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Formation of Oxidative-Stress Resistant Phenotypes of Listeria Monocytogenes Serotypes 1/2A and 4B and their Stability at 37oC and 4oC

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Formation of oxidative-stress resistant phenotypes of *Listeria monocytogenes* serotypes 1/2a and 4b and their stability at 37°C and 4°C

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The purpose of this study was to induce an oxidative-stress adaptation in *Listeria monocytogenes* Bug600 (serotype 1/2a) and F1057 (serotype 4b) by pre-exposing to sublethal H_2O_2 and alkali-stress either singly or sequentially. Our findings show that the sequential pre-exposure of cells to pH 9 for 30 min treatment followed by 50 ppm H_2O_2 for 30 min at 37°C yielded the highest oxidative-stress resistant phenotypes of *L. monocytogenes* Bug600 and F1057. The sublethal H_2O_2 and sublethal alkali-stress induced oxidative-stress adaptations were completely reversible within 60 min at 37°C in the absence of such sublethal stress. However, the oxidative-stress adaptation induced at 37°C was stable at 4°C over a 24 h test period in both *L. monocytogenes* Bug600 and F1057. Future studies will focus on the potential cross-resistance of oxidative-stress adapted *L. monocytogenes* serotypes 1/2a and 4b to commonly used disinfectants and GRAS antimicrobials.

DEDICATION

This work is dedicated to my loving parents Mr. Edmen Abeysundara and Mrs. Cindrella Silva and my husband Mr. Suranga Rajapaksha.

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CHAPTER I

INTRODUCTION

Listeria monocytogenes can lead to the development of a life-threatening disease called listeriosis in individuals with immune-compromised systems (Ramaswamy et al., 2007a). It is a gram positive, facultative anaerobic, and rod shaped non-spore forming bacterium (Jemmi and Stephan, 2006). *L. monocytogenes* is ubiquitous in nature and has the ability to tolerate a wide range of environmental conditions, including 0-45°C, pH 4.1-9.6 and salt concentrations as greater as 14-20% (McClure et al., 1989, Jemmi and Stephan, 2006). Even though listeriosis occurs less frequently than other foodborne illnesses, it is recognized as the most devastating foodborne disease with a high mortality and hospitalization rate (Jemmi and Stephan, 2006, Wang et al., 2013). There are 13 serotypes of *L. monocytogenes* in nature (Datta et al., 2012). Among them, the most virulent serotypes are 1/2a, 1/2b and 4b which are responsible for a high percentage of human listeriosis outbreaks (Pan et al., 2009). Recently, most listeriosis outbreaks are frequently caused by 1/2a and 1/2b that are found in foods such as fruits and cheese (Laksanalamai et al., 2012).

In food processing environments, *L. monocytogenes* may be exposed to various physiological stresses such as heat, acid, salt, alkali, oxidative, and radiation while preparing the food products of desired quality. Such processing stresses exert a great

influence on bacterial cell physiology and may directly affect the virulence response of *L. monocytogenes* serotypes (Roche et al., 2005).

All living organisms produce reactive oxygen species (ROS) (for example, superoxides and peroxides) which are highly toxic to microbial cell components such as proteins, lipid and DNA (Lushchak, 2011). In addition, microorganisms may be exposed to ROS during cleaning and sanitation in food processing environments. However, microorganisms have different mechanisms to detoxify and control ROS levels to prevent damage to cell components (Lushchak, 2011). Also, when there is an imbalance between production and detoxification of ROS, microbial cells may undergo a condition called oxidative-stress (Mehdizadeh Aghdam et al., 2012, Lushchak, 2011).

Stress hardening is a phenomenon in which microorganisms modify their internal metabolic activities under sublethal stress conditions to become more resistant to subsequent lethal stresses than the non-adapted cells (Lou and Yousef, 1997a). Such stress adaptation in *L. monocytogenes* under sublethal acid, heat and salt is well documented but understanding the oxidative-stress adaptation is fairly limited. Lou and Yousef (1997a) have reported that *L. monocytogenes* Scott A is capable of inducing an oxidative-stress adaptation upon exposure to 500 ppm of H_2O_2 for 1h with a cell density of 9 log CFU/ml. Also, such 500 ppm H_2O_2 pre-treated *L. monocytogenes* Scott A cells were more heat resistant than non-adapted cells (Lou and Yousef, 1996).

Besides oxidative-stress, *L. monocytogenes* may also be exposed to alkaline-stress during cleaning and sanitation treatments. Koga and others (2002) have reported that *Vibrio parahaemolyticus* is capable of inducing cross-protection against oxidative stress under sublethal alkaline condition. However, the induction of cross-protection against

oxidative stress in *L. monocytogenes* that is exposed to sublethal alkali-stress conditions is unknown.

The purpose of this study was to determine if an oxidative-stress adaptation is induced in *L. monocytogenes* Bug600 (serotypes 1/2a) and F1057 (serotype 4b) when cells were pre-exposed to sublethal H_2O_2, sublethal alkali-stress at 37°C or a sequential exposure to H_2O_2 and alkali stress at 37°C . I have determined the effect of pre-exposure to H_2O_2 concentration, contact time and cell density for inducing oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057. I have also determined the effect of alkali pH and contact time and different alkaline reagents on the induction of an oxidative stress adaptation in these *L. monocytogenes* strains. I then determined the effect of sequential sublethal exposure to H_2O_2 and alkali stresses on an oxidative stress adaption in *L. monocytogenes*. Finally, we evaluated the stability of each oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057 at 37°C and 4°C after their induction at 37°C in the presence of sublethal H_2O_2 or alkali-stresses.

CHAPTER II

LITERATURE REVIEW

2.1 *Listeria monocytogenes*

Listeria monocytogenes is a gram positive foodborne pathogen which is widely distributed in nature. It is capable of tolerating a wide range of environmental conditions such as temperatures from 0°C to 45°C, pHs from 4.1 to 9.6 and salt concentrations up to 20% (Jemmi and Stephan, 2006, McClure et al., 1989). It can survive and grow effectively inside the gastrointestinal tract and macrophages where it encounters various stresses such as acid, oxidation and high salinity (Lungu et al., 2009, Camejo et al., 2009).

L. monocytogenes can cause listeriosis in immune compromised people, which leads to severe complications particularly among pregnant women, elderly people, newborn babies and individuals who have undergone organ transplantation and cancer therapies (Wonderling et al., 2004, Rahimi et al., 2012). Listeriosis occurs less frequently than other foodborne illnesses, but it has been recognized as a foodborne disease with a high mortality and hospitalization rate (Jemmi and Stephan, 2006, Wang et al., 2013). Symptoms of listeriosis include fever, muscle ache, nausea, diarrhea, headache, stiff neck, loss of balance and convulsions. It also causes fetal brainstem infections which can lead to meningitis and meningoencephalitis (Mukherjee et al., 2013). In addition, *L. monocytogenes* infections during pregnancy may lead to abortions (Lundén et al., 2004).

L. monocytogenes has a long incubation period in humans as compared to other foodborne pathogens which ranges from 1 to 67 days depending on the clinical type of the microorganism (Goulet et al., 2013).

The genus *Listeria* includes several species including *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. gray* etc. Among these, only *L. monocytogenes* and *L. ivanovii* are pathogenic (Volokhov et al., 2002, Ramaswamy et al., 2007b). In *L. monocytogenes,* 13 serotypes were recognized based on the somatic and flagella antigen differences (Borucki and Call, 2003, Datta et al., 2013) and there is a great variation in virulence of these serotypes (Roche et al., 2012). These 13 serotypes of *L. monocytogenes* were further classified into four main lineages (I, II, III and IV) based on their evolutionary differences. A high frequency of human listeriosis cases were caused by linage I and lineage II (Orsi et al., 2011). Lineage III and IV are considered rare in nature (Chen et al., 2012).

There is a zero-tolerance for all 13 serotypes of *L. monocytogenes* (Roche et al., 2012) on ready-to-eat products but serotype 4b has been responsible for a high percentage of human listeriosis outbreaks (Pan et al., 2009). However it was reported in Finland the number of 4b related listeriosis cases have decreased while 1/2a and 1/2b related number of listeriosis cases have increased during the period from 1990 to 2001 (Lukinmaa et al., 2003). Similarly in the United States, 1/2a and 1/2b serotypes have been reported to be responsible for most of the recent multistate listeriosis outbreaks (Laksanalamai et al., 2012).

2.2 Occurrence of *L. monocytogenes* in foods

L. monocytogenes is found in a wide range of food products which include fresh fruits, vegetables, fish, meat, milk and many types of raw and processed foods. Recently, contaminated fruits and vegetables have been identified as one of the major sources of foodborne outbreaks in many parts of the world (Franz et al., 2010, Park et al., 2012), (Mercanoglu Taban and Halkman, 2011, Franz et al., 2010). The growth rate of *L. monocytogenes* is influenced by the type of the fruit or the vegetable (Jacxsens et al., 1999). For example, growth rate of *L. monocytogenes* on sliced apples is less than sliced honeydew melon stored at 10°C (Leverentz et al., 2003).

Fruits and vegetables can be contaminated with *L. monocytogenes* at the farm by contact with decaying vegetation, feces and effluents containing contaminants (Beuchat, 1996). Recently, research has focused on avoiding *L. monocytogenes* contamination in fruits and vegetables at the farm level especially through reducing contaminants in irrigation water (Park et al., 2012). Fresh fruits and vegetables can also be contaminated with *L. monocytogenes* through non-hygienic handling practices during food processing (Kovacevic et al., 2013). Meat or meat based products can be contaminated with *L. monocytogenes* through contamination of carcasses or by cross-contamination during processing. However, the growth rate of *L. monocytogenes* on meat products depends on the pH and the type of meat product (Glass and Doyle 1989).

L. monocytogenes is also associated with milk and milk based food products. It is reported that approximately 50 % of listeriosis outbreaks in Europe are linked to diary products (Lundén et al., 2004). Even though, *L. monocytogenes* is mainly associted with raw milk, improperly pasturized milk and related dairy products are also linked to

listeriosis outbreaks throughout the world. Among various dairy products, soft cheese is more vulnerable to *L. monocytogenes* as compared to hard cheese (Lobacz et al., 2013). In feremented dairy products, survival and growth of *L. monocytogenes* is inhibited due to competitive growth of starter culture (Morgan et al., 2001).

2.3 *L. monocytogenes* outbreaks

Since *L. monocytogenes* is capable of growing at low temperature, most of listeriosis outbreaks are associated with ready-to-eat (RTE) food products which are normally stored under refrigerated conditions for months or years (Poulsen and Czuprynski, 2013), (Derra et al., 2013).

According to the Centers for Disease Control (CDC), the frequency of listeriosis outbreaks in the USA decreased by 44% during the period of 1989 to 1993 due to enforced regulations. From 1998 to 2008, there were 24 confirmed listeriosis outbreaks with 359 illnesses, 215 hospitalization and 38 deaths. Most of these listeriosis cases have been associated with *L. monocytogenes* serotype 4b (Cartwright et al., 2013). From 2009 to 2011, 12 listeriosis outbreaks have been reported with 1651 human infections. Most of the listeriosis victims during this period have been reported to be over 65 years old (58%) or pregnant (14%) (2013). The largest listeriosis outbreak in the United States in 2011 was linked to whole cantaloupe produced by Jensen farm Colorado that resulted into 33 deaths. Other recent listeriosis outbreaks in the United States were linked to imported cheese brands such as Frescolina Marte and Ricotta Salata that led to 20 illnesses including 4 deaths in 13 states (CDC).

Wide spread human listeriosis outbreaks have also been reported in other countries than the United States. For example, during 2009-2010, a widespread listeriosis

outbreak occurred in five countries including, Australia, Germany, The Czech republic, Poland and Slovakia through a brand of acid curd cheese. Due to this outbreak there were 34 hospitalizations including 8 deaths due this outbreak (Schoder et al., 2012). In 2008, another listeriosis outbreak occurred in Canada that was linked to a cheese brand, which was made with improperly pasteurized milk and resulted 38 confirmed illnesses (Gaulin et al., 2012).

In England, Wales and Northern Ireland, from1985 to 1989, listeriosis incidence that was associated with pates doubled. However, due to government health warnings on pate consumption, a sharp decline in listeriosis cases was reported after 1989 (McLauchlin et al., 1991). Furthermore, during the period from 2005 to 2009, Sweden experienced several listeriosis outbreaks that eventually led authorities to examine the level of *L. monocytogenes* in RTE foods in 2010. Results of those studies revealed that 0.4% of 525 soft and semi soft cheese samples, 1.2% of 507 meat product samples, and 12% of 558 fish samples were positive for *L. monocytogenes* (Lambertz et al., 2012).

More studies detected the presence of *L. monocytogenes* in a variety of foods in other parts of the world that caused listeriosis. For example, a Chinese study, *L. monocytogenes* was detected in many imported foods in China from 2005 to 2008 (Wang et al., 2012). In Egypt, *L. monocytogenes* was commonly found in street-vendor RTE food products where 57% and 39% of 576 street-vendor RTE food samples were positive for *L. monocytogenes* and *L. innocua,* respectively. About 49% of these *Listeria* positive samples had more than 10^3 CFU/g of *L. monocytogenes* cells (El-Shenawy et al., 2011). In Ethiopia, randomly selected dairy and meat products were 27.5% positive for *Listeria* species (Derra et al., 2013). In Japan, Hasegawa and others (2013) reported that out of

210 bovine milk samples tested from 21 different dairy farms, 16 were found to be positive for *L. monocytogenes*. Another survey conducted during 2000 to 2009 in Colombia reported that 1424 out of 1599 food samples were positive for *L. monocytogenes* (Munoz, 2012). All these findings indicate that *L. monocytogenes* is highly prevalent in a diverse range of food products and in many environments.

