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Influence of Temperature on Acid-Stress Adaptation in Listeria Monocytogenes

Qian Shen

Influence of temperature on acid-stress adaptation in *Listeria monocytogenes*

By

Qian Shen

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By

Qian Shen

Approved:

Ramakrishna Nannapaneni Associate Research Professor Food Science, Nutrition and Health Promotion (Director of Thesis)	M. Wes Schilling Associate Professor Food Science, Nutrition and Health Promotion (Committee Member)
Taejo Kim Assistant Research Professor Food Science, Nutrition and Health Promotion (Committee Member)	Zahur Z. Haque Professor Food Science, Nutrition and Health Promotion (Graduate Coordinator)
George M. Hopper Dean of the College of Agriculture and Life Sciences	

Name: Qian Shen

Date of Degree: August 17, 2013

Institution: Mississippi State University

Major Field: Food Science and Technology

Major Professor: Ramakrishna Nannapaneni

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Candidate for Degree of Master of Science

Acid-stress adaptation in *Listeria monocytogenes* (*Lm*) serotype 4b and 1/2a occurred when cells were pre-exposed to pH 5.0 tryptic soy broth supplemented with yeast extract (TSB-YE) at 22°C or 37°C but not at 4°C. Prolonged time, varied sublethal acid pH, substitute of acidulants and addition of sodium chloride during 4°C mild acid pre-exposure still did not induce acid-stress adaptation in *Lm*. This finding was also validated using an acidic cheese, similar to what has seen for Gram-negative bacteria *E. coli* and *Salmonella*. Further investigation revealed that major cold shock protein in *Lm* CspL was not responsible for repressed acid-stress adaptation at 4°C. A bead beating treatment prior to mild acid pre-exposure at 4°C partially induced acid-stress adaptation after pre-exposure in 4°C to mild acid stress. Our data suggests that cold processing or cold storage temperature can lower the possibility of activating acid-stress adaptation in *Lm*.

DEDICATION

This work is dedicated to the amazing grace of God Almighty, my parents Mr. Chun Shen and Mrs. Hongxia Shen and my respectable mentor Dr. Kamlesh Soni.

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CHAPTER I

INTRODUCTION

Listeria monocytogenes is an opportunistic foodborne pathogen that is persistent under various food processing conditions. This bacterium can tolerate harsh environmental conditions such as extreme pH (pH 3.5 - 12), high salt concentration (10% sodium chloride) and a wide range of temperature (1 - 45°C) (George and Lund, 1992, Kallipolitis and Ingmer, 2006, Van Der Veen et al., 2008). The capability of *L. monocytogenes* to tolerate lethal stress results from its innate adaptive mechanisms. Different types of stress adaptation in *L. monocytogenes* have been well documented in previous studies. For example, after being adapted to sublethal acid (pH 5.0 - 5.5), alkali (pH 9.0 - 9.5), oxidation (500 ppm H_2O_2), salt (6.0% - 8.0% NaCl) and heat stresses (43°C - 46°C), *L. monocytogenes* exhibited greater survival to the subsequent homogenous lethal inactivation treatments when compared to non-stressed *L. monocytogenes* (Gahan et al., 1996, Giotis et al., 2010, Lou and Yousef, 1997, Bolton and Frank, 1999, Pagan et al., 1997).

In the area of *L. monocytogenes* stress adaptation, acid-stress adaptation receives extensive research attention. The concept of acid-stress adaptation in *L. monocytogenes*, where cells showed much higher resistance to lethal acid inactivation treatment (pH 3.5) after being pre-exposed to mild acid for 1.5 h, was introduced by Hill (1995). Recently, some studies revealed that acid-stress adaptation in *L. monocytogenes* can also induce

cross protection against other physiological stresses (heat, salt, irradiation and HHP) and antimicrobials treatments (Nisin, bile salts and quaternary ammonium compounds) (McKinney et al., 2009, Wemekamp-Kamphuis et al., 2004, van Schaik et al., 1999, Begley et al., 2002, Lin et al., 2011, O'Driscoll et al., 1996). In addition, some other studies proposed that acid-stress adaptation has the potential to increase the virulence of *L. monocytogenes* by improving survival through gastric barriers (Ferreira et al., 2003) and up-regulation of virulence related genes such as *inlA*, *gadD2*, *bsh*, *opuCA* and *opuCB* (Sue et al., 2004, Werbrouck et al., 2009). Therefore, the risk of acid-stress adaptation in *L. monocytogenes* should be carefully considered when acidulants are applied during food processing.

In food processing plants, application of acid is a frequently used intervention technique to ensure food safety. Some acidic compounds (lactic acid, sodium diacetate or potassium lactate) are added to food products as preservatives to extend the bacterial lag phase and increase product shelf life (Oh and Marshall, 1993, Glass et al., 2002, Porto et al., 2002). Alternatively, some acid based formulations (benzoic acid, citric acid or peroxyacetic acid) are used as decontaminating agents to sterilize food products (Hwang and Beuchat, 1995, Bal'a and Marshall, 1998, Bagge-Ravn et al., 2003). In addition, numerous types of foods have naturally acidic pHs including cheese, yogurt, most fruit juices (apple, orange, grapefruit, etc.) and fermented vegetables (pickle). Acidic environments can create undesirable conditions that allow *L. monocytogenes* to have contact with sublethal acid concentrations and eventually trigger the occurrence of acid-stress adaptation in food products or on food contact surfaces.

In the process of inducing acid-stress adaptation, several parameters are critical for the in order to trigger acid-stress adaptation in *L. monocytogenes*. These triggers include mild acid concentration, mild acid pre-exposure time, bacterial growth stage, acidulants used for creating mild acidic conditions and mild acid pre-exposure temperature. Most of them have been well characterized in previous studies. For example, in broth study, pH 5.0 for 1 h was determined to be the optimal condition to induce acid-stress adaptation in *L. monocytogenes* (Davis et al., 1996). Stationary phase *L. monocytogenes* cells are naturally acid resistant due to the mild acidic media after bacterial growth. Compared to stationary phase cells, log phase cells are more acid sensitive and require mild acid pre-exposure to receive acid-stress adaptation (O'Driscoll et al., 1996, Davis et al., 1996, Datta and Benjamin, 1997). Both organic and inorganic acidulants are able to provide *L. monocytogenes* cells with evident acid-stress adaptation, different transcriptional and proteomic profiles were observed after cells were exposed to those two different types of acidulant (O'Driscoll et al., 1997, Tessema et al., 2012).

However, little attention has been devoted to the impact of temperature on the induction of acid-stress adaptation in *L. monocytogenes*. The majority of previous studies has focused on the temperatures (30°C or 37°C) which are optimal for bacterial growth. From the industrial perspective, lower temperatures (4°C to 20°C) are much more applicable because most of processing and storage is carried out within this temperature range. Therefore, this study aimed to accomplish the following targets: (1) Investigate the effect of temperature on the induction of acid-stress adaptation in 37°C or 4°C grown *L. monocytogenes* log phase cells; (2) Determine whether acid-stress adaptation could occur in sublethal acidic conditions at 4°C by extending pre-exposure time, varying mild acid

concentrations, varying acidulant type and adding sodium chloride during pre-exposure; (3) Determine the induction of acid-stress adaptation in *L. monocytogenes* after pre-exposure in pH 5.1 acidic cheese whey; (4) Evaluate the effect of on the induction of acid-stress adaptation in *E. coli* and *Salmonella*; (5) Investigate the role of the cold shock protein CspL on repressed acid-stress adaptation at 4°C; and (6) Evaluate whether bead beating prior to mild acid pre-exposure can facilitate acid-stress adaptation at 4°C.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction to *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, nonsporeforming, facultative anaerobic, catalase-positive and oxidase negative rod (Farber and Peterkin, 1991). This bacterium can grow in a wide range of environmental conditions (pH 4.1-9.5, 0°C-43°C and 0-14% NaCl) (Gill et al., 1997, Shabala et al., 2008, van der Veen et al., 2009). On classification, *L. monocytogenes* is under the genus *Listeria* which typically consists of other 6 *Listeria* species including *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayii*, *L. ivanovii* and *L. murrayi* (MacGowan et al., 1989). Recently, two *Listeria* species, *L. marthii* and *L. rocourtiae*, are newly identified and are both be avirulent (Leclercq et al., 2010, Graves et al., 2010). Among *Listeria* species, only two of them, *L. monocytogenes* and *L. ivanovii*, are pathogenic. *L. monocytogenes* can infect human beings and is responsible for the occurrence of listeriosis. *L. ivanovii* was previously thought to be only specific for ruminants infection (Vazquez-Boland et al., 2001), but recent study reveals that it is also an enteric opportunistic human pathogen (Guillet et al., 2010). Based on somatic and flagellar antigens, *L. monocytogenes* is divided into 13 different serotypes, among which 1/2a, 1/2b and 4b are responsible for most of listeriosis outbreaks (Farber and Peterkin, 1991, Cartwright et al., 2013). From the evolutionary perspective, *L. monocytogenes* is classified into 4 lineages (I, II, III and IV). Lineage I and II includes

serotypes 1/2a, 1/2b and 4b which are closely associated with listeriosis outbreaks, while lineage III and IV strains are not very common and mainly isolated from animals sources (Orsi et al., 2011).

L. monocytogenes infection in human bodies can cause various clinical symptoms such as gastroenteritis, meningitis and meningoencephalitis (Stanley, 1948, Whitty and Macaulay, 1965, Barrie, 1967). The infection process of *L. monocytogenes* has been well characterized using *in vitro* tissue culture assays and *in vivo* animal models. After being ingested, *L. monocytogenes* cells pass through the gastric fluid barrier, reach the large intestine and initiate invasion to M cells, dendritic cells Ramakrishna Nannapaneni and epithelial cells (MacDonald and Carter, 1980, Pron et al., 2001). During invasion of epithelial cells, two *L. monocytogenes* cells surface proteins (internalin A and internalin B) contribute to the interaction with host cell surface and initiate the invasion process (Gaillard et al., 1991). After entering the host cell, the bacterium is captured by host cell intracellular vacuoles. The secreted toxin (hemolysin) and two phospholipases (PlcA and PlcB) allow *L. monocytogenes* cells to rupture vacuole and escape to cytoplasm (Kathariou, 2002, Pizarro-Cerda et al., 2012). Thereafter, *L. monocytogenes* cells start to invade neighboring host cells depending on another cell surface protein (ActA) which is essential for polymerizing host cell actins to form moving tails that facilitate the penetration of host cell membrane by *L. monocytogenes* (Theriot et al., 1994, Cossart, 1995). Subsequently, *L. monocytogenes* are translocated to the liver and spleen, where resident macrophages kill most of the *L. monocytogenes* cells by producing nitric oxide and interferons (Lepay et al., 1985, Stuehr and Marletta, 1985, O'Riordan et al., 2002).

Finally, any *L. monocytogenes* cells that survive liver inactivation are able to infect hepatocytes and induce systemic infection (Kathariou, 2002).

USDA-FSIS has zero tolerance for *L. monocytogenes* in ready-to-eat (RTE) food products due to its severe pathogenicity (Klima and Montville, 1995). The infection of *L. monocytogenes* in human beings can subsequently result in listeriosis with about 30% mortality (Ramaswamy et al., 2007) which is much higher than that caused by other foodborne pathogens such as *Salmonella* spp. (Cummings et al., 2010) and *Campylobacter* spp. (Nielsen et al., 2010, Fernandez-Cruz et al., 2010). According to the CDC (2006), during 1998-2002, though the occurrence of *L. monocytogenes* outbreaks was less than 1.0% of total outbreaks, it was responsible for more than 50% deaths caused by bacterial foodborne pathogens in the United States. Food related listeriosis outbreaks usually starts with contamination of *L. monocytogenes* in food products including unfermented dairy products, cheese, meat, fish and seafood (Ryser and Marth, 2007). Among those RTE food products, meat and dairy products are the most frequent vehicles of *L. monocytogenes* transmission which come either from *L. monocytogenes* cells present on raw food materials or post-processing contamination from processing environments. From 1998 to 2008, 5 out of 24 listeriosis outbreaks were associated with contaminated cheese and 10 of them were caused by contaminated frankfurters, deli meat or grilled chicken (Cartwright et al., 2013). For example, deli meats caused multi-state listeriosis outbreak from early August 1998 through January 1999 that resulted in 6 deaths and 2 abortions (CDC, 1999). In 2000, one listeriosis outbreak was caused by Mexican style cheese made from *L. monocytogenes* contaminated milk (MacDonald et al., 2005). During 2000 to 2002, two multi-state listeriosis outbreaks occurred due to

contaminated turkey meat (Olsen et al., 2005, Gottlieb et al., 2006). Recently, a listeriosis outbreak from fruits was also reported. A multi-state listeriosis outbreak occurred in 2011 due to tainted cantaloupes caused 30 deaths and spread throughout 28 states (CDC, 2011).

2.2 Stress adaptation in *Listeria monocytogenes*

Stress occurs due to environmental conditions (extreme pH, high heat, strong oxidation, freezing, etc.) that are detrimental to bacterial growth or survival. *L. monocytogenes* is equipped with sophisticated stress adaptive mechanisms to counteract unfavorable environmental conditions. Stress adaptation enables *L. monocytogenes* to tolerate much higher level of lethal stress after mild stress pre-exposure. For example, after being pre-exposed to sublethal acid (pH 5.0-5.5), alkali pH (pH 9.0-9.5), oxidative stress (500 ppm H_2O_2), salt (6.0%-8.0% NaCl) and heat stresses (43°C-46°C), *L. monocytogenes* exhibited greater survival in subsequent homogenous lethal inactivation treatments (Gahan et al., 1996, Giotis et al., 2010, Lou and Yousef, 1997, Bolton and Frank, 1999, Pagan et al., 1997). The phenomenon of stress adaptation is universal and its observation in many other model microorganisms such as *Escherichia coli* and *Salmonella* spp. has also been well documented (Foster, 1999, Meury and Kohiyama, 1991, Briolat and Reysset, 2002).

