. *Listeria booriae* sp. nov. and *Listeria newyorkensis* sp. nov., from food processing environments in the USA. Int. J. Syst. Evol. Microbiol. 65, 286–292.

Wemekamp-Kamphuis, H.H., Karatzas, A.K., Wouters, J.A., Abee, T., 2002. Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. Appl. Environ. Microbiol. 68, 456–463.

Wemekamp-Kamphuis, H.H., Wouters, J.A., de Leeuw, P.P.L.A., Hain, T., Chakraborty, T., Abee, T., 2004. Identification of sigma factor σB-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogenes* EGD-e.

Appl. Environ. Microbiol. 70, 3457–3466.

Wemmenhove, E., van Valenberg, H.J.F., Zwietering, M.H., van Hooijdonk, T.C.M., Wells-Bennik, M.H.J., 2016. Minimal inhibitory concentrations of undissociated lactic, acetic, citric and propionic acid for *Listeria monocytogenes* under conditions relevant to cheese. Food Microbiol. 58, 63–67.

Wen, J., Anantheswaran, R.C., Knabel, S.J., 2009. Changes in barotolerance, thermotolerance, and cellular morphology throughout the life cycle of *Listeria monocytogenes*. Appl. Environ. Microbiol. 75, 1581–1588.

Wersto, R.P., Chrest, F.J., Leary, J.F., Morris, C., Stetler-stevenson, M., Gabrielson, E., 2001. Doublet Discrimination in DNA Cell-Cycle Analysis. Cytom. (Communications Clin. Cytom. 46, 296–306.

Wesche, A.M., Gurtler, J.B., Marks, B.P., Ryser, E.T., 2009. Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. J. Food Prot. 72, 1121–1138.

Wiedmann, M., Arvik, T.J., Hurley, R.J., Boor, K.J., 1998. General stress transcription factor σB and its role in acid tolerance and virulence of *Listeria monocytogenes*. J. Bacteriol. 180, 3650–3656.

World Health Organization (WHO). 2018. Listeriosis – South Africa, Disease outbreak news. http://www.who.int/csr/don/28-march-2018-listeriosis-south-africa/en/ (accessed 15.04.18).

Wood, B.R., 2016. The importance of hydration and DNA conformation in interpreting infrared spectra of cells and tissues. Chem. Soc. Rev. 45, 1980–1998.

Wood, J.M., 1999. Osmosensing by bacteria: signals and membrane-based sensors. Microbiol. Mol. Biol. Rev. 63, 230–262.

Wood, J.M., Bremer, E., Csonka, L.N., Kraemer, R., Poolman, B., Van der Heide, T., Smith, L.T., 2001. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 130, 437–460.

Wood, T.K., Knabel, S.J., Kwan, B.W., 2013. Bacterial persister cell formation and dormancy. Appl. Environ. Microbiol. 79, 7116–7121.

Wu, D., Rasco, B., Vixie, K.R., Ünlü, G., Swanson, B., Liu, Y., 2013. Using Fourier transform infrared (FT-IR) spectroscopy to detect sublethally- or lethally-stressed *Listeria innocua* treated with acetic acid. LWT - Food Sci. Technol. 54, 456–462.

Wu, S., Yu, P.-L., Flint, S., 2017. Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. Food Control 77, 343–250.

Wu, V.C.H., 2008. A review of microbial injury and recovery methods in food. Food Microbiol. 25, 735–744.

Wulff, G., Gram, L., Ahrens, P., Vogel, B.F., 2006. One group of genetically similar *Listeria monocytogenes* strains frequently dominates and persists in several fish slaughter and smokehouses. Appl. Environ. Microbiol. 72, 4313–4322.

Xuan, X.-T., Ding, T., Li, J., Ahn, J.-H., Zhao, Y., Chen, S.-G., Ye, X.-Q., Liu, D.-H., 2017. Estimation of growth parameters of *Listeria monocytogenes* after sublethal heat and slightly acidic electrolyzed water (SAEW) treatment. Food Control 71, 17–25.

Yamaguchi, Y., Inouye, M., 2011. Regulation of growth and death in *Escherichia coli* by toxin-antitoxin systems. Nat. Rev. Microbiol. 9, 779.

Yang, Y., Xiang, Y., Xu, M., 2015. From red to green: The propidium iodide-permeable membrane of *Shewanella decolorationis* S12 is repairable. Sci. Rep. 5.

Yoon, Y., Lee, H., Lee, S., Kim, S., Choi, K.H., 2015. Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses. Food Res. Int. 72, 25–36.