2.4 Pathogenesis of *Listeria monocytogenes*

L. monocytogenes is a facultative intracellular bacterium with capability to grow in human non-phagocyte cells as well as phagocyte cells (Conlan and North, 1992). It mainly enters to host through intestine. According to Gaillard and others (1987) *L. monocytogenes* can enter to human enterocyte-like cell line Caco-2 cells through directed phagocytosis. However, only pathogenic *Listeria* species (L. *monocytogenes* and *L. ivanovii)* can induce their own phagocytosis by Caco-2 cells to enter host cells and illnesses. Liver is the second closest organ that *L. monocytogenes* may invade next to intestinal cells. Due to this reason listeriosis is closely associated with chronic liver disease (Vázquez-Boland et al., 2001).

The intracellular mechanisms that *L. monocytogenes* uses to enter to host cells has been studied comprehensively by Tilney and Portnoy (1989b). According to them, the first step of the infection is phagocytosis. After that, the membrane of the phagosome is dissolved with the help of hemolysin secreted by *L. monocytogenes*. Within 2 h, *L. monocytogenes* cells are completely encapsulated with actin filaments. However, when the *L. monocytogenes* cells start growing, such actin filaments rearrange in to a tail like structure to enter to adjacent macrophages and subsequently promote cell to cell spread. Several membrane components have been suggested as important during phagocyte

entrance of *L. monocytogenes* to host cells. C3b is one of such components which are covalently bound to bacterial cell walls and increases bacterial cell uptake by macrophages via complement receptor type 3 (Alvarez-Dominguez et al., 1993).

Listeriolysin O (or hemolysin) is a major virulence factor in *L. monocytogenes* for invasion of host cells and to escape from host vacuoles (Jones and Portnoy, 1994). Listeriolysin is coded by *hlyA* with around 504 amino acids (Mengaud et al., 1988) and found in all *L. monocytogenes* strains (Mengaud et al., 1988). The loss of *hlyA* reduces the survival of *L. monocytogenes* in macrophages during cell invasions (Kuhn et al., 1988). Listeriolysin is also considered as a sulfhydryl- activated toxin. Unlike other bacterial sufhydryl- activated toxins, listeriolysin shows its maximum activity at low pH such as pH 5.5 and no activity at 7.0 pH. Hence listeriolysin can act better under sublethal acidic conditions normally found in phagocytes (Geoffroy et al., 1987).

Internalin is another group of proteins that contribute to the virulence of *L. monocytogenes*. Gene *inlA* and *inlB* are responsible for encoding InlA and InlB two main internalin proteins (Cossart and Bierne, 2001, Bergmann et al., 2013). Maximum production of InlA and InlB proteins occurs at 37°C in the log phase of *L. monocytogenes* when compared to other temperatures and other states (Dramsi et al., 1997). In addition to *inlA* and *inlB*, there are several more homologues genes including *inlC*, *inlD*, *inlE*, *inlF* which also produce proteins belong to the same family and contributing to the virulence of *L. monocytogenes* (Dramsi et al., 1997). Studies suggest that internalin mediates bacterial entrance to mammalian epithelial cells through adhesion to the N-terminal domain of E- cadherin receptor via the leucine rich repeat domain of InlA (Schubert et al., 2002, Lecuit et al., 2001). The functions of Internalin during *L.*

monocytogenes infection depend on the type of the internalin protein as well as the type of the cell that bacteria are invading. For example, InlB is essential for *L. monocytogenes* to enter hepatocyte cells and does not have any function when entering intestinal cells (Dramsi et al., 1995) Furthermore, studies propose that Internalin mediated mechanism that *L. monocytogenes* uses to enter intestinal epithelial cells is different from the mechanisms they use to enter brain cells (Bergmann et al., 2013).

ActA protein which is coded by the *actA* gene is another virulence factor in *L. monocytogenes*. The main function of this protein is to regulate actin polymerization and thereby manage cell movement during host cell invasion (Travier et al., 2013). Deletion of the *actA* gene in *L. monocytogenes* significantly reduces the bacterial virulence during host cell invasion when compared to wild type cells (Tilney and Portnoy, 1989a).

In addition to the above mentioned virulence factors, the availability of iron in host cells is also an important factor during *L. monocytogenes* infections. For example, when mice were treated with iron, *L. monocytogenes* had high invasiveness in mice liver cells. However, when the same mice were treated with iron chelating agents, the invasiveness was reversed, suggesting that high iron availability makes host cells more susceptible to *L. monocytogenes* infections (Sword, 1966).

2.5 Microbial stress response

A stress can be defined as any physical, chemical or biological factor that affects the growth and the survival of microorganisms (Donnelly, 2002). If the stress is strong enough to kill the microorganism, it is considered a lethal stress while the stresses that are not strong enough to kill the microorganism but can damage cells are considered sublethal stresses (Wesche et al., 2009). Under sublethal stress, many microorganisms

including *L. monocytogenes* can undergo a phenomenon called stress hardening and become resistant to subsequent lethal stresses (Lou and Yousef, 1997a, Hill et al., 2002). All three foodborne pathogens, *L. monocytogenes*, *Escherichia coli* and *Salmonella typhimurium,* are capable of acid stress adaptation when exposed to sublethal acidic conditions (around pH 5) at 25 °C to 37 °C. Such adapted cells exhibit higher resistance to lethal acid stress than the non-adapted cells (Alvarez-Ordonez et al., 2010, Tessema et al., 2012). Pre-exposure to low temperatures facilitate microorganisms to change their cell physiology in a way that they can tolerate cold abuse better than non-cold exposed cells (Tasara and Stephan, 2006). Agoston and others (2010) have reported that *L. monocytogenes* adapts to heat stress after pre-exposure to 46 or 48°C for 30 to 60 min. Also, pre-exposure to sublethal bile enhances bile stress resistance in *L. monocytogenes* (Begley et al., 2002). Under sublethal alkali stress such as pH 9.6, *L. monocytogenes* can adapt to gain a greater resistance to lethal alkali stress when compared to non-adapted cells (Giotis et al., 2008a).

2.6 Oxidative stress adaptation of *L. monocytogenes*

Pre-exposure to sublethal oxidative stress induces an adaptation in *L. monocytogenes* to oxidative stress. For example, *L. monocytogenes* Scott A cells when pre-exposed to 500 ppm H_2O_2 for 60 min at a cell density of 9 log CFU/ml had stable survival in a lethal; treatment of H_2O_2 (1000 ppm) lethal treatment for 8 h while the survival of the control was decreased to 1 log CFU/ml within 4 h at 30°C (Lou and Yousef, 1997a).

Adaptation to one sublethal stress may contribute to *L. monocytogenes* gaining resistance to another stress a phenomenon termed cross-protection (Dragosits et al.,

2013). Cold stress adaptation in *L. monocytogenes* can affect different biochemical reactions associated with various protein synthesis and protein folding which may induce oxidative stress resistance (Garnier et al., 2010, Cacace et al., 2010). Some cold shock proteins such as CspBD and CspABD are important for inducing oxidative stress resistance when *L. monocytogenes* exposed to cold stress (Loepfe et al., 2010). *L. monocytogenes* lacks the *kat* gene exhibits impaired growth under cold conditions when compared to wild type cells and also lacks the ability to adapt to oxidative stress (Azizoglu and Kathariou, 2010). In addition to cold stress, sublethal salt stress (6% NaCl at 37°C or at 7°C) induces cross-protection against oxidative stress in *L. monocytogenes* (Bergholz et al., 2012). *Vibrio parahaemolyticus* induces oxidative stress resistance upon pre-exposure to pH 9 sublethal alkali stress (Koga et al., 2002). Furthermore, Glucose or nitrogen starvation condition can induce cross-protection in *E. coli* against lethal hydrogen peroxide stress (Jenkins et al., 1988).

2.7 Oxidative stress regulation

All living organisms produce reactive oxygen species either as a by-product or intermediate product of their metabolic activities and can experience oxidative stress as a result (Lushchak, 2011). However, microbes have different mechanisms to detoxify and maintain ROS at a low level to avoid cellular damages (Lushchak, 2011). Nevertheless, when the rate of ROS production exceeds the rate of ROS detoxification, cells experience oxidative stress (Mehdizadeh Aghdam et al., 2012, Lushchak, 2011).

Even though *L. monocytogenes* enters host cells through directed phagocytosis, this is one of the main defense mechanism that most of mammalian cells employ to protect themselves from microbial infections. During phagocytosis, unwanted microbial

cells are degraded through digestive enzymes, low pH or increased ROS level (Ex: superoxides and peroxides) (Mertens and Samuel, 2012, Hassett and Cohen, 1989). Thus, *L. monocytogenes* is highly exposed to ROS during host cell invasions thus triggering a high expression of genes related to oxidative stress during its invasion of mammalian cells (Camejo et al., 2009). In addition, antibiotics also employ increased level of ROS to destroy unwanted microorganisms (Tkachenko et al., 2012).

Generally, bacterial stress response is a non-stressor specific reaction since bacteria screen and respond to various stresses based on the damage occurred to cellular components such as proteins and DNA regardless of the type of the stressor (Kültz, 2005). Accumulation of stress proteins in cytoplasm will also act as an initial signal for oxidative stress regulation in bacteria (Gomaa and Momtaz, 2007). Mukhopadyay and Schellhorn (1994) have found that in the presence of chloramphenicol (a compound that inhibits protein production in microbial cells), microorganisms do not adapt to oxidative stress suggesting that protein synthesis plays an important role during oxidative stress adaptation. Hence, oxidative stress regulation is considered as an energy and time consuming mechanism (Gusarov and Nudler, 2005).

Microorganisms produce various antioxidant enzymes in order to detoxify ROS (Baptista et al., 2012). Catalase and superoxide dismutase are two of the universal antioxidant enzymes (Dallmier and Martin, 1988). During cell invasion, these antioxidant enzymes help microorganisms overcome oxidative stress that is created by the host immune system (Bishai et al., 1994). Therefore, antioxidant enzymes are very important in determining the virulence of pathogens (Bishai et al., 1994). For example, catalase (also known as hydroperoxidases; HP) is encoded by the *kat* gene and its activity is

influenced by various factors. In *E. coli,* whose catalase activity is deeply studied, two types of catalases have been found, Hydroperoxidase I (HP I) and Hydroperoxidase II (HP II). HP I and HP II are encoded by *katG* and *katE* genes respectively (Milano et al., 1996). Transcription of *katG* gene is induced by exposure to H_2O_2 in an OxyR dependant manner while the transcription of *katE* gene is controlled by alternative sigma factor called KatF. The activity of catalases in *E. coli* increases once cells leave the exponential phase and enter the stationary phase (Mukhopadhyay and Schellhorn, 1994). Activity of catalase is greatly suppressed when the environmental temperature is around 60°C (Dallmier and Martin, 1988). Salt concentration in the environment also plays a significant influence on the production of catalases in *L. monocytogenes* in a strain dependent manner. For example, *L. monocytogenes* 7644 expresses increased catalase activity when exposed up to 2.5% NaCl, and gradually decreases activity beyond that. Whereas in *L. monocytogenes* LCDC 81-861, high catalase activity can be found in NaCl concentrations as high as 4.6% (Dallmier and Martin, 1990).

Superoxide dismutase (SOD) is another important antioxidant enzyme that can convert ROS into harmless end products. This enzyme is coded by gene *sod* under the influence of SoxR and PerR stress regulators in the presence of ROS (Ricci et al., 2002, Greenberg et al., 1990). SOD is comparatively heat liable when compared to catalase and the activity is affected by environmental NaCl in a strain dependant manner (Dallmier and Martin, 1990). Increased resistance to oxidative stress of microorganisms is directly correlated with a high activity of SOD enzyme (Scott et al., 1987). However, in *L. monocytogenes,* unlike catalase, the activity of the SOD is down regulated when the cells enter into the stationary phase due to serine/threonine phosphorylation of SOD protein

(Archambaud et al., 2006). Recent findings show that there is a very close relationship between production of SOD and the biofilm forming capacity of *L. monocytogenes*. A mutant of *sod* had reduced biofilm forming capacity with high ROS production compared to wild type of *L. monocytogenes* (Suo et al., 2012). The deletion of *sod* gene in *L. monocytogenes* 4b, increased the expression of the *kat* gene along with two stress regulator encoding genes (*per*R and *sig*B), and one DNA repair gene (*rec*A) were increased. The activity of SOD in *L. monocytogenes* is closely linked with the activity of the antioxidant enzymes and plays an important role in its biofilm formation.

L. monocytogenes employ various stress regulators, which initiate the production of many antioxidant enzymes in order to combat ROS. Peroxide stress regulator (PerR) is one such regulator that controls the expression of many genes which are responsible for producing a number of antioxidant enzymes (Makthal et al., 2013). Even though PerR is mainly responsible for mediating the antioxidant defense system against peroxide free radicals, it induces the transcription of the *sod* gene under the influence of superoxide free radicals in *Streptococcus pyogenes* (Ricci et al., 2002). Therefore, PerR is an important virulence factor in the genus *Streptococcus* and for group A streptococcus (GAS) to survive in human blood (Le Breton et al., 2013, Makthal et al., 2013). Furthermore, in GAS it has been reported that 76 out of 237 peroxide-regulated genesare PerR dependent (Grifantini et al., 2011). *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Campylobacter jejuni* lack the OxyR regulator depend upon on PerR to regulate oxidative stress (Kim et al., 2011).

OxyR or the peroxide sensor is another widespread oxidative stress regulator found in many bacteria, including *L. monocytogenes*. OxyR initiates the production of

antioxidant enzymes in the presence of ROS, such as HP I in the presence of ROS. The activation mechanism of OxyR is well understood when compared to other oxidative stress regulators. In the absence of ROS, OxyR protein is inactive in all microorganisms. However, in the presence of ROS, OxyR activation is induced through the formation of a disulfde bond between two conserved cysteine residues (C199 and C208) (Chen et al., 2008). Once the disulfde bond is reduced, the OxyR regulator goes back to its natural inactive position (Aslund et al., 1999, Cabiscol et al., 2010).