Acid adaptation in *L. monocytogenes*, where cells showed much higher resistance to low pH lethal inactivation treatment after being pre-exposed to mild acid for 1.5 h, was first proposed by Hill (1995). Additional studies confirmed the validity of this concept and revealed that acid adaptation in *L. monocytogenes* requires protein *de novo* synthesis (Davis et al., 1996, O'Driscoll et al., 1996). Numerous studies have been conducted to

understand the phenomenon of acid adaptation in *L. monocytogenes* due to its relevance to food safety area. In the food industry, one of the most commonly used interventions is acid based. Some acidic compounds (lactic acid, sodium diacetate or potassium lactate) are added to food products as preservatives to extend the bacterial lag phase and increase product shelf life (Oh and Marshall, 1993, Glass et al., 2002, Porto et al., 2002). Alternatively, some acid based formulations (benzoic acid, citric acid or peroxyacetic acid) are used as decontaminating agents to sterilize food products (Hwang and Beuchat, 1995, Bal'a and Marshall, 1998, Bagge-Ravn et al., 2003). Many kinds of food products such as cheese, yogurt, apple cider and orange juice have naturally acidic pHs. Acidic environments in food processing plants increases the chance of allowing *L. monocytogenes* to have contact with sublethal acid concentration and subsequently trigger acid adaptation.

2.3 Genetic mechanisms of acid resistance and acid adaptation in *Listeria monocytogenes*

Several genetic mechanisms have been postulated that explain how *L. monocytogenes* cells have ability to resist acid stress. Arginine deiminase (ADI) system and glutamate decarboxylase (GAD) system are proposed to have critical roles in acid tolerance response of *L. monocytogenes*. The ADI system is involved in the production of ammonia that neutralizes intracellular pH in a variety of bacteria. This system contains three enzymes arginine deiminase, ornithine carbamoyltransferase and carbamate kinase, that are encoded by *arcA*, *arcB* and *arcC*, respectively (Cotter and Hill, 2003). These enzymes catabolize arginine to yield ammonia, carbon dioxide and ornithine (Cunin et al., 1986). In addition, within the ADI system, *argR* encodes a putative activator

controlling the expression of downstream genes of the ADI cascade. Transcriptional analysis showed that *argR*, *arcA*, *arcB* and *arcC* were significantly expressed at a pH of 5.0 TSB-YE (Ryan et al., 2009). At phenotypic confirmation, the absence of the ADI gene cascade in *L. monocytogenes* lead to reduced resistance to acid stress. Compared to the parent strain, the deletion of *argR* or *arcA* resulted in 2-4 log CFU/ml reduced survival in pH 3.5 TSB-YE and approximately 30 h delayed growth in pH 4.8 TSB-YE (Ryan et al., 2009). Compared to the wild type strain, the *arcB* (encoded by *lmo336*) deficient strain exhibited a longer lag phase in sublethal acid stress (pH 4.8) and approximately reduced survival by 1 log CFU/ml synthetic human gastric fluid with a pH of 2.5 (Chen et al., 2011).

The GAD system, which is composed of glutamate decarboxylase and glutamate/γ-aminobutyrate antiporter, utilizes glutamate to consume protons to increase bacterial intracellular pH (Cotter and Hill, 2003). In this system, decarboxylase works as an enzyme to convert glutamate into γ-aminobutyrate by consuming protons. The glutamate/γ-aminobutyrate antiporter pumps γ-aminobutyrate out of the cells to exchange for another glutamate uptake that continues the decarboxylation process in cytoplasm. So far, five GAD genes have been identified in *L. monocytogenes*, encoding three types of glutamate decarboxylase (GadA, GadB and GadD) and two types of antiporter (GadC and GadE) (Wemekamp-Kamphuis et al., 2004, Cotter et al., 2005). GadA and GadE were identified in *L. monocytogenes* LO28 to be responsible for cell growth in pH 5.1 sublethal acidic conditions while GadB and GadC mainly helped cells survive at a pH of 2.8 (Cotter et al., 2005). At phenotypic level, in the presence of monosodium glutamate, wild type *L. monocytogenes* significantly improved its survival in lethal acidic conditions

due to the activity of the GAD system. Addition of 10 mM monosodium glutamate exogenously in culture media increased the survival of *L. monocytogenes* LO28 by 3 log CFU/ml in pH 2.5 gastric fluid (Cotter et al., 2001a) or reconstituted skim milk (Cotter et al., 2001b) in comparison to cell treatment that did not receive glutamate molecules. Moreover, such survival differences in lethal pH by the addition of sodium glutamate were not conferred to the mutant strain that was deficient in GAD activity. Recently, an atypical operation model of the GAD system has been observed in *L. monocytogenes*. Typically, the reaction product of glutamate decarboxylation, γ-aminobutyrate is exported to exchange for glutamate uptake in the GAD system. However, Karatzas et al. (2010) reported that γ-aminobutyrate was accumulated in the cytoplasm instead of being exported out of cell during the lethal acid challenge (pH 2.5) if the *L. monocytogenes* cells grew in chemically defined media which did not contain any glutamate. It is proposed that some components in complex media such as BHI are essential for export γ-aminobutyrate in the GAD system.

Similar to *L. monocytogenes*, the GAD system conferred acid resistance is also observed in gram-negative bacteria such as *E. coli*. The system consists of glutamate decarboxylase (encoded by *gadA* or *gadB*) and antiporter (encoded by *gadC*) (Smith et al., 1992, Blattner et al., 1997). Deletion of *gadB* and *gadC* resulted in 1-2 log CFU/ml reduced survival in pH 2.0 Luria Bertani (LB) broth when compared to the parent strain (Castanie-Cornet et al., 1999). Two regulators, GadE and RcsB, are necessary to induce the expression of *gadA/B* and subsequently provide *E. coli* with acid resistance at lethal acid stress (pH 2.5) (Castanie-Cornet et al., 2007). Another study indicated that the outer membrane channel TolC, a component of the multi-drug resistance efflux pumps

component, was required for the expression of GadA and GadB to help *E. coli* cells grow between pHs 4.5 and 6.0 and survive at pH of 2.0 (Deininger et al., 2011). In *Salmonella* spp., arginine and lysine decarboxylase systems mediated acid resistance is more commonly observed when compared to the GAD system. The composition of those two systems is quite similar to the GAD system which includes arginine/lysine decarboxylase and an antiporter (Park et al., 1996, Kieboom and Abee, 2006). Deletion of any of the genes (*adiA*, *adiC* and *adiY*) in the arginine decarboxylase system in *Salmonella* Typhimurium reduced survival in pH 2.5 broth when compared to wild type strain (Kieboom and Abee, 2006). Another study revealed that lysine decarboxylase CadA not only protected *Salmonella* Typhimurium against lethal acid challenge at a pH of 2.3 but also promoted cell growth in sublethal acid stress at a pH of 4.5 (Viala et al., 2011).

In addition to GAD and ADI systems, various sigma factors also play important roles in the regulation of the acid stress response. Sigma factors are the key components in bacterial gene expression that bind to RNA polymerase and subsequently initiate transcription (Gruber and Gross, 2003). Five sigma factors have been identified in *L. monocytogenes* which are divided into two groups: the σ^{70} family (σ^{B}, σ^{C}, σ^{D} and σ^{H}) and single σ^{54} family (σ^{L}) (Glaser et al., 2001). Among these factors, the alternative σ^{B} is the most well characterized in *L. monocytogenes*. It has been demonstrated by several authors that σ^{B} is required for *L. monocytogenes* to show acid resistance in lethal acid stress (pH 2.5) (Wiedmann et al., 1998, Ferreira et al., 2001, Ferreira et al., 2003, Wemekamp-Kamphuis et al., 2004). Some insightful studies indicated that σ^{B} conferred acid resistance in *L. monocytogenes* mainly attributed to σ^{B}-dependent regulation of acid stress response genes. For example, Kazmierczak et al. (2003) reported that the

expression of *gadB* (encoding glutamate decarboxylase) in wild type strain was 5.3-fold higher than that of Δ*sigB* strain in stationary phase *L. monocytogenes* cells. The expression of ADI system is also under control of σ^B as *arcA* and *argR* were significantly down-regulated in Δ*sigB* strain compared to wild type strain (Ryan et al., 2009). Another study revealed that deletion of σ^B in *L. monocytogenes* reduced the expression of acid tolerance related genes *lmo0913*, *lmo0796* and *lmo2391* (encoding succinate semialdehyde dehydrogenase, YceI-like family protein and putative oxidoreductase, respectively) which provided *L. monocytogenes* with protection against lethal acidic condition at a pH of 2.5 (Abram et al., 2008). In addition to contribution of σ^B to acid resistance, the role of σ^B on activation of acid adaptation in *L. monocytogenes* also has also been well investigated. At the molecular level, *gadB*, *gadC* and *gadD* were significantly induced after 1 h of acid pre-exposure in at a pH of 4.5 for wild type strain, while these differential gene expressions after mild acid pre-exposure were not observed in Δ*sigB* strain. At the phenotypic level, mild acid stress in pH 4.5 BHI broth for 1 h, wild type strain increased about 2 log CFU/ml survival whereas Δ*sigB* strain did not show any significant survival improvement in BHI broth (pH 2.5) (Wemekamp-Kamphuis et al., 2004). Apart from σ^B, σ^L is also another important regulon in *L. monocytogenes* that is critical in acid adaptation (Raimann et al., 2009) as well as bacteriocin resistance, osmotic tolerance and cold adaptation (Dalet et al., 2001, Okada et al., 2006, Chan et al., 2008). Transcription analysis indicated that σ^L was significantly induced in the presence of organic acids during growth (Raimann et al., 2009, Tessema et al., 2012). Another study showed that after 9 weeks growth in mild acidic conditions (pH 5.5 or 6.0) at 4°C, the wild type *L. monocytogenes* strain had 5 log CFU/ml more growth

in comparison to the Δ*sigL* strain (Mattila et al., 2012). The detailed mechanisms of how σ^L regulate acid stress response in *L. monocytogenes* remains elusive and more studies are needed to understand the hierarchy of σ^L mediated direct and indirect regulons of acid adaptations.

Moreover, a two-component system consisting of histidine kinase to sense environmental signal and cytoplasmic response regulons to activate target gene transcriptions appears to have an important role on *L. monocytogenes* acid resistance (Stock et al., 1989). A two-component system encoded by *lisRK* controls the growth-phase-dependent acid resistance in *L. monocytogenes*. Compared to the parent strain, the Δ*lisK* strain exhibited an approximately 10-fold higher sensitivity to pH 3.5 TSB-YE during the exponential phase but showed about 1.5 log CFU/ml greater survival after entering the stationary phase (Cotter et al., 1999). The deletion of LBA1524HPK-LBA1525RR, a two-component system similar to *lisRK*, in *Lactobacillus acidophilus* rendered 2 log CFU/ml reduced survival in MRS with a pH of 3.5 compared to the wild type strain (Azcarate-Peril et al., 2005). In model gram-negative bacteria *E. coli*, a well known two-component system EvgS/EvgA provides cells with acid resistance by up-regulating acid resistance genes *gadABC* and *hdeAB* (Masuda and Church, 2002). Activation of another two-component system PhoQ/PhoP in *E. coli* leads to increased expression of acid resistance genes (*gadW* and *hdeA*) and subsequently enables cells to counteract acid stress (Zwir et al., 2005).

Use of genomic profiling and mutant strains have also identified a few other genes and their protein products that participate in controlling the acid tolerance in *L. monocytogenes*. UvrA, a well known protein that is involved in DNA repair system, was

required for *L. monocytogenes* growth since the growth of Δ*uvrA* strain was significantly delayed at a pH of 5.0 defined minimal medium compared to wild type strain (Kim et al., 2006). Similarly, another DNA repair protein, RecA was essential for *L. monocytogenes* survival when exposed to lethal acidic conditions. When compared to the wild type strain, *recA* deletion in *L. monocytogenes* resulted in about a 2 log CFU/ml reduced survival in a lethal acid challenge with a pH of 1.8-3.4 (van der Veen and Abee, 2011). A putative transcription activator protein (encoded by *lmo0501*) was observed to promote the growth of *L. monocytogenes* under sublethal acidic, osmotic and cold stresses. When compared to the wild type strain, the Δ*lmo0501* strain showed 1-2 log CFU/ml reduced growth after 9 weeks of incubation at 4°C in mild acidic BHI broth with a pH 5.5 or 6.0 (Michel et al., 2011). Recently, gene *thiT* was found to be involved in acetoin production via consumption of protons to help *L. monocytogenes* to combat lethal acid stress. A transposon mutant incorporating a Tn917 insertion in the *thiT* gene rendered about 2 log CFU/ml reduced survival in pH 3.0 BHI broth in comparison to the wild type strain (Madeo et al., 2012).