Yousef, A.E., Courtney, P.D., 2003. Basics of stress adaptation and implications in new-generation foods, in: Yousef, A.E., Juneja, V.K. (Eds.), Microbial Stress Adaptation and Food Safety. CRC Press LLC, Boca Raton.

Zilelidou, E., Karmiri, C.-V., Zoumpopoulou, G., Mavrogonatou, E., Kletsas, D., Tsakalidou, E., Papadimitriou, K., Drosinos, E., Skandamis, P., 2016a. *Listeria monocytogenes* strains which are underrepresented during selective enrichment with the ISO method might dominate during passage through simulated gastric fluid and in vitro infection of Caco-2 cells. Appl. Environ. Mcrobiology AEM-02120.

Zilelidou, E., Manthou, E., Skandamis, P., 2016b. Growth differences and competition between *Listeria monocytogenes* strains determine their predominance on ham slices and lead to bias during selective enrichment with the ISO protocol. Int. J. Food Microbiol. 235, 60–70.

Zilelidou, E.A., Rychli, K., Manthou, E., Ciolacu, L., Wagner, M., Skandamis, P.N., 2015. Highly invasive Listeria monocytogenes strains have growth and invasion advantages in

strain competition. PLoS One 10, 1–17.

Zoumpopoulou, G., Papadimitriou, K., Polissiou, M.G., Tarantilis, P.A., Tsakalidou, E., 2010. Detection of changes in the cellular composition of *Salmonella enterica* serovar Typhimurium in the presence of antimicrobial compound(s) of *Lactobacillus* strains using Fourier Transform Infrared spectroscopy. Int. J. Food Microbiol. 144, 202–207.

Zunabovic, M., Domig, K.J., Pichler, I., Kneifel, W., 2012. Monitoring transmission routes of *Listeria* spp. in Smoked Salmon Production with Repetitive Element Sequence-Based PCR Techniques. J. Food Prot. 75, 504–511.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van't Riet, K., 1990. Modeling of the Bacterial Growth Curve. Appl. Environ. Microbiol. 56, 1875–1881.

PUBLICATIONS AND PRESENTATIONS FROM THIS WORK

PUBLICATIONS

1. Sibanda, T., and Buys, E. M. (2017). Resuscitation and growth kinetics of sub-lethally injured *Listeria monocytogenes* strains following fluorescence activated cell sorting (FACS). *Food Research International*. *100*, 150-158.

ORAL PRESENTATIONS

1. Sibanda T. and Buys E.M. (2015). Stress response in food-borne pathogens: The case of *Listeria monocytogenes*. South African Society of Dairy Technology (SASDT) Northern Division Student Evening. University of Pretoria, South Africa.

2. Thulani Sibanda and Elna M. Buys (2017). Modelling the survival of acid and salt stressed *Listeria monocytogenes* in a lactic soft cheese. 50th Annual South African Society of Dairy Technology (SASDT) symposium. 9 – 11 May 2017. Kievits Kroon Country Estate, Pretoria.

3. Thulani Sibanda and Elna M. Buys (2018). Acid stress-induced persistence of *Listeria monocytogenes* and the differential expression of stress response genes in revived persister cell survivors. 51st Annual South African Society of Dairy Technology (SASDT) symposium. 24 – 25 April 2018, Kievits Kroon Country Estate, Pretoria, South Africa.

4. Thulani Sibanda and Elna M. Buys (2018). Persistence of *Listeria monocytogenes* strains from processing plants. Southern African Society for Veterinary Epidemiology and

Preventative Medicine (SASVEPM) Congress 2018. 18 – 20 June 2018, Birchwood Hotel and Conference Centre, Boksburg, South Africa.

POSTER PRESENTATIONS

1. Thulani Sibanda and Elna M. Buys (2015). Stress response and recovery of sub-lethally injured *Listeria monocytogenes*. 21st South African Association for Food Science and Technology (SAAFoST) Beinnial International Congress. 6th – 9th September 2015. Southern Sun Elangeni, Durban, South Africa.

2. Thulani Sibanda and Elna M. Buys (2016). Modelling the survival of *Listeria monocytogenes* in cottage cheese and the diversity of survivors in mixed strain challenge tests. 2016 International Dairy Federation, World dairy Summit (IDF-WDS). 15th – 21st October 2016. De Doelen, Rotterdam, The Netherlands.

3. Thulani Sibanda and Elna M. Buys (2017). Modelling the effect of acid and salt stress on the survival and diversity of *Listeria monocytogenes* in a lactic soft cheese stored at 4°C. International Association for Food Protection (IAFP 2017) Annual Meeting. 9 -12 July 2017. Tampa, Florida, USA.