Similarly, SoxR, a redox-sensitive transcriptional activator is also triggered by superoxide (O_2^-) ions through oxidation of its iron sulfur center (Hidalgo et al., 1997, Gaudu and Weiss, 1996). Activated SoxR induces transcription of many genes, which are responsible for producing various antioxidant enzymes such as endonuclease iv, glucose 6-phosphate dehydrogenase, Mn superoxide dismutase and paraquat diaphoroase (Greenberg et al., 1990). Since many OxyR controlled genes are not expressed under O_2^- stress, SoxR and OxyR stress regulators must be governed by different control systems.

The alternative sigma factor SigB (σB) is very important as a multi stress regulator in *L. monocytogenes* (Gorski et al., 2011). SigB is encoded by gene *sigB* and mutants of *sigB* have increased susceptibility to many physiological stresses including oxidative stress (Kazmierczak and others 2003). *Bacillus subtilis* expression of *sigB* gene can be initiated through various physiological stresses such as ethanol, NaCl, heat, and cold. SigB can successively induce about 200 genes, which lead to the production of hundreds of stress proteins (Reder et al., 2012). SigB contributes to oxidative stress regulation, by inducing the production of KatE (HP II) (Engelmann and Hecker, 1996).

Universal stress proteins (Usp) are another group of proteins that are important in stress regulation in bacteria (Liu et al., 2007). *E. coli* has six *usp* genes, namely *uspA*, *uspC*, *uspD*, *uspE*, *uspF* and *uspG* which are expressed under various environmental stresses such as oxidation and heat (Liu et al., 2007). Transcription of *usp* genes increases when microbial cells enter stationary phase (Liu et al., 2007). Among all Usp proteins, UspA and UspD, are important in regulating ROS level in bacteria. For example, *L. monocytogenes* UspA protein is strongly involved in oxidative stress regulation while mutants of the *uspA* gene have diminished growth when exposed to sublethal H_2O_2 concentrations (Seifart Gomes et al., 2011). Also, UspD protein is important in controlling the intracellular iron level which in turn affects the intercellular ROS concentration (Nachin et al., 2005).

Dussurget and others (2005) have reported that Ferritin-like proteins have vital contribute to oxidative stress regulation in microorganisms. Dps is a protein that belongs to the ferritin-like protein family and is coded by the *fri* gene under the influence of the PerR regulator as well as SigB alternative sigma factor. Dps is capable of binding iron and therefore regulating the ROS level inside bacterial cells (Olsen et al., 2005). Dps can protect bacterial DNA during oxidative stress as well as nutrition starvation stresses (Calhoun and Kwon, 2011, Zhao et al., 2002).

Manganese plays a vital role as a cofactor for the antioxidant enzyme called Manganese superoxide dismutase (SOD). It can fight with ROS with no Fenton reaction (Aguirre and Culotta, 2012). Also, there is a very close relationship between iron and oxidative stress in bacteria. The free form of iron can increase the formation of ROS and thereby elevate oxidative stress inside bacterial cells (Rangel et al., 2012). However, in

GAS, iron is an essential metal element for optimum response of PerR against ROS (Grifantini et al., 2011). The activity of manganese is vulnerable in the presence of iron since iron can out compete with manganese for manganese superoxide dismutase and diminish the activity of the enzymes (Aguirre and Culotta, 2012).

2.8 Alkali stress regulation in *L. monocytogenes*

Many cleaning agents used in food industry are alkaline in nature and microorganisms can undergo alkaline stress during cleaning and sanitation. *L. monocytogenes* is capable of growing under sublethal alkali stress such as pH 9.6 to 10.5 where its lag phase is prolonged compared to neutral conditions (Giotis et al., 2008a). When such stress adaption is induced, *L. monocytogenes* survives better in lethal alkali stress than non-adapted cells. For example, *L. monocytogenes* is capable of inducing alkaline stress adaptation at pH 9.5 within 1 h at 30°C so that it can survive when exposed to lethal alkali stress (pH 12.0) by more than 3 log CFU/ml when compared to non-adapted cells (Giotis et al., 2008b), (Taormina and Beuchat, 2001). Scanning electron microscopy studies revealed that *L. monocytogenes* cells gain multinucleate filamentous nature at a pH of 9.0 or above. However, after the removal of buffered alkaline stress condition, these morphological changes are reversible (Giotis et al., 2007). Vail and others (2012) reported that stimulation of filamentous nature in *L. monocytogenes* under alkali stress may lead to the underestimating the risk of *L. monocytogenes* in food products. These multinucleate filaments can grow on agar plated as single colonies with lower CFU counts but are capable of dividing in to multiple cells to grow independently once the stress is later removed in food products.

V. parahaemolyticus is also capable of inducing an alkali stress adaptation when exposed to sublethal alkali stress conditions to survive at lethal alkali stress of a pH 10.5 compared to non-stressed cells. Exposure to alkali stress also induces morphological changes in *V. parahaemolyticus* and *L. plantarum* cells (Lee et al., 2011, Koga et al., 2002).

L. monocytogenes induces many genes related to virulence, cell division and morphological changes during alkali stress adaptation (Giotis et al., 2010). *SigB* is one of the genes that contribute to alkaline stress adaptation. Giotis and others (2008a) have observed that there is a significant increment of *sigB* derived mRNA during alkali stress adaptation and *sigB* mutant of *L. monocytogenes* is significantly sensitive to lethal alkaline stress when compared to wild type cells.

L. monocytogenes also induces the expression of genes related to *sig*B, Na+/H+ antiporteres, ATP-binding cassettes transporters, motility and virulence as a rapid response to alkaline stress (Giotis et al., 2010). *Bacillus subtilis* induces about 80 genes related to multiple metabolic pathways during a sudden increase in pH from 6.3 to 8.9 (Wiegert et al., 2001). Under alkali stress, *L. monocytogenes* expresses an elevated ROS level due to a net migration of proton from the cells and induces the production of proteins such as lmo1407 that were also observed under oxidative stress as well (Nilsson et al., 2013).

2.9 Hydrogen peroxide as an antimicrobial agent

Hydrogen peroxide (H_2O_2) is one of the commonly used biocide in food industry(Lillard and Thomson, 1983). It is a clear solution which has a molecular weight of 34.047 g/mol and density of 1.45g/ cm^3. The IUPAC ID for hydrogen peroxide is

dihydrogen dioxide. The pH of the H_2O_2 solution ranges from 4.6 to 5.1 depending on the concentration of the solution (2013). H_2O_2 can be easily degraded in to water and oxygen and the decomposition rate increases with increasing the temperature. In addition, the decomposition rate also increases in the presence of impurities. Since H_2O_2 can be degraded easily, it is considered as an environmental friendly biocide(2013).

H_2O_2 produces hydroxyl free radicals which can oxidize chemical bonds in lipids, proteins and DNA and their by destroy microorganisms (McDonnell and Russell, 1999). Inhibition of enzyme activities, oxidation of nucleosides and disruption of protein synthesis are some of the effects of H_2O_2 against microbial cells that cause their death. H_2O_2 has a wide spectrum of antiseptic activity against viruses, bacteria, yeast and bacterial spores. Anaerobes are more sensitive to H_2O_2 compared to aerobes or facultative anaerobes since they do not produce catalase to break down H_2O_2 (Block, 2001)

The activity of H_2O_2 is affected by various factors including concentration, temperature and pH of the medium. The antimicrobial activity of H_2O_2 is greatly increased with increasing temperature and concentration. However unlike other biocides, sporicidal activity of H_2O_2 is not affected by the presence of organic matter. Also, H_2O_2 makes bacterial spores more sensitive to heat (Block, 2001).

CHAPTER III

FORMATION OF OXIDATIVE-STRESS RESISTANT PHENOTYPES OF *LISTERIA MONOCYTOGENES* SEROTYPES 1/2A AND 4B AND THEIR STABILITY AT 37°C AND 4°C

3.1 Introduction

Listeria monocytogenes accounts for one in four deaths due to foodborne infections in the United States with a reported listeriosis cases of 1600 annually (Ramaswamy et al., 2007a) Listeriosis causes severe complications in immune compromised individuals, including pregnant women, elderly people, newborn babies and those receiving under organ transplant and cancer therapies (Wonderling et al., 2004, Rahimi et al., 2012). *L. monocytogenes* infects susceptible individuals through contaminated food or water (Derra et al., 2013). Since *L. monocytogenes* thrives in high salt and cold conditions, it is frequently associated with refrigerated fully cooked ready-to-eat food products such as luncheon meat, hotdogs, cheese and smoked salmon. However, recently outbreaks of *L. monocytogenes* are frequently associated with fresh fruits and vegetables besides meat and dairy products (Hoffmann et al., 2012).

Since environmental conditions exert a great influence on bacterial cell physiology, it was found that the exposure to different physiological stresses may directly affect the virulence of *L. monocytogenes* (Roche et al., 2005). Many of the foodborne pathogens including *L. monocytogenes* possess adaptive responses to physiological

stresses such as acid, heat, salt, alkali or oxidative that these cells may be exposed during food processing environments (Lou and Yousef, 1997b, Bolton and Frank, 1999, O'Driscoll et al., 1996) . As a result of this, *L. monocytogenes* cells may become stress-hardened and resistant to harsh environmental conditions compared to non-adapted cells. Such stress-adapted cells of *L. monocytogenes* may escape the effect of food preservation measures resulting into serious foodborne illness with long-term consequences.

L. monocytogenes cells may be exposed to oxidative-stress during food processing environments when they encounter different cleaning and sanitization compounds. Regulation of oxidative-stress in *L. monocytogenes* is mainly accomplished through a variety of antioxidant enzymes (Baptista et al., 2012). Lou and Yousef (1997a) reported that *L. monocytogenes* is capable inducing oxidative-stress adaptation upon pre-exposure to 500 ppm of H_2O_2 for 1 h. Such adapted cells exhibited as high as 9 log CFU/ml survival for almost 8 h while the survival of non-adapted cells decreased to 1 log CFU/ml within 4 h under the same lethal oxidative stress treatments. Loepfe and others (2010) found that cold stressed *L. monocytogenes* cells may also become resistant to lethal oxidative stress. Bergholz and others (2012) observed that a short exposure to sublethal salt-stress can also significantly enhance the oxidative stress resistance in *L. monocytogenes* compared to non-exposed control cells. While a pre-exposure to sublethal alkali-stress was found to induce lethal oxidative stress adaptation in *V. parahaemolyticus*, but such a phenomenon was not reported in *L. monocytogenes*.

The purpose of this study is to determine the effect of pre-exposure to sublethal oxidative-stress and sublethal alkali-stress either singly or sequentially on the formation of oxidative-stress resistant phenotypes of *L. monocytogenes* serotypes 1/2a and 4b. In

addition, the stability of such oxidative-stress adaptation in *L. monocytogenes* serotypes 1/2a and 4b at 37°C and 4°C was determined.

3.2 Materials and methods

3.2.1 Bacterial strains and culture condition

Two *Listeria monocytogenes* (serotype 1/2a) Bug600 and (serotype 4b) F1057 were used in present study. In order to prepare overnight cultures, one loop of working stock culture was inoculated into 10 ml of fresh Tryptic soy broth (Difco^{TM}) supplemented with 0.6% yeast extract (TSB-YE) and incubated at 37°C for 18 to 20 h (Imperial III incubator, Lab-Line instrument inc, IL, USA). Log phase cells were prepared by transferring 200 µl of overnight culture into 20 ml fresh TSB-YE and incubation at 37°C in a shaker incubator (C24 Classic series incubator shaker, New Brunswick Scientific, Inc., Edison, NJ, USA) until the optical density (OD_{630}) reached to 0.2-0.25.

3.2.2 Effect of H_2O_2 concentration on the induction of oxidative stress adaptation in *L. monocytogenes*

In this assay, survival of *L. monocytogenes* 1/2a (Bug600) and 4b (F1057) in lethal oxidative stress (1000 ppm H_2O_2) was determined after pre-exposure to different H_2O_2 concentrations (0 ppm to 500 ppm) for 30 min. Initially, 35% (w/w) stock solution (Acros organics, New jersey, USA) of H_2O_2 was diluted to 1% in de-ionized water and 0, 10, 50, 100, 200, 300, 400 and 500 µl of this diluted solution were added in different tubes containing TSB-YE broth to make the final volume up to 9 ml. Thereafter, 1 ml of exponential phase *L. monocytogenes* Bug600 or F1057 cell culture was transferred into these tubes to get the desired H_2O_2 concentrations of 0, 10, 50, 100, 200, 300, 400 and 500 ppm. Pre-exposure in sublethal H_2O_2 stress was performed at 37°C for 30 min. At the

end of pre-exposure period, 1ml samples of H_2O_2 stressed and non-stressed treatments were centrifuged at 13,000 rpm for 5 min (Eppendorf- Netheler- Hinz, GmbH, Brink mann Instruments. Inc, Westbury, N.Y) at 37°C to remove the pre-exposure medium. The resulting cell pellets were then re-suspended in TSB-YE that contain 1000 ppm H_2O_2 which was prepared by adding 1.428 µl of 5 % H_2O_2 to 8.57 ml of fresh TSB-YE. The post-exposure was carried out at 37°C for up to 60 min for the Bug600 strain and 105 min for the F1057 strain. At the end of lethal challenge, for enumeration of survival, 1ml samples from each treatment were centrifuged at 13,000 rpm for 5 min at 37°C and the resulting cell pellet was re-suspended in 0.85% physiological saline. Aliquots of the resulting cell suspensions were serially diluted using 0.85% physiological saline and plated on TSA-YE agar plates. Plates were incubated at 37°C for 48 h before counting Colony Forming Units (CFU).