2.4 Acid adaptation leads to altered cell envelope of *Listeria monocytogenes*

Research results suggest that alteration of cell envelope in *L. monocytogenes* occurs during the process of acid adaptation. Fatty acid profile of cell membranes is modulated after acid adaptation in *L. monocytogenes* cells. Compared to non-adapted cells, mild acid (pH 5.0-5.5) stressed cells down-regulated the biosynthesis of branch chain fatty acids in cell membrane (Giotis et al., 2007, Mastronicolis et al., 2010). Another study showed that after acid adaptation in pH 5.5 for 1 h, *L. monocytogenes* cells yielded increased production of straight-chain fatty acids (C14:0 and C16:0) in the cell

membrane (van Schaik et al., 1999). In addition, decreased membrane fluidity was also observed in acid-adapted cells. Sublethal concentrations of acid decontaminants (acidified sodium chloride, citric acid and peroxyacids) were used to treat *L. monocytogenes* and cells exhibited higher anisotropy values than untreated cells, which indicates that pre-exposure in these acidic decontaminant compounds decreased their cell membrane fluidity (Alonso-Hernando et al., 2010). Moorman et al. (2008) reported that acid adaptation in *Listeria innocua* decreased the cell membrane fluidity due to reduced production of C_{15} unsaturated fatty acids which are more fluid compared to other long chain fatty acids. Similar to *L. monocytogenes*, modulated composition of cell membrane fatty acids after mild acid exposure has also been confirmed in model studies with *E. coli*. Acid-adapted *E. coli* O157:H7 cells decreased membrane fluidity by producing more palmitic acid and less cis-vaccenic acid (Yuk and Marshall, 2004). During acid habituation in pH 5.0 TSB, *E. coli* converted monosaturated fatty acids into cyclopropane derivatives or saturated fatty acids. It is proposed that increased synthesis of cyclopropane fatty acids in the *E. coli* cell membrane protected the cells against lethal acid inactivation at a pH of 3.0 (Brown et al., 1997).

Besides the altered fatty acid composition in the cell membrane, modifications of other components of the cell envelope are also involved during mild acid exposure. After being exposed to mild acid (pH 5.0) and sublethal sodium chloride concentration (10%) simultaneously, cell filamentation was formed in *L. monocytogenes* and the cell surface became hydrophilic. This change in cell surface properties could subsequently influence the cell adhesion capacity (Bereksi et al., 2002). Acid adaptation also induces differential expression of *L. monocytogenes* cell surface protein. After cultivation in TSB-YE (pH

5.0), down-regulation of flagellin and up-regulation of P60 protein (invasion associated protein) was observed (Tresse et al., 2006).

2.5 Acid adaptation of *Listeria monocytogenes* induced responses to other physiological stresses

A multi-hurdle approach is the new norm for the food industry to safeguard food products from persistence of pathogenic microorganisms. In this approach, more than one lethal stressor from heat, acid, alkali, antimicrobials and physical treatments such as HHP or irradiation are used as it has been well recognized that a single hurdle approach is not very reliable for the inactivation of foodborne pathogens (Tokarskyy and Marshall, 2008, Brown et al., 2011, Kang et al., 2012). Interestingly, this multi-hurdle approach also renders the possibility of stressed hardening in which the surviving population from first stress exposure was protection against subsequent inactivation treatments.

Several studies have attempted to understand how pre-exposure to sub-lethal acid stress protects the *L. monocytogenes* cells against other lethal inactivation treatment by inducing a cross resistance response. Sodium chloride is most widely used preservative approach in food industry. Compared to non-adapted cells, acid-adapted *L. monocytogenes* cells exhibited about 3 log CFU/ml more survival after 10 days incubation in 2.5M sodium chloride (O'Driscoll et al., 1996). Likewise, it was reported that mild acetic acid (pH 5.5) was able to provide *Salmonella* spp. cells with about 100-folds greater resistance to 2.5M of sodium chloride or potassium chloride (Greenacre and Brocklehurst, 2006). Four out of 9 tested *L. monocytogenes* strains exhibited 1-3 log CFU/ml greater survival in 3.4M of sodium chloride after acid adaptation in pH 5.5 for 2-4 h (Faleiro et al., 2003).

In addition, enhanced heat tolerance in *L. monocytogenes* after acid adaptation has been observed in several research articles. For example, at lethal heat temperatures (54°C), acid-adapted *L. monocytogenes* cells exhibited about 2 log CFU/ml more survival than control cells (O'Driscoll et al., 1996, Marron et al., 1997). Another study showed that acid adaptation in pH 5.0 for 1.0 or 1.5 h assisted *L. monocytogenes* cells to survive about 1.5 log CFU/ml more at 57°C lethal inactivation (Skandamis et al., 2008). The increased thermal tolerance due to acid adaptation also occurred in other foodborne pathogens such as *E. coli* O157:H7 and *Salmonella* spp. cells. Cheng et al. (2002) reported that 1-2 log CFU/ml increased survival at 52°C was observed in *E. coli* O157:H7 after pre-adaptation in pH 5.0 TSB for 4 h. Recently, another study found that acid-adapted *E. coli* O157:H7 cells showed enhanced thermal tolerance in fresh compost at 55°C (Singh and Jiang, 2012). For *Salmonella* Typhimurium cells, acid adaptation increased the decimal reduction times (D-value) at 59°C or 61°C by 0.16 and 0.05 min, respectively (Bacon et al., 2003). Growth in acidified media (pH≈4.5) protected *Salmonella* spp. cells against inactivation by cooking at 62°C (Singh et al., 2006) and increased the D-value at 58°C by about two times in fruit juice (Alvarez-Ordonez et al., 2009).

Apart from increasing thermal tolerance, acid adaptation can also confer *L. monocytogenes* with enhanced resistance to non-thermal processing inactivation treatments. Mild acid-stressed (pH 5.0) *L. monocytogenes* cells exhibited about 2 log CFU/ml higher survival than non-stressed cells after 5 min UV light inactivation (McKinney et al., 2009). In addition, Wemekamp-Kamphuis et al. (2004) reported that acid adaptation helped *L. monocytogenes* cells resist high hydrostatic pressure and

freezing. Prior adaptation in pH 4.5 for 1 h conferred *L. monocytogenes* cells with 2 log CFU/ml more survival after 20 min inactivation by 350 MPa or 5 cycles of freeze-thaw, respectively.

Since acid-stressed resistant phenotypes of *L. monocytogenes* appear resistant to most physiological stresses, it is imperative to understand the mechanisms behind this phenomenon in order to inactivate this pathogen under food processing conditions. Unfortunately, the exact molecular mechanisms conferring cross protection are not well studied. There are limited evidences indicating that some of the acid adaptation induced proteins may also have critical and overlapping function against other lethal inactivation treatments. For example, the *opuCABCD* operon in *L. monocytogenes* is significantly induced in mild acidic conditions (Sue et al., 2004, Werbrouck et al., 2009, Tessema et al., 2012). This operon encodes compatible solutes transporter proteins which contribute to osmotolerance in *L. monocytogenes* (Cetin et al., 2004, Okada et al., 2006). Proteomic analysis revealed that the oxidative stress response regulon LexA mediated SOS DNA repair response was activated in pH 4.5 TSB-YE (Bowman et al., 2012). LexA contributes to single strand DNA repair that is caused by mutagenesis inducing treatments such as antibiotics and irradiation (Aburatani and Horimoto, 2005, van der Veen et al., 2010). Apart from acid adaptation induced protective proteins, it is plausible that enhanced σ^B activity after acid adaptation also protects the cells from other lethal treatments as σ^B is a master regulator that contributes to numerous stress responses such as osmotic (Becker et al., 1998) and heat stress (Somolinos et al., 2010).

2.6 Acid adaptation of *Listeria monocytogenes* induced responses to antimicrobials treatments

Since the formation of acid adapted *L. monocytogenes* during plant equipment cleaning processes and subsequent transfer to food products is a probable scenario, understanding of how acid-adapted cells behave in the presence of chemical preservatives is an important area of investigation. In one study, 80% of acid-adapted *L. monocytogenes* strains could recover from Nisin containing fermented broth after 30 days whereas non-adapted cells were inactivated completely during the fermentation process (Bonnet and Montville, 2005). Similarly, Van Schaik et al. (1999) reported that *L. monocytogenes* cells exhibited increased resistance to Nisin by 10-fold after being adapted to pH 5.5 for 1 h. Similar to *L. monocytogenes*, sublethal acid stress induced cross protection against antimicrobial agents that also occurs in *E. coli* and *Salmonella* spp. Mild acid pre-exposure also conferred *E. coli* and *Salmonella* cells with cross protection from nisin, curvacin A and lactoperoxidase inactivation (Ganzle et al., 1999, Parry-Hanson et al., 2010, Leyer and Johnson, 1993).

Apart from GRAS antimicrobials, some information is also available on the behavior of acid-adapted *L. monocytogenes* in disinfectants. Acid-adapted *L. monocytogenes* exhibited a 2 to 3 log CFU/ml higher survival than non-adapted cells in 15% ethanol or 100 ppm crystal violet, respectively (O'Driscoll et al., 1996, Marron et al., 1997). Adaptation in pH 5.5 increased about 3 log CFU/ml survival in 0.3% bile salt (Begley et al., 2002). Acid adaptation in *L. monocytogenes* enhanced cells survival by 1-2 log CFU/ml in 0.2% quaternary ammonium compounds (Lin et al., 2011). Similarly, *L. innocua* became more resistant to quaternary ammonium compound cetrimide after acid adaptation (Moorman et al., 2005).

There are also some other noteworthy scenarios in which acid adaptation in *L. monocytogenes* does not result in increased resistance against antimicrobial agents. For example, acid-adapted and non-adapted *L. monocytogenes* cells did not show different sensitivity to 200 ppm sodium hypochlorite and 150 ppm peroxyacetic acid in beef washings (Stopforth et al., 2002). Compared to non-adapted cells, acid-adapted cells of *L. monocytogenes* showed similar or increased susceptibility to sodium hypochlorite inactivation (0.1 to 1.0%) (Adriao et al., 2008). In *Salmonella* spp., after acid adaptation, cells showed about 1000-fold increased sensitivity to hypochloric acid or hydrogen peroxide (Leyer and Johnson, 1997, Greenacre et al., 2006). Those research works indicate that utilization of oxidative antimicrobial treatments could be an efficient approach to remove acid-resistant phenotypes of *L. monocytogenes* during food processing.

Acid adaptation induced cross resistance to antimicrobials agents in *L. monocytogenes* raises concern of the efficacy of commonly used antimicrobial agents in the food industry. In order to improve the efficacy of antimicrobial agents, it is necessary to understand the mechanisms behind acid adaptation induced antimicrobial resistance. Increased expression of sigma factors after acid adaptation could be a major explanation for this phenomenon as it has been demonstrated by several authors that sigma factors are actively involved in the antimicrobial resistance of *L. monocytogenes*. Compared to the wild type strains, the Δ*sigB* strain exhibited 1-3 log CFU/ml reduced survival after lethal challenge in 40 ppm benzalkonium chloride, 3 ppm cetylpyridinium chloride, 1.0% sodium docecyl sulfate and 20 ppm peracetic acid (Ryan et al., 2008, van der Veen and Abee, 2010). Another study revealed that deletion of σ^B or σ^L in *L. monocytogenes* caused

increased sensitivity to antimicrobial peptides SdpC and nisin (Palmer et al., 2009). On the other hand, most of the antimicrobials need to interact with the cell membrane of *L. monocytogenes* to show listericidal activity, it is postulated that the altered cell membrane in *L. monocytogenes* after acid adaptation could reduce antimicrobial uptake or change the target sites where antimicrobials can bind to the cell surface. For example, increased nisin resistance and altered membrane fatty acid composition has been observed in acid-adapted *L. monocytogenes* cells. (van Schaik et al., 1999). Decreased membrane fluidity in *L. innocua* after acid adaptation resulted in increased resistance to quaternary ammonium compound (Moorman et al., 2008). Based on these scientific findings, more than one type of antimicrobial agent are recommended to be applied together in the food industry to improve inactivation efficacy against acid-resistant phenotypes of *L. monocytogenes*.

2.7 Influence of acid adaptation on the virulence of *Listeria monocytogenes*

Several studies have demonstrated that acid adaptation indivertibly confers *L. monocytogenes* with higher virulence potential. The main postulation for this observation is that acid adaptation ultimately confers better survivability of *L. monocytogenes* cells in the lethal gastric fluid barrier or human immune systems. Using lab assay models, several authors have validated that acid-adapted *L. monocytogenes* as well as other foodborne pathogens had greater survival in simulated gastric fluid juice medium with pH 1.5-2.5 (Ferreira et al., 2003, Yuk and Schneider, 2006, Yuk and Marshall, 2005, Yuk and Marshall, 2004). In an *in vitro* assay model, Cataldo et al. (2007) reported that acid adapted cells had 4-fold higher invasion efficiency when compared to non-adapted control cells. Similarly, another *in vitro* study found that acid-adapted *L. monocytogenes*

cells survived better than non-adapted cells in activated human macrophage (Conte et al., 2002). An *in vivo* experimental model using murine intragastric infection showed that acid adaptation of *L. monoctyogenes* improved the cell survival during gastric fluid inactivation and increased the number of live cells that reached intestinal compartments (Saklani-Jusforgues et al., 2000). A few other *in vivo* studies indicated that acid-adapted *L. monocytogenes* cells had better survivability in mice spleen in comparison to non-adapted cells (O'Driscoll et al., 1996) or cells deficient in acid tolerance response (Marron et al., 1997).