3.2.3 Effect of pre-exposure contact time in sublethal H_2O_2 on the induction of oxidative stress adaptation in *L. monocytogenes*

In this assay, *L. monocytogenes* Bug600 and F1057 cells were pre exposed to 50 ppm of H_2O_2 concentration for time periods ranging from 5 to 120 min. One ml of log phase *L. monocytogenes* Bug600 or F1057 culture (OD_{630} 0.2-0.25) was mixed with 9 ml of fresh TSB-YE and 50 µl of 1% H_2O_2 was added to the sample to make a 50 ppm H_2O_2 solution. Cells were exposed to sublethal H_2O_2 stress for 5, 15, 30, 60, 90, and 120 min at 37°C and at the end of each pre-exposure period, a lethal challenge (1000 ppm) was introduced following centrifugation. The control for this experiment was also prepared at the same time by mixing 1ml of log phase culture with 9 ml of fresh TSB-YE and incubated for 30 min at 37°C before going into the 1000 ppm H_2O_2 lethal treatment. Post-

exposure for all the treatments and control was carried out up to 60 min for the Bug600 and 105 min for the F1057 strain at 37°C. At the end of the lethal challenge, survival was enumerated on TSA-YE plates as previously described.

3.2.4 Death curves of *L. monocytogenes* in a lethal oxidative stress challenge after pre-exposure to 50 ppm H_2O_2 for 30 min

The objective of this assay was to study the effect of 50 ppm H_2O_2 pre-exposure for 30 min on the survival of *L. monocytogenes* Bug600 and F1057 during a lethal oxidative stress challenge. One ml of log phase culture was added to 9 ml of fresh TSB-YE and 50 µl of 1% H_2O_2 solution was added and cells were pre-exposed to 50 ppm H_2O_2 for 30 min at 37°C. As a control, 1 ml of log phase culture was mixed with 9 ml of fresh TSB-YE and incubated at 37°C for 30 min. At the end of the 30 min pre-exposure period, the 1000 ppm H_2O_2 lethal challenge was introduced following centrifugation and incubated at 37°C. Survival was enumerated at every 30 min until 120 min for the Bug600 strain and 150 min for the F1057 strain on TSA-YE plates as previously described.

3.2.5 Effect of cell density in sublethal H_2O_2 on an oxidative stress adaptation of *L. monocytogenes*

The purpose of this assay was to study the effect of initial cell density on the induction of an oxidative stress adaptation in *L. monocytogenes* under sublethal H_2O_2 stress conditions. In this experiment, log phase *L. monocytogenes* Bug600 and F1057 strains were pre-exposed to different sublethal H_2O_2 concentrations (0 to 100 ppm) with either a high cell density (7 log CFU/ml) or a low cell density (5 log CFU/ml) and then exposed to 1000 ppm H_2O_2 for a lethal oxidative stress challenge. For the high cell

density assay, 0, 10, 20, 30, 40, 50, 70 and 100 µl volumes of 1% H_2O_2 were added to fresh TSB-YE such that the final volume was 9 ml and 1 ml of log phase Bug600 or F1057 culture was added to each tube such that the final H_2O_2 concentrations were 0, 10, 20, 30, 40, 50, 70 or 100 ppm. For the low cell density assay, above mentioned volumes of 1% H_2O_2 were mixed with fresh TSB-YE such that the final volumes reach 9.9 ml and that 100 µl log phase culture was added to each tube. The pre-exposure for both assays was carried out at 37°C for 30 min. At the end of the pre-exposure period, a 1000 ppm H_2O_2 lethal challenge was introduced following centrifugation.

The lethal challenge for the high cell density assay was continued for up to 60 min for Bug600 or 105 min for F1057 at 37°C. In the low cell density assay, the lethal challenge was continued for up to 45 min for Bug600 or 60 min for F1057 at 37°C. At the end of lethal challenge, survival was enumerated on TSA-YE plates as previously described.

3.2.6 Effect of pre-exposure to sublethal alkali-stress on the induction of an oxidative stress adaptation in *L. monocytogenes*

The effect of pre-exposure to sublethal alkali-stress (pH 8 to pH 10) for 30 min on the survival of *L. monocytogenes* Bug600 and F1057 in a 1000 ppm H_2O_2 lethal treatment at 37°C was studied. In order to adjust the required alkaline pH in 10 ml of fresh TSB-YE, the amounts of 4M NaOH required were predetermined using a pH meter (model- AB15, Fisher scientific, Hampton, NH) and dissolved in 9 ml of fresh TSB-YE. Thereafter, 1 ml of log phase culture was added to each tube. As the control, the same amount of log phase culture was mixed with 9 ml of fresh TSB-YE. At the end of 30 min pre-exposure at 37°C, 1000 ppm of H_2O_2 lethal challenge was introduced following

centrifugation. The lethal challenge was carried out up to 90 min for the Bug600 and 105 min for the F1057 strain at 37°C following enumeration of survival on TSA-YE plates as described earlier.

3.2.7 Effect of pre-exposure time to sublethal alkali-stress on the induction of an oxidative stress adaptation in *L. monocytogenes*

In this assay, *L. monocytogenes* cells were pre-exposed to pH 9.0 sublethal alkali-stress for 5 to 120 min to study the effect of pre-exposure contact time on the survival of *L. monocytogenes* Bug600 and F1057 serotypes prior to exposure to 1000 ppm H_2O_2 at 37°C. The required volume of 4M NaOH to adjust the pH of 10 ml fresh TSB-YE was first dissolved in 9 ml of fresh TSB-YE. One ml of log phase culture was added to the same tube such that the final pH was 9.0. Cells were then incubated in pH 9 sublethal alkali-stress for 5, 15, 30, 60 and 120 min at 37°C. As a control, 1 ml of log phase culture was added to 9 ml of fresh TSB-YE broth and incubated for 30 min at 37°C. At the end of each pre-exposure time period, 1000 ppm of H_2O_2 was introduced following centrifugation as described earlier. The lethal challenge was carried out at 37°C for 60 min for the Bug600 and 105min for the F1057 strain and survival was enumerated on TSA-YE plates.

3.2.8 Effect of pre-exposure to different alkali reagents on the induction of an oxidative stress adaptation in *L. monocytogenes*

The objective of this assay was to study the effect of alkaline reagents that increase the pH of the medium on the sublethal alkali-stress induced oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057. The amounts of 4M NaOH, 4M-KOH or 7.5M- NH_4OH required to adjust the pH of 10 ml TSB-YE to pH 9 were

predetermined using a pH meter. The predetermined amount of alkaline reagent was first dissolved in 9 ml of fresh TSB-YE prior to the addition of 1 ml of log phase culture. As a control, 1 ml of log phase culture was mixed with 9 ml of fresh TSB-YE. At the end of 30 min pre-exposure at 37°C, 1000 ppm H_2O_2 was introduced following centrifugation and later incubated at 37°C. Survival was enumerated every 30 min for up to 120 min for the Bug600 strain and 150 min for the F1057 strain on TSA-YE plates as previously described.

3.2.9 Effect of single and sequential pre-exposure to sublethal H_2O_2 and alkali-stress on the induction of an oxidative stress adaption in *L. monocytogenes*

The effect of sequential pre-exposure to sublethal alkali and H_2O_2 stresses on the survival of *L. monocytogenes* Bug600 and F1057 in 1000 ppm H_2O_2 was studied. Three types of controls were used for proper comparison of effects made by each sublethal stress for the final oxidative stress resistance of sequentially stressed phenotypes. In the first control (Negative control) no stress was added. For this, 1 ml of log phase culture was added to 9 ml of fresh TSB-YE and incubated for 30 min at 37°C. The second control was the sublethal alkali control. The log phase cells were pre- exposed to pH 9.0 (adjusted by adding NaOH) for 30 min. In the third control, log phase cell were pre-exposed only to 50 ppm of H_2O_2 for 30 min. In sequential exposures, two sequences of sublethal alkali and oxidative stresses were carried out. In the first sequence, log phase cells were pre-exposed to 50 ppm H_2O_2 for 30 min followed by pH 9 sublethal alkali stress for 30 min at 37°C. In the second sequence, log phase cells were pre-exposed to pH 9 sublethal alkali stress for 30 min followed by 50 ppm H_2O_2 stress for 30 min at 37°C. During, sequential pre-exposure, the medium with the first stress was completely

removed through centrifugation at 13000 rpm for 5 min at 37°C before introducing the second stress to avoid transferring any residuals from the first stress into the second stress. At the end of each pre-exposure treatment, 1000 ppm H_2O_2 lethal challenge was introduced following centrifugation which was later incubated at 37°C. The survivals were enumerated at every 30 min up to 120 min for the Bug600 strain and 150 min for the F1057 strain as previously described.

3.2.10 Stability of the oxidative stress adaptation of *L. monocytogenes* at 37°C and 4°C after their induction by sublethal H_2O_2 or alkali-stress at 37°C

The purpose of this experiment was to study the stability of 50 ppm H_2O_2 or pH 9 sublethal stresses induced oxidative stress adaptation of *L. monocytogenes* Bug600 and F1057 at 37°C and 4°C when the corresponding sublethal stress is no longer available. To begin with, log phase *L. monocytogenes* Bug600 and F1057 cells were pre-exposed to pH 9.0 (NaOH) or 50 ppm H_2O_2 for 30 min at 37°C as described in previous experiments. As a control, log phase cells were incubated in fresh TSB-YE for 30 min at 37°C. In order to study the stability of each adaptation at 37°C, at the end of 30 min pre-exposure, 1 ml volumes of control and treatments were centrifuged at 13000 rpm for 5 min and resulting cell pellets were re-suspended in fresh TSB-YE medium following incubation for 0, 30, 60, and 120 min at 37°C. At the end of 0 and 30 min incubation in fresh TSB-YE at 37°C, 1000 ppm H_2O_2 was introduced following centrifugation as mentioned in earlier experiments. At the end of 60 min incubation, to adjust the cell number to 7 log CFU/ml (to avoid the effect of cell growth that *L. monocytogenes* underwent during 60 min), each treatment and control were diluted 1:2 times using fresh TSB-YE and then subjected to the same lethal challenge. At the end of 120 min incubation period,

treatments and controls were diluted 1:10 times using fresh TSB-YE and then subjected to the same lethal challenge.

In order to study the stability of each adaptation at 4°C, after pre-exposure to 50 ppm H_2O_2 or pH 9 sublethal stress, 1 ml samples of each treatments were centrifuged and cell pellets were re-suspended in fresh TSB-YE that was pre-chilled to 4°C. After that, cells were kept in fresh TSB-YE at 4°C for 1, 4 and 24 h and then directly subjected to 1000 ppm H_2O_2 following centrifugation at 4°C without any dilution step. The lethal challenge for both stability experiments (at 37°C and 4°C) was carried out at 37°C for up to 60 min for the Bug600 strain and 105 min for the F1057 strain and survival were enumerated on TSA-YE plates as previously described.

3.2.11 Statistical analysis

All assays were performed using a complete randomized structure design. Each assay was replicated three times and data present as the average of three independent experiments. In order to compare D-values and protection area between the control and treatments (Fig 3, 7, 8, 9) the student's t-test was used ($p < 0.05$). For all the other experiments, in order to compare survival among the control and treatment effects the ANOVA test with Tukey's Honestly Significant Difference test ($p < 0.05$) was performed using SPSS (version 19).

3.3 Results

3.3.1 Survival of *L. monocytogenes* in lethal oxidative-stress after pre-exposure to sublethal H_2O_2 concentrations

The effect of pre-exposure to different concentrations of sublethal H_2O_2 on the survival of *L. monocytogenes* in lethal oxidative-stress at 37°C is shown in Fig. 3.1. The

oxidative-stress adaptation in *L. monocytogenes* Bug600 and F1057 was found to be sublethal H_2O_2 concentration dependent with 50 ppm H_2O_2 for 30 min being the optimum pre-exposure for inducing the oxidative stress adaptation. For example, the survival of *L. monocytogenes* Bug600 (serotype 1/2a) decreased to 1 log CFU/ml from the initial 7 log CFU/ml within 60 min exposure to lethal oxidative stress at 37°C. By contrast, the survival of *L. monocytogenes* Bug600 was decreased to 3 or 5 log CFU/ml from the initial 7 log CFU/ml after 60 min in lethal oxidative stress when cells were pre-exposed to sublethal H_2O_2 (50-100 ppm for 30 min) (Fig. 3.1A). There were no differences in survival of *L. monocytogenes* Bug600 observed in lethal oxidative-stress when cells were pre-exposed to 10 or 200 ppm H_2O_2 which were similar to control cells at 1 log CFU/ml. When *L. monocytogenes* F1057 (serotype 4b) was exposed to lethal oxidative stress at 37°C, the survival of the 50 ppm H_2O_2 pre-exposure treatment was approximatly 2 log CFU/ml higher than that of control cells (Fig. 3.1B). No differences in survival of *L. monocytogenes* F1057 cells were observed when cells were pre-exposed 10, 100, 200 ppm H_2O_2 when compared to control prior to lethal oxidative stress. On the other hand, when *L. monocytogenes* Bug600 and F1057 cells were pre-exposed to much higher concentrations of H_2O_2 (300-500 ppm), all cells died without triggering any oxidative-stress adaption.

3.3.2 Influence of pre-exposure time in sublethal H_2O_2 on the oxidative-stress adaptation in *L. monocytogenes*

The survival of *L. monocytogenes* Bug600 and F1057 in lethal oxidative-stress after 5 to 120 min pre-exposure time in 50 ppm H_2O_2 at 37°C is shown in Figure 3.2. *L. monocytogenes* Bug600 control cells exhibited 1 log CFU/ml survival from the initial 7

log CFU/ml after 60 min in lethal oxidative stress at 37°C (Fig 3.2A). When cells were pre-exposed to 50 ppm H_2O_2 for 15, 30 and 60 min, survival was increased by 2-4 log CFU/ml when compared to that of control. Similarly, *L. monocytogenes* F1057 control cells had 2 log CFU/ml survival from the initial 7 log CFU/ml after 105 min exposure to lethal oxidative stress at 37°C (Fig 3.2B). By contrast, its survival in lethal oxidative stress was 4.5 log CFU/ml which was 2.5 log CFU/ml higher than the control when pre-exposed to 50 ppm H_2O_2 for 30 min. With a few exceptions, the survival of *L. monocytogenes* in all the other pre-exposure treatments was not different from that of control ($P > 0.05$). Overall, a 30 min pre-exposure in H_2O_2 was optimum for inducing a significant oxidative-stress adaptation in *L. monocytogenes* Bug600 and F1057.

3.3.3 Death curves of *L. monocytogenes* exposed to lethal oxidative-stress after pre-exposure to sublethal H_2O_2

The death curves of *L. monocytogenes* Bug600 and F1057 when exposed to lethal oxidative-stress at 37°C with and without pre-exposure to sublethal H_2O_2 (50 ppm for 30 min) are shown in Figure 3.3. Survival of *L. monocytogenes* Bug600 decreased rapidly from 7 log CFU/ml to 1 log CFU/ml within the first 60 min of exposure to lethal oxidative-stress and were non-detectable after 90 min exposure (Fig 3.3A). When cell were pre-exposed to 50 ppm H_2O_2 for 30 min, the survival of *L. monocytogenes* was approximately 5 log CFU/ml after 60 min exposure to lethal oxidative stress which was 4 log CFU/ml higher than the control and then cell numbers gradually decreased to 1 log CFU/ml in 90 min and was non-detectable at 120 min. For *L. monocytogenes* F1057, the survival in lethal oxidative-stress at 37°C was gradually decreased from the initial 7 log CFU/ml to 5.5 log CFU/ml at 60 min exposure and 2.5 log CFU/ml at 90 min exposure

and was undetectable at 120 min (Fig 3.3B). By contrast, when cells were pre-exposed to 50 ppm H_2O_2 at 37°C, the survival of *L. monocytogenes* F1057 during lethal oxidative stress was 4.5 log CFU/ml in 90 min and 2.5 log CFU/ml in 120 min and was non-detectable after 150 min. In summary, the pre-exposure to 50 ppm H_2O_2 for 30 min significantly increased the survival of *L. monocytogenes* Bug600 and F1057 by 2 to 4 log CFU/ml compared to the control cells during 60 to 120 min in lethal oxidative-stress at 37°C. For both strains of *L. monocytogenes,* the pre-exposure to 50 ppm H_2O_2/30 min yielded the protection area values greater than zero ($P < 0.05$) and higher D-values indicating a significantly higher survival than the control (Table 3.1) during lethal oxidative-stress treatment at 37°C.

3.3.4 Influence of cell density on the oxidative-stress adaptation in *L. monocytogenes*

The effect of cell density in pre-exposure to sublethal H_2O_2 on the survival of *L. monocytogenes* Bug600 and F1057 exposed to lethal oxidative stress is shown in Figure 3.4. From the initial cell density of 7 log CFU/ml, *L. monocytogenes* Bug600 survival was decreased to 1.5 log CFU/ml in lethal oxidative stress at 37°C (Fig 3.4A). At the same cell density, the survival of *L. monocytogenes* Bug600 was increased to 5 log CFU/ml when cells were pre-treated with 50 ppm H_2O_2 for 30 min prior to lethal oxidative stress. The other pre-exposure concentrations of H_2O_2 (except 40 and 70 ppm) did not have any effect on the survival of *L. monocytogenes* at this cell density when exposed to lethal oxidative stress. At an initial cell density of 5 log CFU/ml *L. monocytogenes* Bug600, control cells were non-detectable within 45 min of exposure to lethal oxidative stress at 37°C (Fig 3.4C) while those pre-exposed to 30 ppm H_2O_2 had

the highest survival (3.5 log CFU/ml) compared to all other pre-exposure treatments. From 7 log CFU/ml of initial cell density, *L. monocytogenes* F1057 survival in the control decreased to 2 log CFU/ml when exposed to lethal oxidative stress at 37°C (Fig 3.4B) but for those pre-treated with 50 or 70 ppm H_2O_2, survival was only decreased to 3.5-4 log CFU/ml. At 5 log CFU/ml initial cell density, *L. monocytogenes* F1057 cells pre-treated with 30 ppm H_2O_2 exhibited the highest survival rate in lethal oxidative stress when compared to all other treatments (Fig 3.4D). Overall, the optimum pre-exposure concentration of H_2O_2 that was required for an oxidative-stress adaptation was dependent on the initial cell density and increased with as cell density increased (for example, 30 ppm at 5 log CFU/ml or 50 ppm at 7 log CFU/ml) for *L. monocytogenes* Bug600 and F1057.

3.3.5 Survival of *L. monocytogenes* in lethal oxidative-stress after pre-exposure to sublethal alkali-stress

The effect of pre-exposure to sublethal alkali-stress on the survival of *L. monocytogenes* Bug600 and F1057 exposed to lethal oxidative stress at 37°C is shown in Figure 5. *L. monocytogenes* Bug600 (7 log CFU/ml) exposed to pH 7 (control) were non-detectable after 90 min exposure to the lethal oxidative-stress treatment (Fig 3.5A) while those pre-treated to pH 8 or 8.5 had 1.5 log CFU/ml survival and those pre-treated to pH 9, 9.5 or 10 had 4 log CFU/ml survival after exposure to lethal oxidative stress at 37°C. *L. monocytogenes* F1057 survival decreased to 2 log CFU/ ml from the 7 log CFU/ml inoculums level when exposed to lethal oxidative stress while survival of those pre-treated to sublethal alkali 3 to 4 log CFU/ml greater than the control (Fig 3.5B). Overall,

the survival of *L. monocytogenes* Bug600 and F1057 exposed to lethal oxidative stress increased significantly ($P < 0.05$) after pre-exposure to sublethal alkali-stress.

3.3.6 Influence of pre-exposure time in sublethal alkali-stress on the oxidative-stress adaptation in *L. monocytogenes*

Effect of pre-exposure time in pH 9 on the survival of *L. monocytogenes* Bug600 and F1057 when exposed to a lethal oxidative stress at 37°C is shown in figure 3.6. The survival of *L. monocytogenes* Bug600 and F1057 controls were decreased to 1-2 logs CFU/ml from the 7 log CFU/ml after exposure to lethal oxidative stress for 60 or 105 min (Fig 3.6A & 3.6B). However, the survival of these strains were 2 to 3 log CFU/ml greater than the control when cells were pre-exposed to a pH of 9 for 5 min and their survival was only decreased to 4-5 log CFU/ml when cells were pre-exposed to pH 9 for 60-120 min prior to exposure to the lethal oxidative stress. Overall, a 60 min pre-exposure to pH 9 yielded the highest survival rate for *L. monocytogenes* Bug600 and F1057 after exposure to lethal oxidative stress.

3.3.7 Death curves of *L. monocytogenes* in lethal oxidative-stress after pre-exposure to sublethal alkali-stress

Survival of sublethal alkali (NaOH) pre-treated *L. monocytogenes* Bug600 and F1057 when exposed to lethal oxidative stress at 37°C is shown in Figure 7. *L. monocytogenes* Bug600 survival was quickly decreased from the initial 7 log CFU/ml to 1 log CFU/ml within the first 60 min of exposure to the lethal challenge and was undetectable after 90 min exposure (Fig 3.7A). In contrast, the survival of *L. monocytogenes* Bug600 cells pre-treated with pH 9 for 30 min was decreased to 4.5 log CFU/ml after 60 min exposure to lethal oxidative stress when compared to 1 log CFU/ml

for the control. After 90 and 120 min exposure to lethal oxidative stress, their survival was approximately 3 and 1.5 log CFU/ml, respectively, while the control was non-detectable. *L. monocytogenes* F1057 survival was 2.5 log CFU/ml after 90 min exposure to lethal oxidative stress and was undetectable in 120 min (Fig 3.7B). However, when pre-exposed to pH 9 for 30 min, *L. monocytogenes* F1057 survival was decreased to 5 log CFU/ml with 90 min exposure lethal oxidative stress decreased to 4.5 log CFU/ml at 120 min and 3 log CFU/ml at 150 min, while the control was non-detectable. In summary, 30 min pre-exposure to pH 9 using NaOH led to a significant induction of resistance to lethal oxidative stress in both *L. monocytogenes* strains.

The effect of sublethal alkali pre-exposure using KOH and NH_4OH on the survival of *L. monocytogenes* Bug600 and F1057 exposed to lethal oxidative stress at 37°C are shown in Figure 3.8 and 3.9, respectively. The survival of both strains of *L. monocytogenes* Bug600 and F1057 increased significantly after exposed to lethal oxidative stress when pre-exposure to sublethal alkali-stress for 30 min using KOH or NH_4OH. With sublethal alkali treatments of *L. monocytogenes* Bug600 , there were approximtly 5-6 log CFU/ml survival after 60 min of lethal oxidative stress from the initial 7 log CFU/ml while the control contained 1 log CFU/ml after exposure to the same lethal conditions (Fig 3.8A & 3.9A). After 120 min in lethal oxidative stress, survival in sublethal alkali treatments were 1.5 log CFU/ml while the control cells were undetectable. Similarly in *L. monocytogenes* F1057, survival in the control was decreased to 2.5 log CFU/ml from the initial 7 log CFU/ml within the first 90 min of exposure and was non-detectable after 120 min exposure (Fig 3.8B and 3.9B). In contrast, the survival of cells that were treated with sublethal alkali stress (induced by KOH or NH_4OH) was

approximately 2 log CFU/ml greater than the control after 90 min of lethal oxidative stress. These sublethal alkali adapted cells still survived after 150 min of lethal oxidative stress while the controls were non-detectable.

L. monocytogenes Bug600 and F1057 protection areas were greater than zero ($P < 0.05$) and D-values were significantly higher for all sublethal alkali-stress treatments compared controls that were exposed to a lethal oxidative-stress challenge at 37°C (Table 3.2).

3.3.8 Influence of single or sequential pre-exposure to sublethal stresses on the oxidative-stress adaptation in *L. monocytogenes*

The effect of pre-exposure to sublethal alkali-stress and sublethal oxidative-stress either singly or sequentially on the survival of *L. monocytogenes* Bug600 and F1057 when exposed to lethal oxidative stress is shown in Figure 3.10. Survival of *L. monocytogenes* Bug600 was decreased from the initial inoculums of 7 log CFU/ml to 1 log CFU/ml within first 60 min and was undetectable after 90 min of exposure to lethal oxidative stress at 37°C (Fig 3.10 A). By pre-exposure of cells to 50 ppm H_2O_2 for 30 min, there was an increase in *L. monocytogenes* survival to 5 log CFU/ml after exposure to lethal oxidative stress which was approximately 4 log CFU/ml higher than the control after 60 min exposure. Also, *L. monocytogenes* survival was 2 log CFU/ml higher for 50 ppm H_2O_2 pre-treated cells after 90 min exposure to lethal oxidative stress which was non-detectable at 120 min. Similarly, when cells were treated for 30 min at pH 9, they had 4 log CFU/ml higher survival than the control at 60 min exposure to lethal oxidative stress which gradually decreased to 3.5 log CFU/ml after 90 min exposure and 1.5 log CFU/ml after 120 min exposure. When cells were pre-treated with 50 ppm H_2O_2 followed

by pH 9, their survivals was 5.5 and 3 log CFU/ml at 60 and 90 min, respectively and was non-detectable after 120 min exposure to lethal oxidative stress at 37°C. However, when cells were treated with pH 9 followed by 50 ppm H_2O_2, *L. monocytogenes* Bug600 survivals were increased to 6.5, 5 and 2 log CFU/ml at 60, 90 and 120 min in lethal oxidative stress which was approximately 1-2 log higher than the other sequential pre-exposure treatment. Similar patterns of survival were observed for *L. monocytogenes* F1057 in lethal oxidative stress after pre-exposure to single or sequential sublethal stresses (Fig 3.10 B). Cells treated with either 50 ppm H_2O_2 had 5.5, 4, 2 log CFU/ml survival respectively at 60, 90, and 120 min and was undetectable after 150 min exposure to lethal oxidative stress at 37°C. Similarly, cells that were pre-exposed to pH 9 for 30 min had approximately 6, 5, 3.5 and 3 log CFU/ml survival, respectively, after 60, 90, 120 and 150 min exposure to lethal oxidative stress. There were no additional increases in *L. monocytogenes* F1057 survivals after expose to lethal oxidative stress when cells were first pre-treated with 50 ppm H_2O_2 for 30 min followed by pH 9 for 30 min when compared to those pre-treated at pH 9 alone. However, when the cells were treated with pH 9 followed by 50 ppm H_2O_2 for 30 min each, their survival was increased to 7, 6.5, 5.5 and 4.5 log CFU/ml, respectively, after exposure 60, 90, 120 and 150 min of lethal oxidative stress. Overall, the highest survival of both *L. monocytogenes* Bug600 and F1057 were found in cells that were sequentially pre-exposed to pH 9 for 30 min followed by 50 ppm H_2O_2 for 30 min when compared to cells that were treated with 50 ppm H_2O_2 for 30 min first followed by pH 9 for 30 min. These differences were clearly noticeable in protection area and D-value calculations for these treatments (Table 3.3).