Apart from the acknowledged advantage of survivability in lethal gastric pH and human immune systems, other molecular mechanisms have also been presented for increased virulence potential for acid-adapted *L. monocytogenes* cells. Compared to non-adapted cells, acid-adapted *L. monocytogenes* cells have been shown to have higher expression of virulence related genes such as *inlA*, *gadD2*, *bsh*, *opuCA* and *opuCB* (Sue et al., 2004, Werbrouck et al., 2009). *InlA* encodes invasion proteain InlA which is primarily responsible for the initiation of *L. monocytogenes* in host epithelial cells (Gaillard et al., 1991). *GadD2* and *bsh* encode glutamate decarboxylase and bile salt hydrolase, respectively (Cotter et al., 2005, Dussurget et al., 2002). The expression of these two enzymes helps *L. monocytogenes* cells counteract the antimicrobial activities of gastric fluid and bile salts to enhance their survivability in the gastro-intestinal tract. *OpuCA* and *opuCB* are the two major components of the *opuC* operon which encodes the L-carnitine transporter in *L. monocytogenes* (Fraser et al., 2000) and helps cells to colonize onto animal's small intestines (Sleator et al., 2001). These observations suggest that increased virulence potential in *L. monocytogenes* after acid adaptation could be a

complex event with the potential for initial mild acid adaptation being the activator of key virulence genes that subsequently confer advancement of *L. monocytogenes* in host invasion. So far, not much work has been done with practically relevant organic acid based preservatives but one study reported that sodium diacetate and sodium lactate exposure increased the invasion capacity of *L. monocytogenes* to Caco-2 cells but could not provide protection against gastric fluid (Garner et al., 2006).

Some additional insight into acid adaptation induced virulence is also available from other model gram negative microorganisms such as *E. coli* and *Salmonella*. Similar to *L. monocytogenes*, acid adaptation also appears to confer increased virulence potential in these two pathogens. For example, *E. coli* O157:H7 cells subjected to sequential sublethal (pH 5.0) and lethal acid stress (pH 3.0) showed enhanced adhesion potential in *in vitro* assay model (House et al., 2009). Likewise, an *in vivo* study demonstrated that acid tolerant *Salmonella* Enteritidis tended to be more virulent in mice and showed higher invasiveness in the reproductive tissues of laying hens (Humphrey et al., 1996). There are also some unique findings observed in *E. coli* that have not been reported for *L. monocytogenes* yet. This include that increased virulence potential after acid exposure is not always linked to the up-regulation of key virulence determinants. For example, low pH acid stress (pH 3.0-5.0) enhanced host cell adhesion capacity of enteropathogenic *E. coli*, though little alteration of the expression of well known adhesins (bundle forming pilus, intimin and flagella) was observed between acid-stressed and non-stressed cells (de Jesus et al., 2005). Another study reported that long-term acid adaptation in pH 5.5 BHI for enteropathogenic *E. coli* repressed the expression of virulence genes but conferred this bacterium with improved adhesion capacity to Caco-2 cells (Olesen and Jespersen,

2010). Hence, the complex link between acid adaptation and expression of various direct and indirect regulators of pathogenicity for observed increased invasion capacity is not clear. Additional work such as using microarray or proteomics that look at sequential changes (i.e. during initial acid adaptation, during lethal gastric pH exposure and during invasion process) in expression profile for various gene or protein products are needed.

2.8 Factors controlling the induction of acid adaptation in *Listeria monocytogenes*

During the process of acid adaptation, several variables such as type of acidulants used for pre-exposure, bacterial strains, growth phase of bacteria, mild acid pH, pre-exposure time and pre-exposure temperatures can significantly influence the acquirement of acid adaptation in *L. monocytogenes*. Therefore, with a clear understanding of the conditions that induce acid adaptation, it is possible to reduce the occurrence rate of acid-resistant phenotypes of *L. monocytogenes* in the food industry.

According to the literature, organic and inorganic acids can differentially induce acid adaptation in *L. monocytogenes*. For example, it has been observed that *L. monocytogenes* showed different protein expression profiles after being exposed to lactic acid and hydrochloric acid (O'Driscoll et al., 1997). At the transcriptional level, compared to mild hydrochloric acid pre-exposure, lactic acid mild pre-exposure triggered an increased expression level of acid adaptation related genes (Tessema et al., 2012). Unlike *L. monocytogenes*, the induction of acid adaptation for *Salmonella* spp. was independent of the type of acidulants used for mild acid pre-exposure as there were no survival differences among lactic, citric and hydrochloric acid-adapted cells after lethal acid exposure (Arvizu-Medrano and Escartin, 2005, Alvarez-Ordonez et al., 2010). Different *L. monocytogenes* strains exhibited various adaptive responses to mild acidic

conditions. Out of four *L. monocytogenes* strain isolates from the dairy industry, two were isolated from raw ewe's milk that was used to make cheese and exhibited appreciable acid adaptation whereas the other two isolated from cheese made from raw cow's milk were naturally acid resistant (Adriao et al., 2008). The growth phase of *L. monocytogenes* influences the occurrence of acid adaptation since stationary phase cells are naturally acid resistant, potentially due to generation of an acidic environment in culture medium as a result of bacterial growth; whereas log phase cells required mild acid pre-exposure to get improved acid tolerance (O'Driscoll et al., 1996, Davis et al., 1996, Datta and Benjamin, 1997). The induction of acid adaptation also has requirements for pre-exposure pH and pre-exposure time. Davis et al. (1996) reported that pre-exposure in pH 5.0 BHI yielded the best acid adaptation and 1 h in pH 5.0 BHI was required for inducing acid adaptation in *L. monocytogenes*. So far, the only parameter during acid adaptation that has not been fully investigated is temperature. Since temperature is the most controllable condition during food processing, more efforts need to be devoted into understanding the effect of temperature on the induction of acid adaptation in future work.

There are many different ways to generate acid-stress resistant phenotypes of *L. monocytogenes*. In the food industry, mild acid exposure could be short-term, long-term or even combined with other stressors such as salt or heat. In the majority of published findings, acid adaptation in *L. monocytogenes* is activated by being exposure to mild acidic broth for 1 h. Recently, instead of a short period of pre-exposure, long-term mild acid pre-exposure (e.g. growth in pH 5.5 broth or on mild acidic agar) has been used to induce acid adaptation in *L. monocytogenes* (Skandamis et al., 2012). Buchanan and

Edelson first reported that the addition of 1.0% glucose could gradually decrease the pH media due to acid production from glucose metabolism, subsequently induced acid adaptation in *E. coli* (Buchanan and Edelson, 1996). This method was subsequently used by several other researchers to generate acid-stress resistant phenotypes of *L. monocytogenes* (Koutsoumanis et al., 2003, Chorianopoulos et al., 2011). Additionally, repetitive exposure to lethal acidic conditions also enables *L. monocytogenes* to have greater survival than non-adapted cells in lethal acid inactivation with pH 3.5 (Rajkovic et al., 2009). Similar observations also occurred for *Salmonella enteric*. A cyclic exposure in pH 2.5 increased the acid tolerance of *Salmonella enteric* by 3-4 fold (Karatzas et al., 2008). Apart from inducing acid adaptation by single mild acid stress, it is reported that sequential multiple mild stresses pre-exposure (e.g., NaCl then pH 5.04 or pH 5.04 then NaCl) or combined multiple mild stresses pre-exposure simultaneously (e.g., NaCl-acid or NaCl-acid-heat) also conferred *L. monoctytogenes* cells with pronounced acid adaptation. The acid adaptation conferred by multi-sublethal stressors was similar to that induced by mild acid stress alone (Skandamis et al., 2008, Skandamis et al., 2009).

2.9 Impact of acid adaptation of *Listeria monocytogenes* on food products and food processing conditions

In the food industry, *L. monocytogenes* comes from two main sources: the transfer of cells present on raw food materials to finished products and post-processing contamination from processing environments. Usually, cells present on raw food materials are inactivated completely during processing and HACCP based risk control approaches help to ensure that processing parameters are performed properly. Therefore,

post-processing contamination is the major way to introduce *L. monocytogenes* into food products. With respect to low pH, acidic foods (orange juice, yogurt or apple cider etc), have the possibility of post processing *L. monocytogenes* contamination did not receive adequate attention in acidic food products as *L. monocytogenes* does not grow or survive very well in those food products. However, more and more studies indicate that acid adaptation enables *L. monocytogenes* to be more persistent in various types of acidic food products. One study observed that 1 h of acid adaptation in pH 5.5 TSB-YE subsequently improved 2-5 log CFU/ml greater survival than non-adapted cells in commercially available acidic food products (yogurt, cheese, orange juice and salad dressing) or during active milk fermentation by *Streptococcus thermophilus* (Gahan et al., 1996). Another study reported that acid-adapted (pH 5.1 for 1 h) *L. monocytogenes* cells showed about 3 log CFU/ml growth in Crescenza cheese (pH 5.0-5.6) within 14 days storage whereas non-adapted cells did not show any growth (Cataldo et al., 2007). Similar to *L. monocytogenes*, acid adaptation induced enhanced survival in acidic food products also occurs in gram-negative foodborne pathogens *E. coli* O157:H7 and *Salmonella* spp. Adaptation in pH 5.0 broth for 4-5 h conferred *E. coli* O157:H7 with 2-3 log CFU/ml higher survival in apple cider (pH 3.46) or during sausage fermentation (Leyer et al., 1995). Compared to neutral media grown cells, acidified media grown *Salmonella* Typhimurium cells showed 3-4 log CFU/ml greater survival during milk fermentation or cheese storage under refrigerated temperature (Leyer and Johnson, 1992, Shen et al., 2007). Hence, the acid-stress resistant phenotypes of *L. monocytogenes* should be carefully considered when mild acidic conditions are involved during food processing.

Using a broth model, it has been concluded that acid adaptation in *L. monocytogenes* contributes to its extended survival during various types of lethal inactivation treatments (Ferreira et al., 2003, Lin et al., 2011). However, several studies confirmed that no significant survival difference between acid-adapted and non-adapted cells is observed when the lethal treatment is performed in food substrates. For example, on fresh beef, no survival difference was measured between acid-adapted and non-adapted cells after dipping treatment in hot water (55°C or 75°C), 2% lactic acid, 2% acetic acid or the combination of 55°C and 2% acid (Ikeda et al., 2003). On beef jerky, similar survival of *L. monocytogenes* acid-adapted and non-adapted cells was observed after being treated with marinade, acetic acid plus marinade or marinade combined with Tween 20 and acetic acid (Calicioglu et al., 2003a). On bologna slices, prior acid adaptation in acidified broth did not help *L. monocytogenes* cells to show enhanced resistance to simulated gastric fluid with a pH of 1.0 (Formato et al., 2007). Another study showed that acid-adapted *L. monocytogenes* did not survive better than non-adapted cell after being inactivated at 57°C in cantaloupe or watermelon juice (Sharma et al., 2005). No significant gamma irradiation resistance was observed between *L. monocytogenes* acid-adapted and non-adapted cells in a seafood salad (Foley et al., 2005). The same phenomenon also occurs in *E. coli* O157:H7 and *Salmonella* spp. when the lethal treatment is performed on the food matrix (Calicioglu et al., 2003b, Calicioglu et al., 2003c, Berry and Cutter, 2000). One possible explanation for those contradictory results in food substrates when compared to model broth studies could be attributed to the protective effect of the food matrix which counteracts protection provided by acid

adaptation. Thus, in the future, more studies should be performed using real food products to enhance their applicability to the food industry.

During food processing, *L. monocytogenes* has the opportunity to attach to food contact surfaces and subsequently form biofilms in the processing environment. *L. monocytogenes* biofilms are naturally more resistant to antimicrobial treatments when compared to planktonic cells (Robbins et al., 2005, Pan et al., 2006). Improved antimicrobial resistance in biofilms provides *L. monocytogenes* with an enhanced ability to survive through the decontamination procedure and such surviving cells increase the likelihood of post processing contamination that occurs in food products. Therefore, removal of *L. monocytogenes* biofilm on food contact surfaces receives extensive attention. There is some evidence that acid adaptation enhances the ability of *L. monocytogenes* to attach and form biofilms. Compared to non-adapted cells, acid-adapted *L. monocytogenes* cells yielded increased biofilm production on plastic microtitre plates (Cataldo et al., 2007, Adriao et al., 2008). Growth in acidified media (pH 4.5-5.7) promoted *L. monocytogenes* attachment to microtitre plates (Nilsson et al., 2011) and stainless steels (Chorianopoulos et al., 2011). Moreover, acid exposure enhanced the adherent capacity of *L .monocytogenes* is also observed in food matrix. The number of *L. monocytogenes* attached to 1.0% lactic acid treated pig skin was about 1 log CFU/ml higher than that attached to untreated pig skin (Morild et al., 2011). However, the detailed mechanisms of how *L. monocytogenes* acid adaptation relates to increased biofilm formation or adherent capacity are still not well characterized except some limited available information on this topic. For example, increased Internalin B (invasion protein) expression was observed in *L. monocytogenes* after acid adaptation (Bowman et

al., 2012). The presence of Internalin B is critical to the adhesion process to abiotic surfaces in *L. monocytogenes* (Chen et al., 2008). In order to efficiently remove biofilm from food contact surfaces, more breakthroughs need to be accomplished to illustrate the mechanism behind the effect of mild acid exposure on *L. monocytogenes* biofilm formation.