3.3.9 Stability of the oxidative-stress adaptation at 37°C and 4°C for *L. monocytogenes* when induced by sublethal H_2O_2 at 37°C

The stability of the oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057 at 37°C when induced by pre-exposure to sublethal H_2O_2 at 37°C is shown in Figure 3.11. These stability experiments were conducted by first pre-exposure to sublethal H_2O_2 (50 ppm for 30 min) followed by different non-stress periods (at pH 7) on the survival of *L. monocytogenes* Bug600 and F1057 after exposure to lethal oxidative stress at 37°C. After, 30, 60 or 120 min non-stress periods at 37 °C, survival in *L. monocytogenes* Bug600 and F1057 cells pre-exposed to sublethal H_2O_2 were almost similar to that of control cells indicating that the oxidative stress adaptation was reversible at 37°C after 2 h.

The stability of the oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057 at 4°C when induced by pre-exposure to sublethal H_2O_2 at 37°C is shown in Figure 3.12. After a 1 h non-stress period at 4 °C, the survival of both *L. monocytogenes* Bug600 and F1057 that were pre-exposed to sublethal H_2O_2 were decreased to 3-4 log CFU/ml from the initial 7 log CFU/ml after exposed to lethal oxidative stress at 37°C but there was no further decline in survival after either 4 or 24 h of non-stress period at 4°C. Notably, the survival of control pH 7 cells of both *L. monocytogenes* strains in lethal oxidative stress treatments increased during 24 h at 4°C and had approximately 2 log CFU/ml higher survival than 50 ppm H_2O_2 treated cells after the 24 h non-stress period.

3.3.10 Stability of oxidative-stress adaptation at 37°C and 4°C for *L. monocytogenes* when induced by sublethal alkali-stress at 37°C

The stability of the oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057 at 37°C when induced by pre-exposure to sublethal alkali-stress at 37°C is shown

in Figure 3.13. After a 30 min non-stress period at 37 °C, the survival of pH 9 pre-treated cells of *L. monocytogenes* Bug600 was decreased to 4 log CFU/ml in comparison to 2.5 log CFU/ml after 2 h non stress period which was similar to the same as that of control cells. After 2 h of non-stress period at 37°C, the survival of pH 9 treated *L. monocytogenes* F1057 cells was gradually decreased to 3.5 log CFU/ml which was similar to the control cells.

The stability of the oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057 at 4°C when induced by pre-exposure to sublethal alkali-stress at 37°C is shown in Figure 3.14. After 1, 4 and 24 h exposure at 4°C, the survival of pH 9 pre-treated *L. monocytogenes* Bug600 was 4, 6 and 6 log CFU/ml after exposure to lethal oxidative stress at 37 °C. This was similar for in *L. monocytogenes* F1057, where the survival of pH 9 pre-treated cells exposed to lethal oxidative stress challenge at 37°C were approximately 4.5, 5 and 6 log CFU/ml after 1, 4 and 24 h of non-stress periods at 4°C. Also, the survival of control (pH 7) cells of both *L. monocytogenes* strains that were exposed to cold stress for 24 h at 4°C was similar to that of pH 9 pre-treated cells.

3.4 Discussion

Oxidative stress is one of the main physiological stresses that *L. monocytogenes* cells undergo during cleaning and sanitation in the food processing environments. Our study examined the induction of an oxidative-stress adaptation of *L. monocytogenes* Bug600 (serotype 1/2a) and F1057 (serotype 4b) under sublethal oxidative-stress and sublethal alkaline-stress conditions either singly or sequentially. Our findings show that pre-exposure to either sublethal H_2O_2 (50 ppm for 30 min) or sublethal alkali (pH 9 for 30 min) readily triggers oxidative-stress adaptation in both *L. monocytogenes* Bug600

and F1057. Both *L. monocytogenes* and *S. typhimurium* were are resistant to lethal oxidative stress when pre-exposed to sublethal H_2O_2 (Lou and Yousef, 1997a, Christman et al., 1985). Increased tolerance of oxidative stress in bacteria is directly associated with a higher activity of antioxidant enzymes (Scott et al., 1987). Presence of H_2O_2 forms ROS such as peroxides and superoxide which can trigger genetic and physiological mechanisms that are responsible for producing various antioxidant enzymes. For example, H_2O_2 is capable of inducing the production of catalase through activating an OxyR regulator in *E.coli* (Aslund et al., 1999). In addition, ROS in the medium also activates the bacterial SoxR transcriptional activator through oxidizing the iron sulfur center and thereby inducing the production of antioxidant enzymes such as endonuclease iv, glucose 6-phosphate dehydrogenase, Mn superoxide dismutase, and paraquat diaphoroase (Greenberg et al., 1990, Hidalgo et al., 1997, Gaudu and Weiss, 1996). Therefore, when exposed to sublethal H_2O_2, *L. monocytogenes* cells may produce more antioxidant enzymes that will enhance their survival during lethal oxidative stress when compared to control cells. We also observed strain differences in oxidative-stress resistance in *L. monocytogenes*. For example, *L. monocytogenes* F1057 cells are more resistant to lethal oxidative stress challenge than *L. monocytogenes* Bug600 under laboratory conditions, suggests that the antioxidant enzymatic activity in *L. monocytogenes* is strain dependant with some strains naturally expressing a higher enzymatic activity (Dallmier and Martin, 1988).

The optimum pre-exposure concentration of H_2O_2 that is required for inducing an oxidative stress adaptation increased with an increasing cell density of *L. monocytogenes*. A pre-exposure concentration of 50 ppm of H_2O_2 was optimum at a cell density of 7 log

CFU/ml of *L. monocytogenes*. Alternatively at a higher cell density (9 log CFU/m) of *L. monocytogenes*, a 10 times concentration of H_2O_2 (500 ppm) was needed to induce the oxidative stress adaptation (Lou and Yousef, (1997a). In contrast, we found that 500 ppm H_2O_2 concentration was too lethal for *L. monocytogenes* to induce an oxidative stress adaptation a cell density of 7 log CFU/ml.

Exposure to alkaline pH is another major physiological stress which is widely used in food industries to inactivate microorganisms (Giotis et al., 2008a). Our findings indicate that sublethal alkali stress ranging from pH 8 to 10 is capable of inducing oxidative-stress adaptation in *L. monocytogenes* Bug600 and F1057. Nilsson and others (2013) have reported that under the influence of sublethal alkali stress, *L. monocytogenes* may experience oxidative stress due to a net migration of protons out of the cells and formation of ROS. Also, some of the stress proteins that *L. monocytogenes* produces under sublethal alkali stress were also found during oxidative stress. For example, lmo1407 (pyruvate formate lyase) was expressed in *L. monocytogenes* under sublethal alkali stress as well as oxidative stress (Nilsson et al., 2013, Giotis et al., 2010). Therefore, intracellular formation of ROS under alkali stress may be responsible for triggering a *L. monocytogenes* oxidative stress-adaptation response. Also, we have observed that as rapid as 5 min pre-exposure to a sublethal alkali-stress was sufficient to induce oxidative-stress adaptation in *L. monocytogenes*. Similarly, transcriptome analysis of cells subjected to 15 min pre-exposure to sublethal alkali triggered many genes related to multiple metabolic pathways in *L. monocytogenes* (Giotis et al., 2010).

Similar to sublethal H_2O_2 exposed cells, the induction of the oxidative-stress adaption response by pre-exposure to a sublethal alkali treatment (pH 9 for 30 min) to *L.*

monocytogenes may potentially trigger antioxidant enzyme production. We have observed the induction of an oxidative stress adaptation response that was triggered by different alkali reagents. All three alkali regents tested (NaOH, KOH and NH_4OH) equally induced oxidative stress adaptation in *L. monocytogenes* Bug600 and F1057**.**

In previous studies, stress adaptation behavior occurred when *L. monocytogenes* cells exposed to a single sublethal stress however in food processing environments, *L. monocytogenes* cells may be exposed to multiple or sequentially sublethal stresses simultaneously. Our findings indicate that *L. monocytogenes* can become more resistant to lethal oxidative stress when cells were pre-exposed to sublethal alkali stress followed by sublethal H_2O_2 stress for a total of 60 min than the cells treated with the same sublethal stress conditions but in an opposite sequence. Such sequential exposure to sublethal alkali followed by sublethal H_2O_2 stress may trigger production of a greater concentration of antioxidant enzymes and further studies are needed to elucidate such mechanism.

The stability of oxidative-stress adaptation in *L. monocytogenes* tested at different temperatures indicate that both sublethal H_2O_2 and sublethal alkali induced oxidative stress adaptations were completely reversible within 60 min in the absence of the corresponding sublethal stress at 37°C. While the cell physiological adaptations under sublethal stress conditions are induced by damaged macromolecules such as DNA and proteins (Kültz, 2005), microorganisms also have corresponding metabolic activities to reverse those damages and restore them into normal condition which may cause sublethal alkali and H_2O_2 stress adapted cells to lose such adaptation under non-stress conditions (Denkel et al., 2011). Since there was a slight increase in *L. monocytogenes* cell numbers

at 37°C by 0.5 and 1 log CFU/ml during the 60 -120 min non-stress period, appropriate dilutions were made in order to a avoid possible interference by cell density on the killing rate when pre-exposed to a lethal oxidative stress challenge at 37°C. On the other hand, when cells that were pre-exposed to sublethal H_2O_2 or alkali at 37°C were transferred to 4°C, there was no cell growth during the 24 h non-stress periods, but the oxidative stress adaptation was quite stable at 4°C as compared to cells at 37°C. Also, the control cells of *L. monocytogenes* Bug600 and F1057 that were not pre-exposed to sublethal H_2O_2 or alkali at 37°C also exhibited increased resistance to a lethal oxidative stress challenge after cold shock for 24 h at 4°C comparison to cells at 37°C without any cold shock treatment. Loepfe and others (2010) have reported that that *L. monocytogenes* expresses Cold Shock Proteins at low temperature that provide additional protection against oxidative stress. Therefore, we are postulating two possibilities for the stability of oxidative-stress resistant phenotypes of *L. monocytogenes* at 4°C which are as follows: (1) Absence of cell growth during 24 h at 4°C helps *L. monocytogenes* cells maintain the oxidative stress adaptation intact; (2) Exposure to cold stress also induces increased resistance to lethal oxidative-stress in *L. monocytogenes* without the need for pre-exposure to sublethal H_2O_2 or alkali.

In conclusion, our findings show that *L. monocytogenes* Bug600 and F1057 cells are capable of inducing oxidative-stress resistant phenotypes when pre-exposed to either sublethal H_2O_2 or alkali stress at 37°C. We also observed that the sequence of pre-exposure to sublethal alkali followed by sublethal H_2O_2 stress plays an important role in creating more stable oxidative-stress resistant phenotypes of *L. monocytogenes* Bug600 and F1057 at 37°C. Finally, both sublethal alkali and sublethal H_2O_2 induced oxidative-

stress resistance in *L. monocytogenes* at 37°C was completely reversible within 1 h at 37°C. However, the oxidative-stress resistant phenotypes induced by pre-exposure to sublethal alkali or sublethal H_2O_2 at 37°C were stable at 4°C during the 24 h testing period. Also, cells of *L. monocytogenes* Bug600 and F1057 when exposed to cold stress alone exhibited increased resistance to lethal oxidative stress with no pre-exposure to sublethal H_2O_2 or alkali-stress.

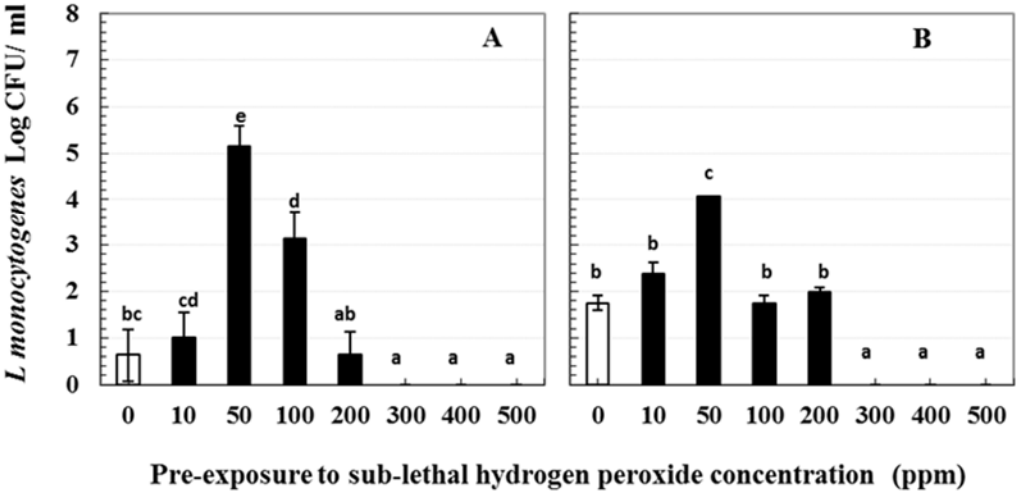

Figure 3.1 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 60-105 min) after 30 min pre-exposure to sublethal hydrogen peroxide at different concentrations at 37°C for two serotypes: (A) 1/2a (Bug600); and (B) 4b (F1057).

Note: Bars with different lowercase letters indicate significant differences between treatments based on Tukeys Honestly Significant Difference test ($P < 0.05$).