CHAPTER III

ACID ADAPTATION IN *LISTERIA MONOCYTOGENES* IS NOT INDUCED AT 4ºC IN THE PRESENCE OF SUBLETHAL ACID

3.1 Introduction

Listeria monocytogenes is the cause of listeriosis that leads to about 30% mortality among infected individuals (Ramaswamy et al., 2007, Siegman-Igra et al., 2002). Listeriosis outbreaks are typically associated with *L. monocytogenes* contamination in ready-to-eat (RTE) food products such as deli meat, Mexican-style cheese, cold smoked fish, salads and fruits (Ryser and Marth, 2007, CDC, 2011, Cartwright et al., 2013). Due to its severe pathogenicity, USDA-FSIS implemented a "zero tolerance" policy for *L. monocytogenes* in ready-to-eat (RTE) food products (Klima and Montville, 1995). Traditional methods for the control of *L. monocytogenes* during food processing include pasteurization, low pH, high salt or strong oxidation (Hwang and Beuchat, 1995, Nerbrink et al., 1999, Siragusa and Johnson, 1989, Baert et al., 2009). Novel inactivation measures such as ozone, high hydrostatic pressure (HHP) and pulsed electric field technology are being evaluated for their ability to inactivate *L. monocytogenes* in food processing environment (Wade et al., 2003, Lopez-Pedemonte et al., 2007, Rowan et al., 1999).

L. monocytogenes possesses sophisticated adaptive mechanisms to counteract unfavorable environmental conditions. For example, *L.* monocytogenes is able to tolerate

higher levels of lethal acid, heat, salt or oxidative stresses after exposure to sublethal concentrations of homogenous stress (Lou and Yousef, 1997, Bolton and Frank, 1999, O'Driscoll et al., 1996). The phenomenon of acid adaptation in *L. monocytogenes*, where cells exhibited enhanced acid tolerance during lethal acid inactivation (pH 3.5) after pre-exposure to mild acidic condition (pH 5.0), was first reported by Hill (1995). Additional studies confirmed this concept and proposed that activation of acid adaptation required protein *de novo* synthesis (Davis et al., 1996, O'Driscoll et al., 1996). Further investigations indentified two enzymatic systems, glutamate decarboxylase (GAD) system and arginine deiminase (ADI) that contribute to acid adaptation in *L. monocytogenes* by neutralizing intracellular pH (Wemekamp-Kamphuis et al., 2004, Ryan et al., 2009, Cotter and Hill, 2003). The general stress response regulon σ^B is also critical for *L. monocytogenes* acid adaptation through positive regulation of GAD and ADI systems and other acid stress response proteins (Lmo0913, Lmo0796 and Lmo2391) (Wemekamp-Kamphuis et al., 2004, Abram et al., 2008, Ryan et al., 2009).

L. monocytogenes typically enters into food processing plants from raw food materials (Lunden et al., 2002, Berrang et al., 2005, Kabuki et al., 2004). In the food processing environments, *L. monocytogenes* cells are able to attach to various types of food contact surfaces (Beresford et al., 2001, Alessandria et al., 2010). After attachment occurs, exposure to sublethal acid concentration from food products (cheese, fruit juice, meat etc.) or exposure to acid based antimicrobial treatments (lactic acid, acetic acid, benzoic acid, etc) can activate acid adaptation in *L. monocytogenes*. Acid-adapted *L. monocytogenes* cells survive better than non-adapted cells in acidic foods including orange juice, salad dressing, yogurt and cheese where lethal acid stress was present

(Gahan et al., 1996). In addition, acid adaptation induced stress hardening enhanced cross resistance against other inactivation treatments such as salt, heat, HHP, bacteriocins and disinfectants (McKinney et al., 2009, Wemekamp-Kamphuis et al., 2004, O'Driscoll et al., 1996, van Schaik et al., 1999, Begley et al., 2002, Lin et al., 2011).

During mild acid exposure, several factors play critical roles in controlling the induction of acid adaptation in *L. monocytogenes*. Most of them (sublethal acid concentration, mild acid exposure time, type of acidulants, bacterial growth stage and bacterial strain) have been sufficiently investigated in previous studies. Davis et al (1996) determined that mild acid pre-exposure for 1 h at pH 5.0 was optimal to confer *L. monocytogenes* with pronounced acid adaptation. With respect to growth stage, it is known that the stationary phase of *L. monocytogenes* cells have better lethal acid tolerance when compared to log phase cells (Davis et al., 1996, O'Driscoll et al., 1996, Datta and Benjamin, 1997, Ferreira et al., 2003). Normally in broth conditions, *L. monocytogenes* cells attain stationary phase within 18-24 h and the pH of the growth medium drops to 5.6-6.0 (Davis et al., 1996). This drop in pH can partially assist in inducing acid adaptation in stationary phase cells. The ability of inducing acid adaptation also varies among *L. monocytogenes* strains. One study showed that out of four *L. monocytogenes* isolates from the dairy industry, two isolates from raw ewe's milk made cheese were able to show acid adaptation after exposure to pH 5.5 whereas the other two isolates from raw cow's milk made cheese cannot (Adriao et al., 2008). Even though both organic and inorganic acid exposure is able to induce acid adaptation in *L. monocytogenes*, the spectrum of targeted gene modulation due to mild acid pre-exposure

appears to be somewhat different based on microarray and proteomic profiling (O'Driscoll et al., 1997, Tessema et al., 2012).

Little attention has been devoted to the influence of temperature on *L. monocytogenes* acid adaptation. In general food processing plants, the processing or storage room temperatures (4°C-20°C) are generally much lower than the model laboratory studies performed at 30-37°C (O'Driscoll et al., 1996, Faleiro et al., 2003, Koutsoumanis et al., 2003, Koutsoumanis and Sofos, 2004). Therefore, it is imperative to investigate the occurrence of acid adaptation in *L. monocytogenes* under low temperatures. In the present study, the effect of temperature on the induction of acid adaptation in *L. monocytogenes* was evaluated.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study included *L. monocytogenes* Scott A (serotype 4b, human clinical, FDA), *L. monocytogenes* Bug600 (serotype 1/2a, Institut Pasteur, Paris, France), wild type *L. monocytogenes* EGD-e and Δ*CspL* EGD-e (serotype 1/2a) (Schmid et al., 2009), *Escherichia coli* ATCC 11775, *E. coli* O157:H7 ATCC 43890 and *Salmonella* Typhimurium ATCC 23564. Overnight cultures were prepared by inoculating one loop of working stock cultures in 10 ml tryptic soy broth supplemented with 0.6% yeast extract (TSB-YE) and incubation at 37°C for 18-20 h. Early log phase culture of these cells were prepared by transferring 200 µl of overnight culture into 20 ml TSB-YE and incubation at 37°C until OD_{600nm} reached ~ 0.15 (Thermon Electron Corporation), which is equivalent to approximately 2×10^8 CFU/ml. Experiments were performed using 37°C grown early log phase cells unless otherwise noted.

3.2.2 Effect of mild acid pre-exposure temperature on the induction of acid tolerance response

The temperature dependent acid adaptation in *L. monocytogenes* Scott A and Bug600 was evaluated by initially pre-exposing the *L. monocytogenes* cells to pH 5.0 (1M lactic acid) or pH 7.2 TSB-YE at three different temperatures for 1 h (37°C, 22°C and 4°C) and subsequently performing a post-lethal acid challenge of the pre-exposed cells in pH 3.5 TSB-YE (3M lactic acid) at 37°C. Control cells were placed at 37°C, 22°C and 4°C in TSB-YE (pH 7.2) for 1 h before being subjected to the lethal acid challenge.

One ml of 37°C grown early log phase cultures were transferred into 9 ml of pre-acidified (mild acid-stressed cells) or neutral TSB-YE (non-stressed cells) for pre-exposure at 37°C, 22°C or 4°C. After 1 h pre-exposure, 1 ml volumes from each of the mild acid-stressed and non-stressed sample tubes were transferred into five eppendorf tubes (to represent five post-exposure sampling time points) and eppendorf tubes were centrifuged (Sorvall Biofuge Fresco, 16060 × g). Samples that were pre-exposed at 4°C were cold centrifuged whereas samples that were pre-exposed at 37°C or 22°C were centrifuged at room temperature. After centrifugation, supernatant was completely decanted and cell pellets were resuspended in 1 ml pH 3.5 TSB-YE for lethal inactivation treatment at 37°C for up to 180 min. *L. monocytogenes* survival was determined at timely intervals using TSA-EF (tryptic soy agar containing 1.0% esculin and 0.05% ferric ammonium citrate) and incubation at 37°C for 48 h. For survival curves of mild acid-stressed and non-stressed cells, protection area and $D_{pH\ 3.5}$ were calculated. Protection area is defined as the area under the curve of mild acid-stressed cells minus the area

under the curve of non-stressed cells. $D_{pH\ 3.5}$ is defined as the time (min) required to inactivate 90% of *L. monocytogenes* cells in pH 3.5 TSB-YE at 37°C.

Similar experiments were also performed using early log phase cold grown cells (OD_{600nm} ~ 0.15). For this experiment, 37°C and 4°C pre-exposure treatments were used and survival of *L. monocytogenes* Scott and Bug600 was enumerated following 22°C post-lethal acid treatments after 90 min and 180 min, respectively.

3.2.3 Determining factors affecting lack of acid adaptation at 4°C

Various factors such as increased of mild acid pre-exposure time, various mild acid concentrations, addition of salt during pre-exposure and acidulant type were analyzed to determine the acid tolerance response of 4°C mild acid-stressed cells. As control experiments, 37°C mild stressed and non-stressed cells were used in all experiments. All experiments were performed using *L. monocytogenes* strain Scott A grown to early log phase at 37°C. Post-lethal acid inactivation treatment was performed in pH 3.5 TSB-YE at 22°C for 60 or 90 min and survivals were enumerated on TSA-EF. Throughout all experiments, cells pre-exposed at 4°C were centrifuged at 4°C while 37°C pre-exposed cells were centrifuged at room temperature. Lactic acid (1M) was used for mild acid pre-exposure unless otherwise noted. Lactic acid (3M) was used for all lethal acid inactivation treatments.

3.2.3.1 Increasing mild acid pre-exposure time

Pre-exposure was conducted as above by transferring 1 ml log phase culture into 9 ml pre-acidified broth (mild acid-stressed cells) or neutral broth (non-stressed cells) that were temperature equilibrated to 37°C or 4°C. Pre-exposure time periods tested at 37°C

were 15, 30, 45 and 60 min, whereas time periods tested for 4°C pre-exposure were 1, 4, 8 and 24 h. At each of those time points, one ml samples of each mild acid-stressed and non-stressed cells were centrifuged and subjected to lethal inactivation.

3.2.3.2 Varying mild acid concentrations

L. monocytogenes Scott A cells were pre-exposed at 37°C or 4°C in various mild pH (4.0, 4.5, 5.0, 5.5, 6.0, and 7.2) TSB-YE for 1 h. At the end of mild acid pre-exposure, samples were centrifuged and cell pellets were subjected to the lethal acid inactivation treatment.

3.2.3.3 Effect of various pre-exposure acidulants

Instead of lactic acid, 1M of hydrochloric acid and acetic acid were used to acidify TSB-YE to pH 5.0. Pre-exposure in mild acid or neutral TSB-YE was performed for 1 h at 37°C or 4°C. After the temperature treatment, cell pellets were obtained and subjected to the lethal acid inactivation treatment.

3.2.3.4 Addition of sodium chloride during mild acid pre-exposure

The TSB-YE that was used in this study already contained 0.5% NaCl. Additional salt concentrations that were tested include 2%, 4%, 6%, 8% and 10% NaCl. Mild acid-stressed cells of *L. monocytogenes* Scott A were generated at 37°C or 4°C by pre-exposing log phase cells to pH 5.0 TSB-YE (lactic acid adjusted) supplemented with various sodium chloride concentrations (0.5%, 2%, 4%, 6%, 8% and 10%) at 37°C or 4°C. Non-acid-stressed cells were pre-exposed to pH 7.2 TSB-YE supplemented with these salt concentrations. After pre-exposure, cells were pelleted and subjected to the lethal acid inactivation treatment.

3.2.4 Acid adaptation of *L. monocytogenes* following mild acid pre-exposure treatment in acidic whey at different temperatures

Fat reduced milk was heated to 85°C and coagulated by 3M lactic acid. Coagulant was strained and acidic whey (pH ~ 5.1) was collected. For mild acid pre-exposure in acidic whey, *L. monocytogenes* Scott A log phase cells were diluted in TSB-YE by 1:10 and centrifuged to collect cell pellets. Thereafter, cell pellets were resuspended in temperature equilibrated acidic whey for 1 h pre-exposure at 37°C, 22°C or 4°C. After 1 h pre-exposure, calculated 3M lactic acid quantity was directly added into those whey samples to attain a final pH of 3.5. Control treatment included cells that were directly resuspended in pH 3.5 acidic whey. The lethal acid challenge for all samples was performed at 22°C and survival was measured on PALCAM agar plates supplemented with 6 mg/L ceftazidime (Difco, Detroit, MI) every 30 min up to 2 h.

3.2.5 Temperature effect on acid adaptation in *E. coli*, *E. coli* O157:H7 and *S.* Typhimurium

One ml of early log phase cultures of these microorganisms were transferred into 9 ml pre-acidified (mild acid-stressed cells, 1M lactic acid) or neutral broth (non-stressed cells) and incubated at either 37°C or 4°C for 1 h. After pre-exposure, cells were pelleted and resuspended into pH 3.5 TSB-YE (3M lactic acid) for lethal acid inactivation treatment at 22°C. Survival was enumerated on tryptic soy agar supplemented with 0.6% yeast extract (TSA-YE) after 30 min, 20 min and 10 min for *E. coli*, *E. coli* O157:H7 and *S.* Typhimurium, respectively.

3.2.6 Effect of *cspL* deletion on mild acid adaptation

Both wild type and its Δ*cspL* strain of *L. monocytogenes* EGD-e were pre-exposed to pH 5.0 (mild acid-stressed cells) or pH 7.2 (non-stressed cells) TSB-YE for 1 h at either 4°C or 37°C. After pre-exposure, samples were centrifuged and the pellets were resuspended in pH 3.5 TSB-YE (adjusted by lactic acid) at 22°C to perform the post-lethal acid treatment. Survival was determined on TSA-EF plates after 1 h lethal acid challenge.