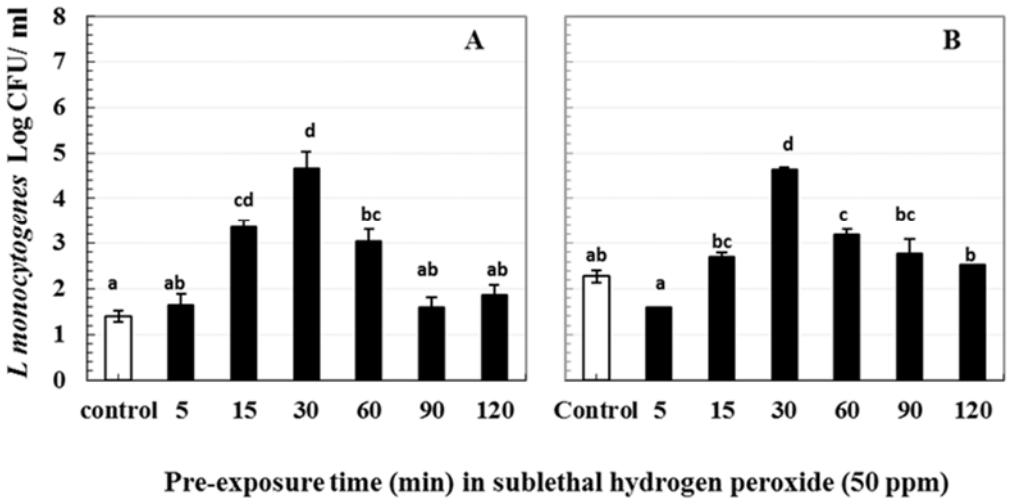

Figure 3.2 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 60-105 min) after pre-exposure to sublethal hydrogen peroxide (50 ppm) for different periods at 37°C for two serotypes: (A) 1/2a (Bug600); and (B) 4b (F1057).

Note: Bars with different lowercase letters indicate significant differences between treatments based on Tukeys Honestly Significant Difference test ($P < 0.05$).

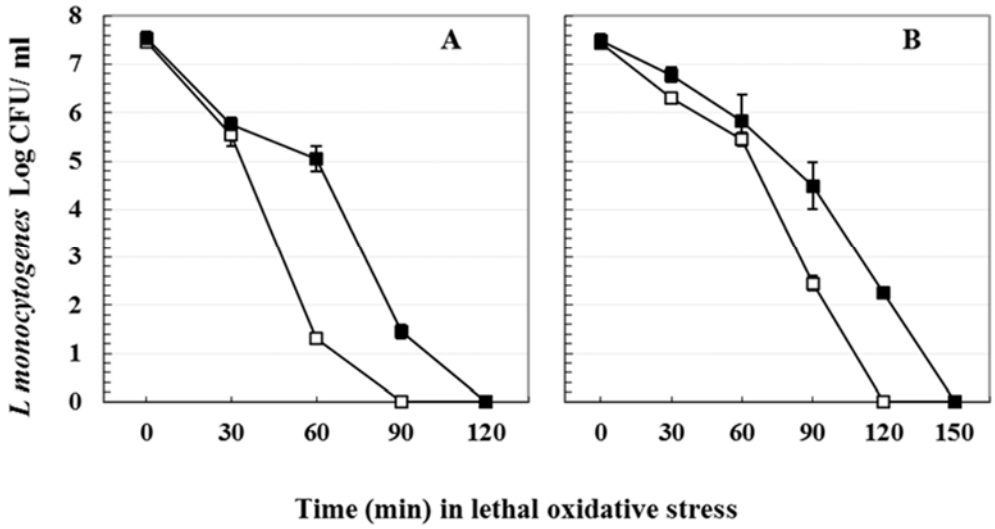

Figure 3.3 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 120-150 min) after pre-exposure to sublethal hydrogen peroxide (50 ppm for 30 min) (■) compared to control cells that were not pre-exposed to sublethal hydrogen peroxide (□) at 37°C for two serotypes: (A) 1/2a (Bug600); and (B) 4b (F1057).

Table 3.1 Protection area and D-values of sublethal H_2O_2 (50 ppm for 30 min) stress adapted and non-adapted *L. monocytogenes* Bug600 (serotype 1/2a) and F1057 (serotype 4b) exposed to lethal oxidative stress at 37ºC.

L. monocytogenes **strain**	**Pre-exposure treatment**	**Protection area value**	**D-value (min)**
Bug600	Control	0	9.2 ± 1.1
	50 ppm H_2O_2	197 ± 31.9*	15.9 ± 0.5*
F1057	Control	0	16.1 ± 0.1
	50 ppm H_2O_2	135 ± 10.2*	22.3± 1.7*

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

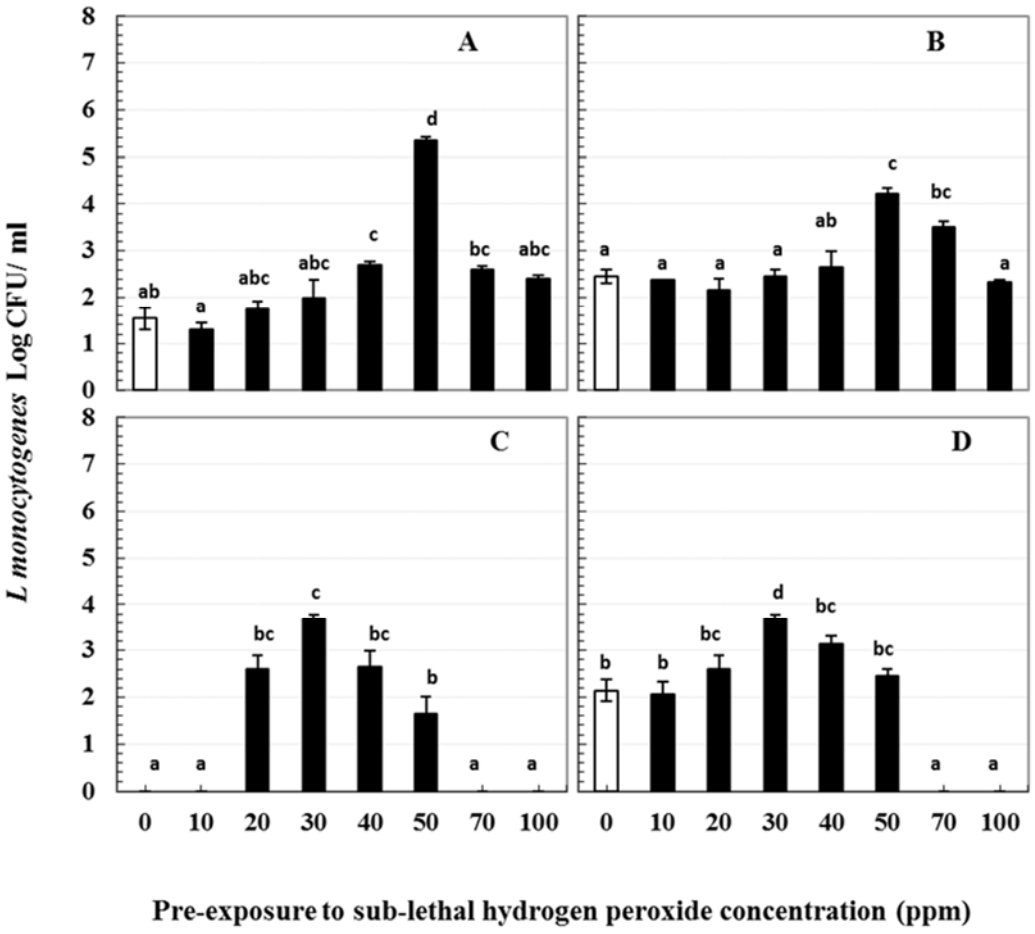

Figure 3.4 Survival of *L. monocytogenes* in lethal oxidative stress (1000 ppm of hydrogen peroxide for 45-105 min) after 30 min pre-exposure to sublethal hydrogen peroxide at different concentrations at 37°C for two serotypes: (A, C) 1/2a (Bug 600); and (B, D) 4b (F1057) at initial cell densities of 7 log CFU/ml (A, B) or 5 log CFU/ml (C, D).

Note: Bars with different lowercase letters indicate significant differences between treatments based on Tukeys Honestly Significant Difference test ($P < 0.05$).

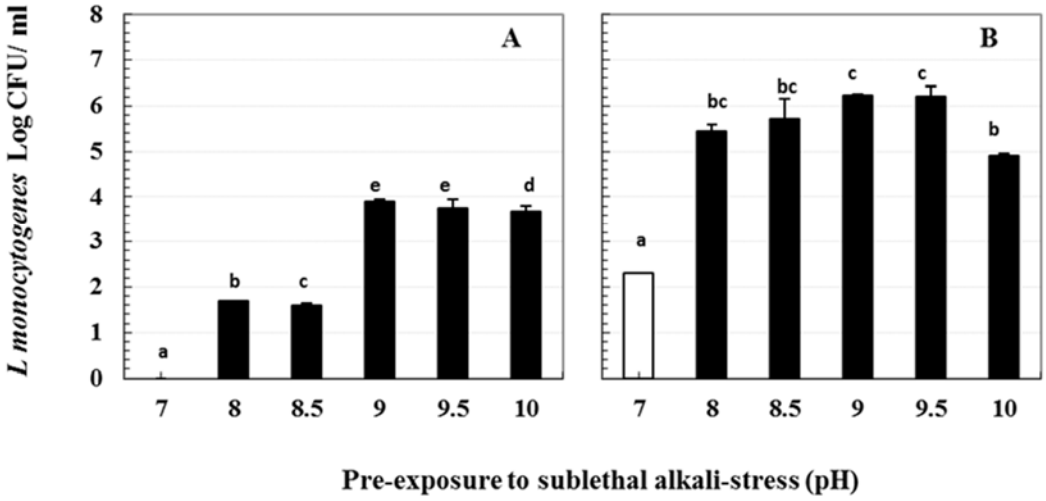

Figure 3.5 Survival of *L. monocytogenes* in lethal oxidative stress (1000 ppm of hydrogen peroxide for 90 min-105 min) after 30 min pre-exposure to sublethal alkali-stress (pH 8 to 10) at 37°C for two serotypes: (A) 1/2a (Bug 600); and (B) 4b (F1057).

Note: Bars with different lowercase letters indicate mean significant differences based on Tukey ANOVA test ($P < 0.05$).

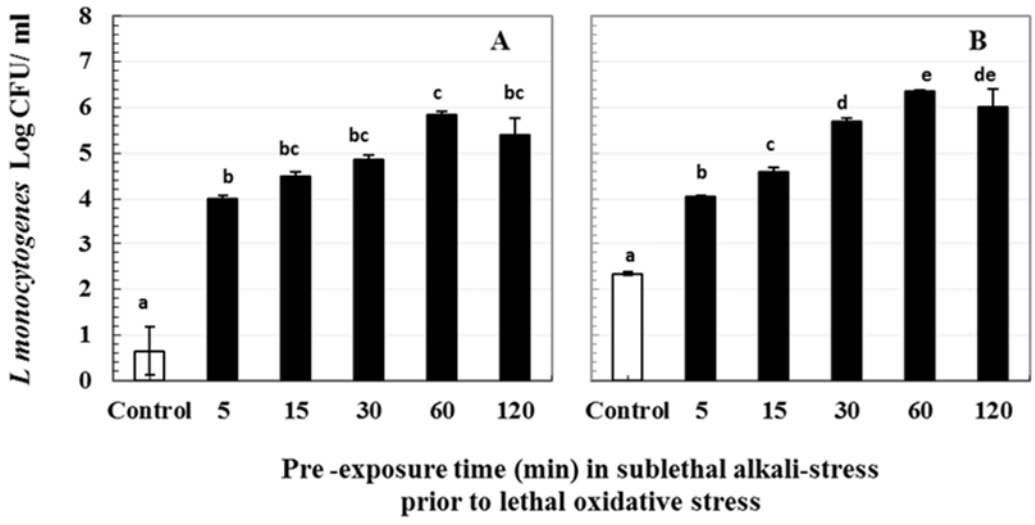

Figure 3.6 Survival of *L. monocytogenes* in lethal oxidative stress (1000 ppm of hydrogen peroxide for 60-105 min) after pre-exposure to sublethal alkali-stress (pH 9) for different periods at 37°C for two serotypes: (A) 1/2a (Bug 600); and (B) 4b (F1057).

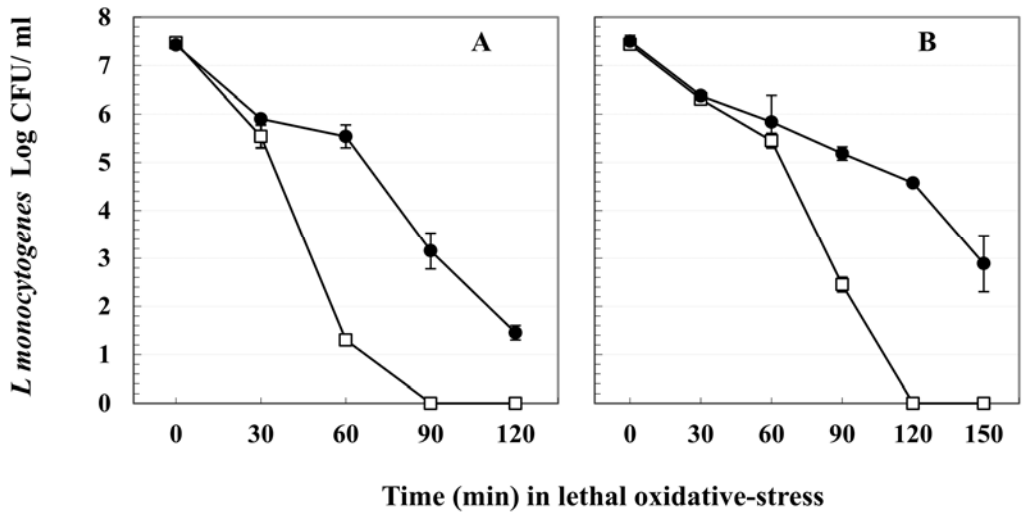

Figure 3.7 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 120-150 min) after pre-exposure to sublethal alkali-stress by NaOH (pH 9 for 30 min) (●) compared to control cells that were not pre-exposed to sublethal alkali-stress (□) at 37°C for two serotypes: (A) 1/2a (Bug 600); and (B) 4b (F1057).