3.2.7 Effect of bead beating on the induction of acid adaptation in *L. monocytogenes* at 4°C

Early log phase cultures grown at 37°C (1.2 ml) were transferred into screw-cap tubes containing 2 g of 0.1 mm zirconium (BioSpec Products, Inc, OK, USA) bead. After 2 min of bead beating (4800 oscillations/min) at room temperature (22°C), 100 µl of cultures were transferred into 900 µl pre-acidified (mild acid-stress) or neutral broth (non-stress) that was initially kept at 37°C or 4°C for temperature equilibration. Pre-exposure was allowed for 1 h at 37°C or 4°C after which pellets were recovered and resuspended in 1 ml pH 3.5 TSB-YE (3M lactic acid). The lethal acid challenge was carried out at 22°C and *L. monocytogenes* Scott A survival was measured every 30 min up to 90 min.

3.2.8 Statistical analysis

All experiments were performed in three replicates with a completely randomized design structure. Data shown here are the mean (± SE) of three individual trials. The student *t*-test ($P < 0.05$) was performed to determine significant mean separation between survival of mild acid-stressed and non-stressed cells (SPSS version 12.0, Chicago, Ill).

3.3 Results

3.3.1 Mild acid stress at 4°C did not induce acid tolerance response in *L. monocytogenes*

Figure 3.1 and Figure 3.2 showed the lethal acid tolerance response of 37°C grown cells of *L. monocytogenes* Scott A and Bug600 cells, respectively, that were mild acid-stressed and non-stressed for 1 h at pre-exposure temperatures of 37°C, 22°C and 4°C prior to exposure to pH 3.5 lethal inactivation treatment at 37°C. Two distinct patterns for *L. monocytogenes* Scott A acid adaptation were observed: (I) conditions where acid adaptation response occurred; and (II) conditions where acid adaptation response did not occur. For both *L. monocytogenes* Scott A and Bug600 strains, cells pre-exposed in mild acid stress at 22°C or 37°C for 1 h exhibited about 2 to 5 log CFU/ml better survival compared to non-stressed cells (Fig. 3.1A, 3.1B, 3.2A, 3.2B). In contrast, no difference in survival was observed between mild acid-stressed and non-stressed cells if pre-exposure was performed at 4°C (Fig. 3.1C, 3.2C). The mild acid-stressed treatment at 4°C had 2 to 3 log CFU/ml lower survival as compared to 22°C and 37°C mild acid-stressed *L. monocytogenes* Scott A cells after 90 min exposure to the pH 3.5 lethal acid inactivation treatment. For *L. monocytogenes* Bug600 cells this difference was approximately 2 log CFU/ml. The same pattern was also apparent in the protection area and $D_{pH\ 3.5}$ of the survival curves (Table 3.1). The protection area of 37°C or 22°C was greater ($P < 0.05$) than zero, and $D_{pH\ 3.5}$ of mild acid-stressed cells was greater ($P < 0.05$) than that of non-stressed cells which indicated that acid adaptation was induced. At 4°C, the protection area was close to zero and no appreciable difference was observed between $D_{pH\ 3.5}$ of mild acid-stressed and non-stressed cells indicating that acid adaptation did not occur. This conceptual finding of no acid adaptation if mild acid pre-exposure is

performed at 4°C was also true if post-lethal acid treatment was performed at 4°C or 22°C (data not shown).

Lack of mild acid adaptation in 4°C mild acid-stressed cells was also evident in cold grown *L. monocytogenes* Scott A (Fig. 3.3A) and Bug600 (Fig. 3.3B) cells. For both strains, no difference ($P > 0.05$) existed between survival of mild acid-stressed and non-stressed cells if mild acid pre-exposure was performed at 4°C. However, for 37°C pre-exposed cells, mild acid-stressed cells had about 2 log CFU/ml higher survival when compared to non-stressed cells. There is also a 2 log CFU/ml higher survival between 37°C mild acid-stressed and 4°C mild acid-stressed cells.

3.3.2 Increase in pre-exposure time, varying pH of mild acid exposure, change of acidulants and addition of salt did not induce acid adaptation in *L. monocytogenes* at 4°C

Increase pre-exposure time in pH 5.0 from 1 h to 4, 8 or 24 h also failed to induce the acid adaptation response in 4°C pre-exposed cells (Fig. 3.4B). *L. monocytogenes* Scott A survival in both non-stressed and pH 5.0 mild acid-stressed cells was approximately 4 log CFU/ml. In contrast, 30 min of pre-exposure at 37°C in mild acid stress induced acid tolerance response as survivals following pH 3.5 lethal inactivation treatments were 5 log CFU/ml in mild-acid stressed cells and 3.5 log CFU/ml in non-stressed cells (Fig. 3.4A).

Different mild acid pre-exposure pH also did not induce acid adaptation in *L. monocytogenes* at 4°C. *L. monocytogenes* Scott A survival for pH 4.0, 4.5, 5.0, 5.5 and 6.0 mild acid-stressed cells followed by lethal inactivation acid treatment were 2-3.5 log CFU/ml which was not appreciably higher than that observed in non-stressed cells (3 log CFU/ml) (Fig. 3.5B). On the contrary, pH 5.0 and pH 5.5 pre-exposure at 37°C revealed

that *L. monocytogenes* had 2-4 log CFU/ml greater survival than non-stressed cells in pH 3.5 TSB-YE (Fig. 3.5A).

Different pre-exposure acidulants other than lactic acid were also tested for their effect on induction of acid adaptation at 37°C or 4°C. There was about 3 log CFU/ml survival for both 4°C mild hydrochloric or acetic acid-stressed cells after lethal acid challenge, which was similar to that of 4°C non-stressed cells (Fig. 3.6B). However, 37°C mild hydrochloric or acetic acid-stressed cells exhibited about 4 log CFU/ml higher survival than 37°C non-stressed cells (Fig. 3.6A).

Addition of different concentrations (2% to 10%) of NaCl during pre-exposure did not induce acid adaptation in *L. monocytogenes* cells at 4°C as survival of non-acid-stressed control cells and mild acid-stressed cells was similar (Fig. 3.7B). In contrast, pronounced acid adaptation was observed when the cells were pre-exposed in pH 5.0 TSB-YE supplemented with sodium chloride at 37°C. In the presence of low concentrations (0.5% to 6%) of NaCl, mild acid-stressed cells exhibited at least 3 log CFU/ml greater survival than non-acid-stressed control cells (Fig. 3.7A). Interestingly, the presence of 2% or 4% NaCl also helped the cells to even receive better acid tolerance. Compared to 0.5% NaCl mild acid-stressed cells, presence of 2% to 4% NaCl resulted in about 1.5 to 2 log CFU/ml greater survival after the lethal acid challenge. Addition of NaCl at higher concentrations (8% and 10%) reduced the acid tolerance response of mild acid-stressed cells. Also, 8% or 10% of salt stress alone induced some acid adaptation in 37°C pre-exposed control cells. As seen from Figure 3.7A, cells that were pre-exposed at pH 7.2 with 8% sodium chloride yielded about 4 log CFU/ml survival in pH 3.5 TSB-YE

whereas control cells pre-exposed to 0.5% sodium chloride had survival less than 2 log CFU/ml.

3.3.3 No acid adaptation of *L. monocytogenes* in acidic whey at 4°C

Cells pre-exposed in 22°C or 37°C acidic whey showed at least 2 log CFU/ml greater survival compared to control cells that were directly subjected to pH 3.5 lethal acid inactivation treatment. In contrast, 4°C acidic whey pre-exposed cells exhibited limited impaired survival in comparison to non-stressed cells (Fig. 3.8). This indicated that *L. monocytogenes* acid adaptation occurred in 37°C or 22°C acidic whey but not in 4°C acidic whey.

3.3.4 *E. coli, E. coli* O157:H7 and *S.* Typhimurium did not induce acid adaptation at 4°C

Non-pathogenic *E. coli* (ATCC 11775) exhibited appreciable acid adaptation after being pre-exposed to 37°C pH 5.0 TSB-YE as mild acid-stressed cells exhibited about 3 log CFU/ml greater survival than non-stressed cells. At 4°C, acid adaptation did not occur as both mild acid-stressed and non-stressed cells had about 2 log CFU/ml survival after the lethal acid challenge (Fig. 3.9A). Likewise, the same pattern was observed in both pathogenic *E. coli* O157:H7 (ATCC 43890) and *S.* Typhimurium (ATCC 23564). For both of them, 4°C mild acid-stressed cells did not survive better than non-stressed cells whereas 37°C mild acid-stressed cells had approximately 3-4 log CFU/ml greater survival after the lethal acid challenge (Fig. 3.9B, 3. 9C).

3.3.5 CspL is not responsible for repressed acid adaptation at 4°C

The role of CspL during mild acid pre-exposure at 4°C was examined using *L. monocytogenes* EGD-e wild type and its Δ*cspL* strain. Results showed that for both wild type and mutant strains, 4°C mild acid-stressed cells did not show any better survival than non-stressed cells in pH 3.5 TSB-YE. On the other hand, 37°C mild acid-stressed cells exhibited at least 2 log CFU/ml greater survival than non-stressed cells after the lethal acid challenge (Fig. 3.10). Therefore, the failed induction of acid adaptation at 4°C did not correlate to the presence of CspL.

3.3.6 Bead beating of cells induced partial acid adaptation at 4°C in *L. monocytogenes*

In this assay, we limited the bead beating time for 2 min which reduced the cell viability from the initial 7.2 to 6.2 log CFU/ml. Bead beating of the log phase of *L. monocytogenes* cells grown at 37°C induced acid adaptation when exposed to pH 5.0 TSB-YE at 4°C. There was an approximately 2 log CFU/ml survival difference between mild acid-stressed and non-stressed cells (Fig. 3.11B). For bead beated cells that were pre-exposed at 37°C, 4-5 log CFU/ml greater survival was observed in mild acid-stressed cells in comparison to non-stressed cells (Fig. 3.11A). These results indicated that bead beated cells can partially induce acid adaption in *L. monocytogenes* at 4°C at pH 5.0.

3.4 Discussion

Our findings indicate that the typical acid adaptation in *L. monocytogenes* that occurs at optimal growth temperature (30ºC or 37ºC) did not occur at refrigeration temperature in the presence of sublethal acid. Initial studies with *L. monocytogenes* Scott A (serotype 4b) and Bug600 (serotype 1/2a) indicated no acid adaptation at 4ºC after 1 h

mild acid pre-exposure (Fig. 3.1 and 3.2). This proof of concept was tested under the following pre-exposure conditions at 4 ºC: (a) extension time of mild acid pre-exposure condition (Fig. 3.4); (b) we evaluated a range of pH in mild acid pre-exposure (Fig. 3.5); (c) we changed acidulants from lactic acid to hydrochloric acid or acetic acid (Fig. 3.6); (d) we investigated the effect of addition of NaCl during mild acid pre-exposure (Fig. 3.7). Under all above conditions, there was no induction of acid adaptation in *L. monocytogenes*. To further confirm, acidic cheese whey was used in the place of laboratory broth media and confirmed that no acid adaptation occurred in acid whey at 4ºC (Fig. 3.8). Later, we tested if the same finding is true for Gram-negative model microorganisms (*Salmonella* and *E. coli*). Both microorganisms did not induce acid adaptation at 4ºC (Fig. 3.9). Therefore, a common fundamental physiological modulation must occur at 4ºC to prevent bacterial cells from getting acid adaptation.

In the present study, log phase *L. monocytogenes* cells were used instead of stationary phase cells. Since stationary phase cells become partially acid adapted due to the drop of pH during their growth into stationary stage (Davis et al., 1996). In addition, after entering the stationary phase, cells become naturally resistant due to the activation of stringent response (mediated by (p)ppGpp) and general stress response (mediated by σ^B) (Godfrey et al., 2002, Hecker and Volker, 1998, Ferreira et al., 2001). Such typical responses that occur in stationary phase cells can make *L. monocytogenes* resistant to various types of physiological stresses including lethal acid stress. Therefore, acidified media and activation of the stringent response and the general stress response in the stationary phase culture enables *L. monocytogenes* cells to acquire enhanced acid tolerance without the need to perform mild acid pre-exposure. Therefore, in present study,

we re-grew the cells in neutral TSB-YE to log phase for the sake of eliminating acquired acid adaptation in the stationary phase culture.

In recent publications, acid tolerance of cold grown *L. monocytogenes* cells was compared with that of optimal temperature grown cells (Samelis et al., 2003, Ivy et al., 2012). Both of these articles reported that *L. monocytogenes* stationary phase cells propagated in cold environments and had less acid tolerance than optimal temperature grown stationary phase cells. As the major focus of these two studies was to look at acid tolerance directly at lethal pH, they did not include the sublethal acid pre-exposure step prior to the lethal acid inactivation treatment. However, both studies used *L. monocytogenes* cells that were grown to the stationary phase in which growth medium pH drops to about 5.6 - 6.0 due to glucose metabolism (Davis et al., 1996). This drop of pH in media can simulate the condition of mild acid pre-exposure in the stationary phase. Such reduced acid tolerance in cold grown stationary cells that observed in these studies could be partially due to repressed acid adaptation at cold temperatures (4°C to 10°C). In addition, one study confirmed that 7°C cold grown log phase cells (no acidification of growth medium) had about 1.0-1.5 log CFU/ml reduced survival when compared to 37°C grown log cells in pH 3.5 BHI broth. It indicates that apart from lack of acid adaptation in cold environments, the cold grown cells may also be naturally deficient in counteracting lethal acid stress (Ivy et al., 2012). Collectively, diminished acid tolerance in cold grown stationary phase *L. monocytogenes* may be attributed to: (1) inability to induce acid adaptation at cold temperatures (4°C to 10°C); and (2) physiological cellular changes that provide inheriting diminished acid tolerance capacity that is independent of the acid adaptation response.

Several authors have proposed that the activation of acid adaptation in bacteria requires *de novo* protein synthesis (Davis et al., 1996, O'Driscoll et al., 1996, Cebrian et al., 2010, Koga et al., 1999). It is possible that acid shock proteins (ASPs) are not synthesized during 4°C mild acid pre-exposure. There are currently no specific gene expression studies that evaluated mild acid treatment modulated genes at variable temperature. Chan et al. (Chan et al., 2007) and Ivy et al. (Ivy et al., 2012) investigated the differential expression of 4°C, 7°C and 37°C grown stationary phase *L. monocytogenes* cells. Since stationary phase cells can cause some inherent acid adaptation due to decrease in growth medium pH, changes in the gene expression profile in that physiological state may provide some additional explanation of the temperature dependent behavior of acid shock genes or proteins. Interestingly, these studies suggested down-regulation of glutamate decarboxylase (GAD) and arginine deiminase (ADI) genes in cold grown stationary phase cells (Chan et al., 2007, Ivy et al., 2012). In another study, it was observed that GAD and ADI genes were not induced when cells were exposed to organic acids at 7°C (Stasiewicz et al., 2011). Both glutamate decarboxylase and arginine deiminase have a critical role in conferring *L. monocytogenes* with acid adaptation (Cotter et al., 2001a, Cotter and Hill, 2003, Ryan et al., 2009). Together, these findings support the notion that there may be lack of synthesis of some critical acid shock genes and proteins at a pH of 5.0 in TSB-YE at 4°C.

Exposure to pH 5.0 at 4°C, *L. monocytogenes* encounters two different types of physiological stress, namely acid stress and cold stress. It has been well characterized that *L. monocytogenes* can adapt to cold environments by expressing a different protein pattern which contributes to its modulated metabolism pathway, nutrient uptake, protein

folding and lipid biosynthesis at cold temperatures (Cacace et al., 2010). Hence, it is likely that between acid and cold adaptation, cold adaptation becomes the priority task for the bacterium to deal with. In cold environments, synthesis of cold shock proteins (Csps) is a major cellular response to assist *L. monocytogenes* cells so that they can adapt to low temperature conditions. Cold shock protein L (CspL) is the primary protein that is responsible for cold adaptation since *L. monocytogenes* strain lacking this gene becomes completely defective in cold growth (Schmid et al., 2009). Hence, we hypothesized that deletion of genes encoding Csps could terminate the process of cold adaptation and subsequently divert cell energy to trigger acid adaptation. In one assay, we pre-exposed Δ*cspL L. monocytogenes* EGD-e to 4°C mild acidic condition (Fig. 3.10) and expected to observe occurrence of acid adaptation in this mutant strain since its cold adaptation process was hampered due to the deletion of *cspL*. But the Δ*cspL* strain still did not show any acid adaptation. From another perspective, it is possible that *L. monocytogenes* could start to respond to acid stress after cells were fully cold adapted. So we tested whether cold grown log phase cells which are fully cold acclimated are able to adapt to acid at 4°C (Fig. 3.3). However, cold grown log cells were not able to adapt to acid at 4°C. Therefore, data from the above two experiments indicate that the actual cold adaptation event may not be the sole factor for the lack of acid adaptation at 4ºC.

The cold stress environment may block the penetration of acid into cytoplasm and subsequently fails to trigger intracellular responses to acid stress. To test this, we used a simple bead beating approach as an alternative for sonication. Bead beating is routinely applied in proteomic or microarray work to break bacterial cell envelope to yield more protein or mRNA extraction. In the bead beating assay, our data demonstrated that *L.*

monocytogenes were able to induce acid adaptation partially at 4°C (Fig. 3.11B), where the magnitude of acid adaptation was still less at 4°C when compared to 37°C (Fig. 3.11A). We did not investigate the molecular changes by which the bead beading treatment was able to partially reverse the non-acid adaptation at 4°C. Theoretically, it is possible that blockage of acid entry into cytoplasm of *L. monocytogenes* cells with an intact cell envelope could be one of the factors responsible for repressed acid adaptation at 4°C. One proteomic analysis on cold adapted *L. monocytogenes* showed that cell wall synthesis was up-regulated at 4°C (Cacace et al., 2010). Another morphology study on *Staphylococcus* spp. revealed that 4°C cold stressed phenotypes possessed thicker cell walls when compared to normal cells (Onyango et al., 2012). Such cell morphological modifications could build the physiological barriers to prevent acid from entering cytoplasm in cold environments and eventually impede the process of acid adaptation. On the other hand, since cell wall blockage partially prevents the induction of acid adaptation, it is necessary to investigate other possible mechanisms to have a comprehensive understanding of the cold temperature effect on acid adaptation in *L. monocytogenes*. Future studies can be focused on illustrating the complete mechanisms using microarray and proteomics to compare the transcription or protein expression profiles between 4°C mild acid-stressed cells and 37°C mild acid-stressed cells.

Table 3.1 Protection area and $D_{pH3.5}$ of *L. monocytogenes* Scott A and Bug600 in pH 3.5 at 37°C

***Lm* Strains**	**Pre-exposure temperature**	**Protection area**	**$D_{pH\ 3.5}$ (min)**	
			Control	**Treatment**
Lm Scott A	37°C	6.30 ± 0.39*	7.92 ± 2.61	20.46 ± 4.70*
	22°C	2.95 ± 0.74*	5.19 ± 0.38	11.01 ± 1.61*
	4°C	0.27 ± 0.10	7.39 ± 2.10	7.22 ± 1.90
Lm Bug600	37°C	7.87 ± 0.12*	9.06 ± 0.73	24.26 ± 0.54*
	22°C	8.54 ± 0.21*	10.22 ± 2.11	27.77 ± 0.30*
	4°C	0.26 ± 0.17	16.32 ± 0.32	19.37 ± 1.46

Note: (a) Protection area values followed with asterisk mark were significantly higher than zero based on Student *t*-test ($P < 0.05$). (b) $D_{pH3.5}$ values followed with asterisk mark indicated significant mean separation between survival of treatment and control based on Student *t*-test ($P < 0.05$).

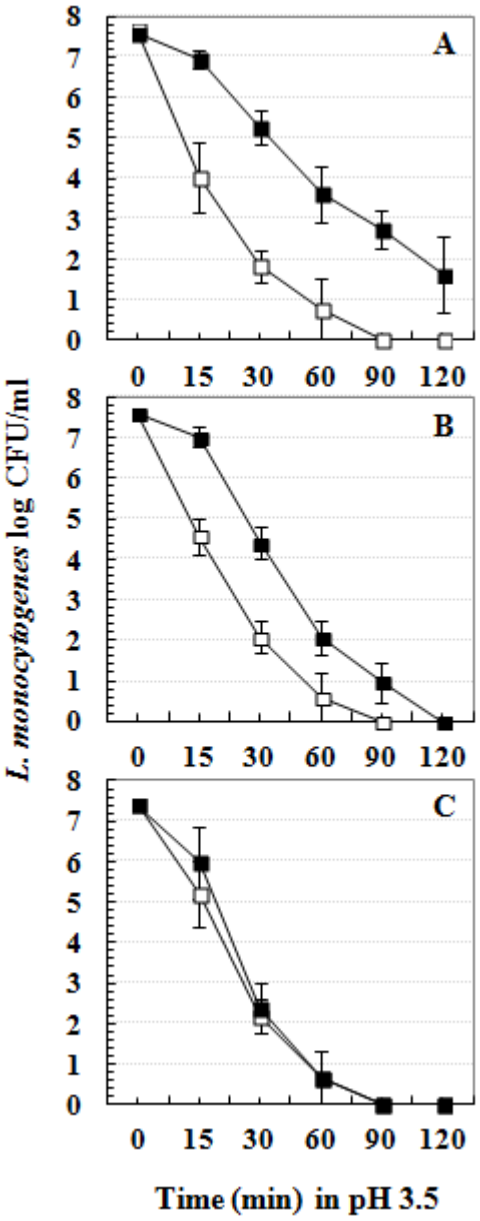

Figure 3.1 Survival of *L. monocytogenes* Scott A (serotype 4b) in lethal pH 3.5 TSB-YE at 37°C after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at 37°C (A), 22°C (B) or 4°C (C).

Note: Detection limit is 1 log CFU/ml

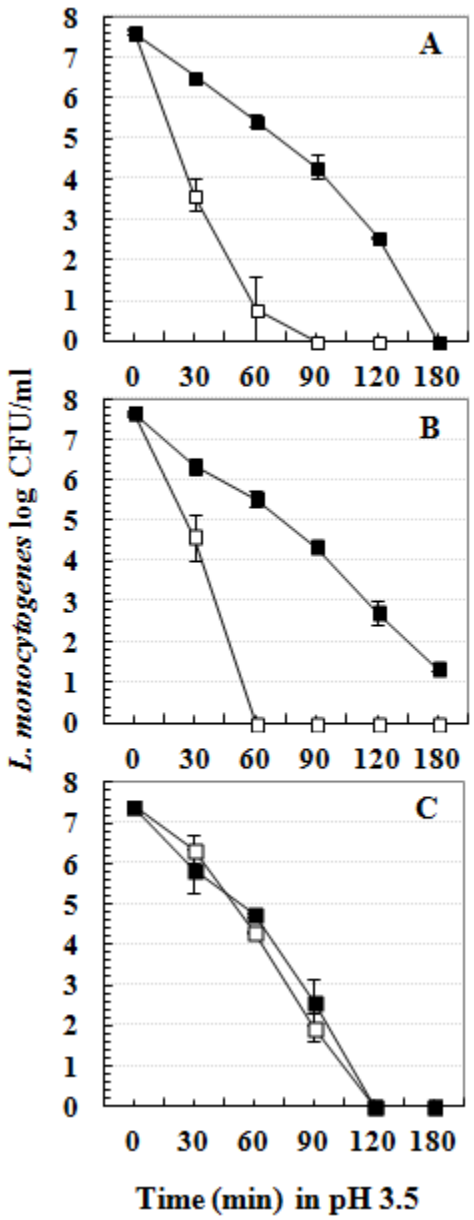

Figure 3.2 Survival of *L. monocytogenes* Bug600 (serotype 1/2a) in lethal pH 3.5 TSB-YE at 37°C after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at 37°C (A), 22°C (B) or 4°C (C).

Note: Detection limit is1 log CFU/ml

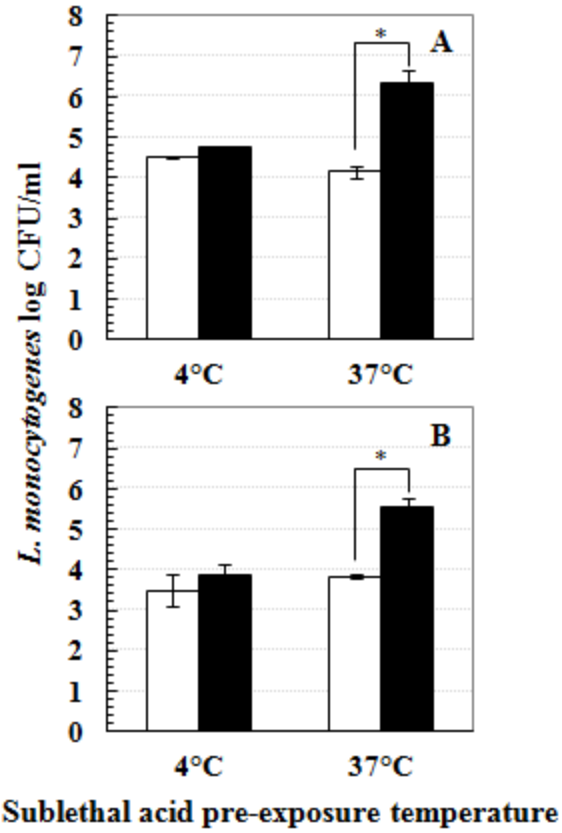

Figure 3.3 Survival of *L. monocytogenes* Scott A (A) and Bug600 (B) cold grown (4°C) log phase cells in lethal pH 3.5 TSB-YE at 22°C after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at 37°C or 4°C.

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. Detection limit is 1 log CFU/ml.

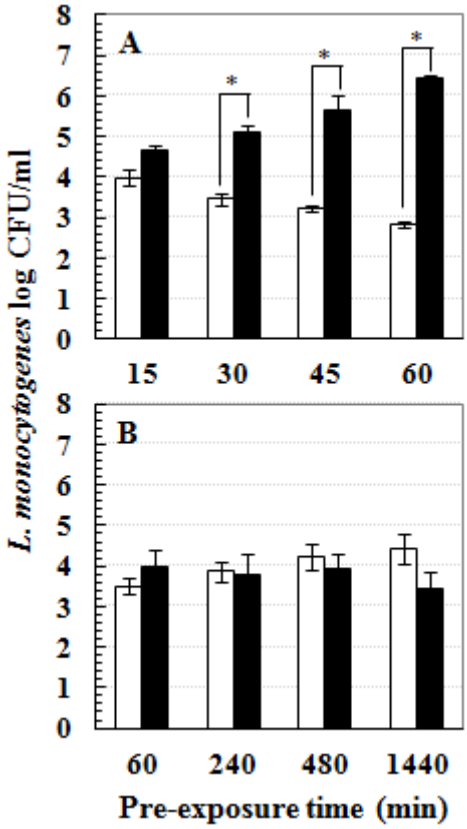

Figure 3.4 Survival of *L. monocytogenes* Scott A in lethal pH 3.5 TSB-YE at 22°C for 60 min after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) for 1 h at 37°C (A) or for 24 h at 4°C (B).

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. Detection limit is 1 log CFU/ml.

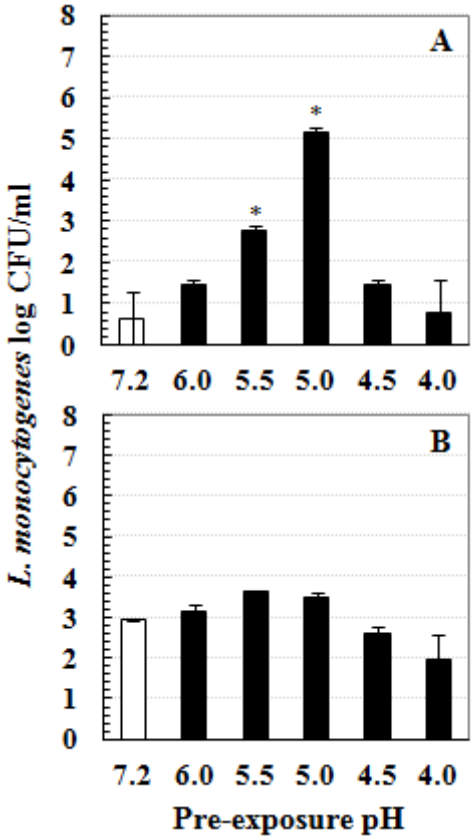

Figure 3.5 Survival of *L. monocytogenes* Scott A in lethal pH 3.5 TSB-YE at 22°C for 90 min after 1 h pre-exposure to different sublethal pH at 37°C (A) or 4°C (B).

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between mild acid-stressed (■) and non-stressed (□) cells. Detection limit is 1 log CFU/ml.

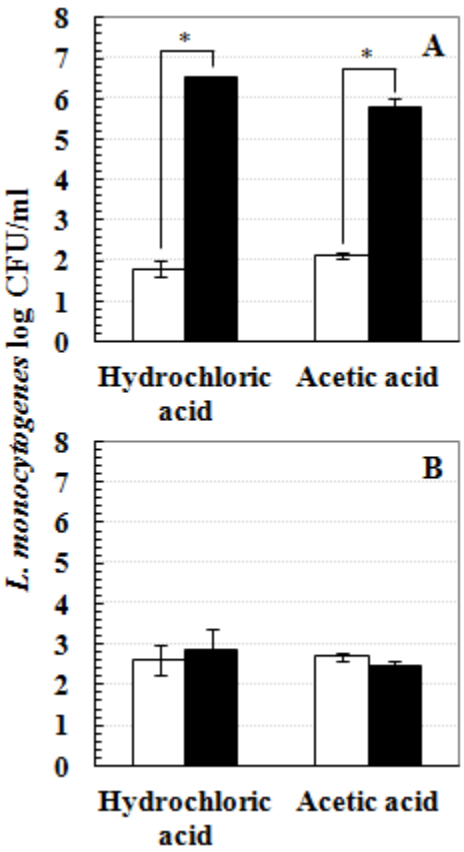

Figure 3.6 Survival of *L. monocytogenes* Scott A in lethal pH 3.5 TSB-YE at 22°C for 90 min after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at 37°C (A) or 4°C (B). Pre-exposure with pH 5.0 was adjusted by hydrochloric acid or acetic acid.

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. Detection limit is 1 log CFU/ml.

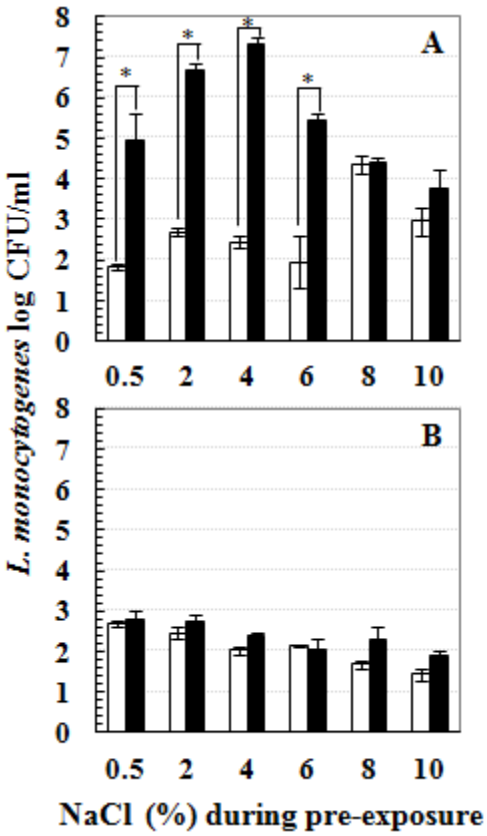

Figure 3.7 Survival of *L. monocytogenes* Scott A in lethal pH 3.5 TSB-YE at 22°C for 90 min after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) in the presence of different concentrations of NaCl at 37°C (A) or 4°C (B).

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. Detection limit is 1 log CFU/ml.

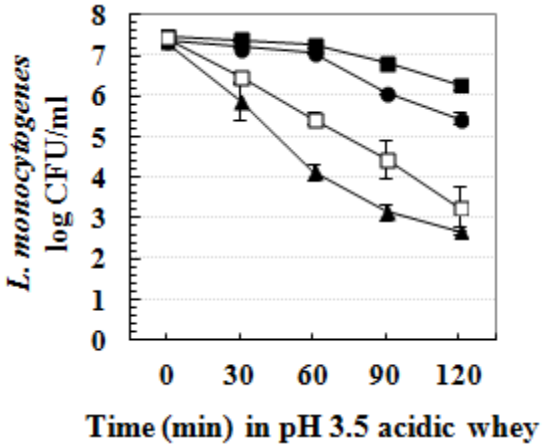

Figure 3.8 Survival of *L. monocytogenes* Scott A in lethal pH 3.5 acidic whey at 22°C. Cells were pre-exposed to acidic whey (pH 5.1) for 1 h at 37°C (■), 22°C (●) or 4°C (▲). Control cells (□) were added to acidic whey and immediately inactivated by lethal pH 3.5 at 22°C.

Note: Detection limit is 1 log CFU/ml

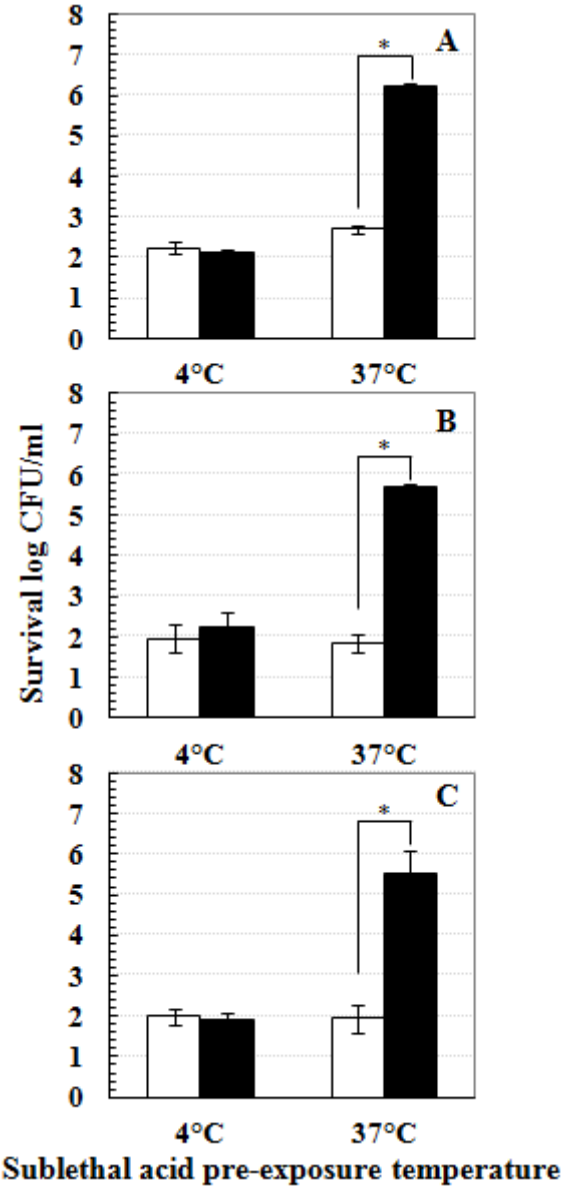

Figure 3.9 Survival of *E. coli* ATCC 11775 (A), *E. coli* O157:H7 ATCC 43890 (B) and *S.* Typhimurium ATCC 23564 (C) in lethal pH 3.5 TSB-YE at 22°C after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at 37°C or 4°C.

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. Detection limit is 1 log CFU/ml.

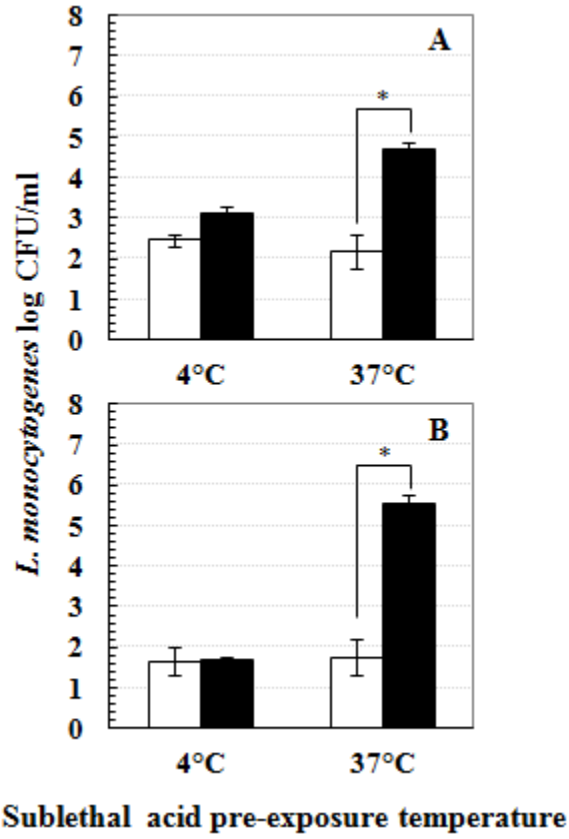

Figure 3.10 Survival of wild type *L. monocytogenes* EGD-e (A) and its Δ*cspL* strain (B) in lethal pH 3.5 TSB-YE at 22°C after 1 h pre-exposure to pH 5.0 (■) or pH 7.2 (□) at 37°C or 4°C.

Note: Bars marked with an asterisk indicate significant survival differences ($P < 0.05$) between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. Detection limit is 1 log CFU/ml.

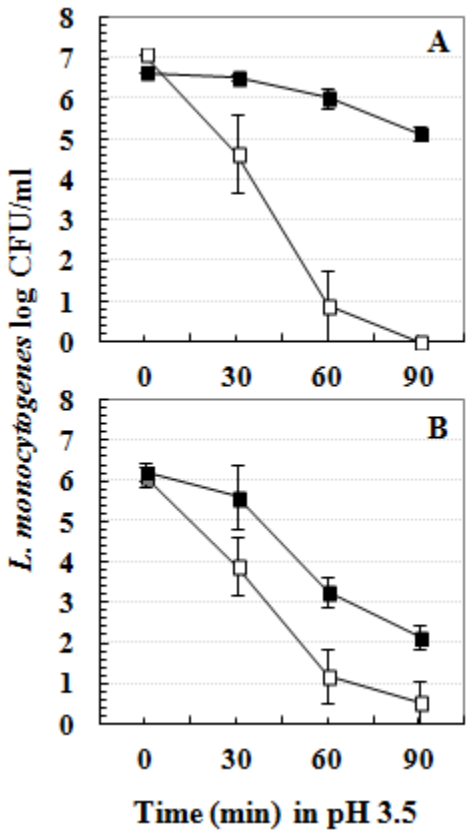

Figure 3.11 Survival of 2 min bead-beated *L. monocytogenes* Scott A cells in lethal pH 3.5 TSB-YE at 22°C after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at 37°C (A) or 4°C (B).

Note: Detection limit is 1 log CFU/ml

3.5 Summary and Conclusions

Our data demonstrated that acid-stress adaptation that occurs in *L. monocytogenes* at 22°C or 37°C was not induced when cells were pre-exposed to pH of 5.0 at 4°C. Extending time, varying mild acid concentration, changing acidulants and addition of sodium chloride during mild acid pre-exposure at 4°C did not induce acid adaptation in *L. monocytogenes*. This finding was further confirmed using an acidic cheese whey model. In addition, the temperature dependent acid-stress adaptation was true for the Gram-negative bacteria *E. coli* and *Salmonella*. The major cold shock protein CspL was not

responsible for repressed acid-stress adaptation at 4°C. *L. monocytogenes* cells treated by bead beating prior to 4°C mild acid pre-exposure partially assisted in inducing the acid-stress adaptation at 4°C. Further studies will focus on illustrating the mechanism behind repressed acid-stress adaptation at 4°C by performing the following assays: (1) Compare transcriptional and proteomic profiles between 37°C mild acid-stressed cells and 4°C mild acid-stressed cells; and (2) Compare fatty acid composition of the cell membrane after 37°C mild acid stress and 4°C mild acid stress.

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