Note: Bars with different lowercase letters indicate significant differences between treatments based on Tukeys Honestly Significant Difference test ($P < 0.05$).

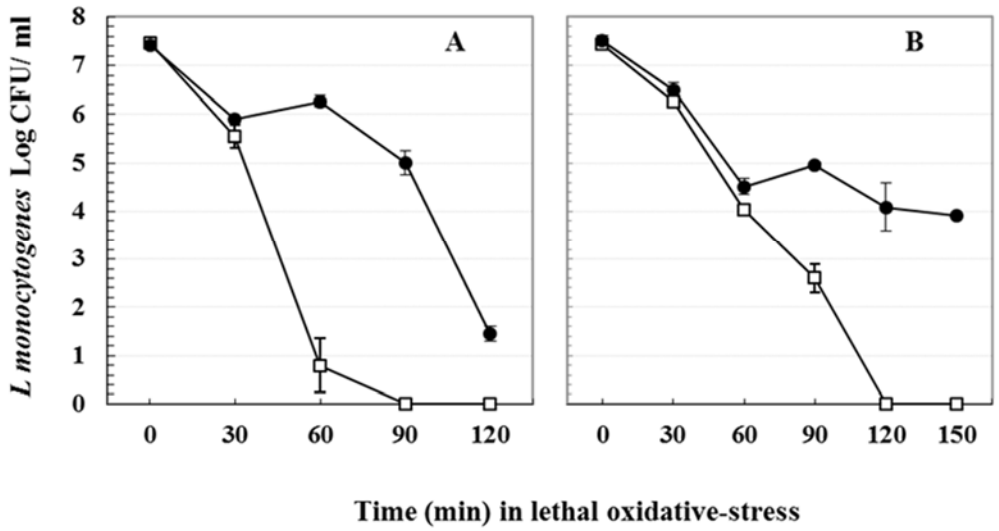

Figure 3.8 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 120-150 min) after pre-exposure to sublethal alkali-stress by KOH (pH 9 for 30 min) (●) compared to control cells that were not pre-exposed to sublethal alkali-stress (□) at 37°C for two serotypes: (A) 1/2a (Bug 600); and (B) 4b (F1057).

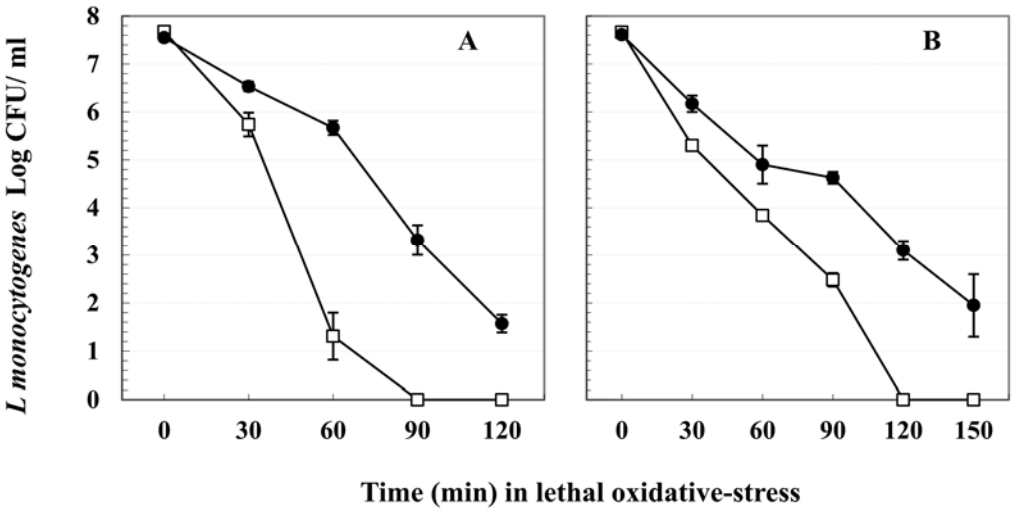

Figure 3.9 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 120-150 min) after pre-exposure to sublethal alkali-stress by NH_4OH (pH 9 for 30 min) (●) compared to control cells that were not pre-exposed to sublethal alkali-stress (□) at 37°C for two serotypes: (A) 1/2a (Bug 600); and (B) 4b (F1057).

Table 3.2 Protection area and D-values of sublethal alkali-stress adapted and non-adapted *L. monocytogenes* Bug600 and F1057 exposed to lethal oxidative stress at 37ºC.

***L. monocytogenes* strain**	**Pre-exposure treatment**	**Protection area**	**D-value (min)**
Bug 600	Control	0	9.8 ± 1.7
	pH 9.0 (NaOH)	281 ± 9.3*	19.9 ± 0.6*
	pH9.0 (KOH)	330 ± 2.4*	22.5 ± 0.8*
	pH 9.0 (NH_4OH)	252 ± 38.5*	19.2 ± 0 *
F1057	Control	0	16.5 ± 0.1
	pH 9.0 (NaOH)	277 ± 11.8*	36.7 ± 3.3*
	pH9.0 (KOH)	275 ± 20.5*	43.1 ± 4.6*
	pH 9.0 (NH_4OH)	252 ± 38.6*	28.2 ±1.2*

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

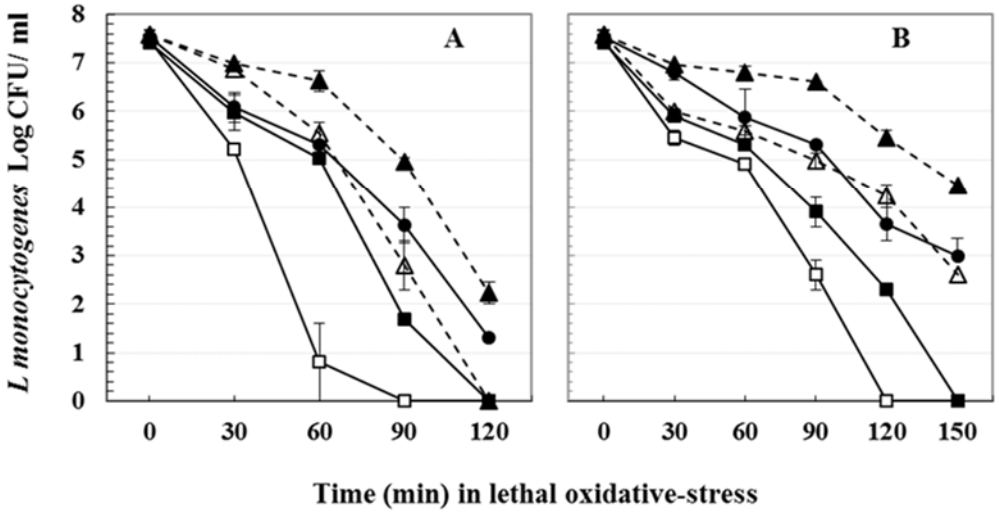

Figure 3.10 Survival of *L. monocytogenes* in lethal oxidative-stress (1000 ppm of hydrogen peroxide for 120-150 min) after sequential pre-exposure to sublethal hydrogen peroxide (50 ppm for 30 min) followed by sublethal alkali-stress (pH 9 for 30 min) (Δ), or sublethal alkali-stress (pH 9 for 30 min) followed by sublethal hydrogen peroxide (50 ppm 30 min) (▲) when compared to cells pre-exposed to sublethal hydrogen peroxide alone (50 ppm for 30 min) (■), or sublethal alkali-stress alone (pH 9 for 30 min) (●), or control cells that were not pre-exposed to sublethal hydrogen peroxide or sublethal alkali-stress (□) at 37°C for two serotypes: (A) 1/2a (Bug600); and (B) 4b (F1057).

Table 3.3 Protection area and D-values of single and sequential sublethal stressed *L. monocytogenes* Bug600 and F1057 in lethal oxidative stress at 37°C.

***L. monocytogenes* strain**	**Pre-exposure Treatment**	**Protection area**	**D-value (min)**
Bug 600	Control	0.0 ± 0.0 ^{a}	10.3 ± 1.1 ^{a}
	50 ppm H_2O_2 for 30 min	199 ± 34.1 ^{b}	16.9 ± 1.8 ^{b}
	pH 9 for 30 min	290 ±23.0 ^{bc}	18.8 ±1.2 ^{b}
	50 ppm H_2O_2/30 min followed by pH 9/30 min	278 ± 17.5 ^{b}	15.9 ± 0.4^{b}
	pH 9/30 min followed by 50 ppm H_2O_2/ for 30 min	412 ± 21.8 ^{c}	24.0 ± 0.4^{c}
F1057	Control	0.0 ± 0.0 ^{a}	17.1 ± 0.2 ^{a}
	50 ppm H_2O_2 for 30 min	182 ± 2.2 ^{b}	21.75± 0.7 ^{b}
	pH 9 for 30 min	271 ± 6.7 ^{c}	30.7 ± 0.2 ^{c}
	50 ppm H_2O_2/30 min followed by pH 9/30 min	266 ±13.5 ^{c}	33.4 ±0.5 ^{c}
	pH 9/30 min followed by 50 ppm H_2O_2/30 min	392 ± 3.6 ^{d}	54.6 ±0.8 ^{d}

Note: Values with different lowercase letters in the same column indicate mean significant differences based on Tukey ANOVA test ($P < 0.05$).

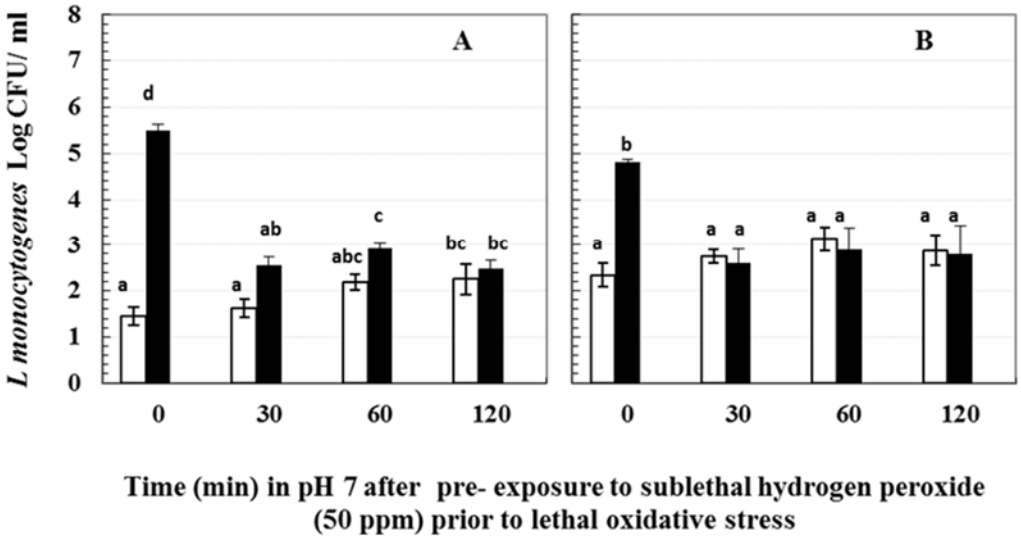

Figure 3.11 Stability of oxidative stress adaptation induced by sublethal hydrogen peroxide (50 ppm for 30 min) (■) after 30-120 min of non-stress (pH 7) at 37°C prior to lethal oxidative stress challenge (1000 ppm of H_2O_2 at 37 °C) when compared to non-adapted control cells (□) for two serotypes: (A) 1/2a (Bug600); and (B) 4b (F1057) .

Note: Bars with different lowercase letters indicate significant differences between treatments based on Tukeys Honestly Significant Difference test ($P < 0.05$).

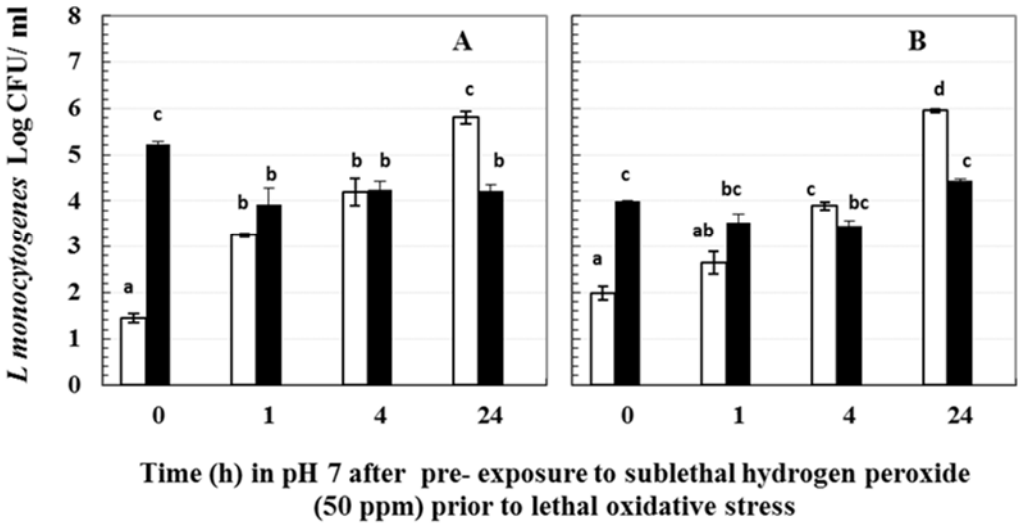

Figure 3.12 Stability of oxidative stress adaptation induced by sublethal hydrogen peroxide (50 ppm for 30 min) (■) after 1-24 h of non-stress (pH 7) at 4°C prior to lethal oxidative stress challenge (1000 ppm of H_2O_2 at 37°C) compared to non-adapted control cells (□) for two serotypes: (A) 1/2a (Bug 600) ; and (B) 4b (F1057) .

Note: Bars with different lowercase letters indicate significant differences between treatments based on Tukeys Honestly Significant Difference test ($P < 0.05$).

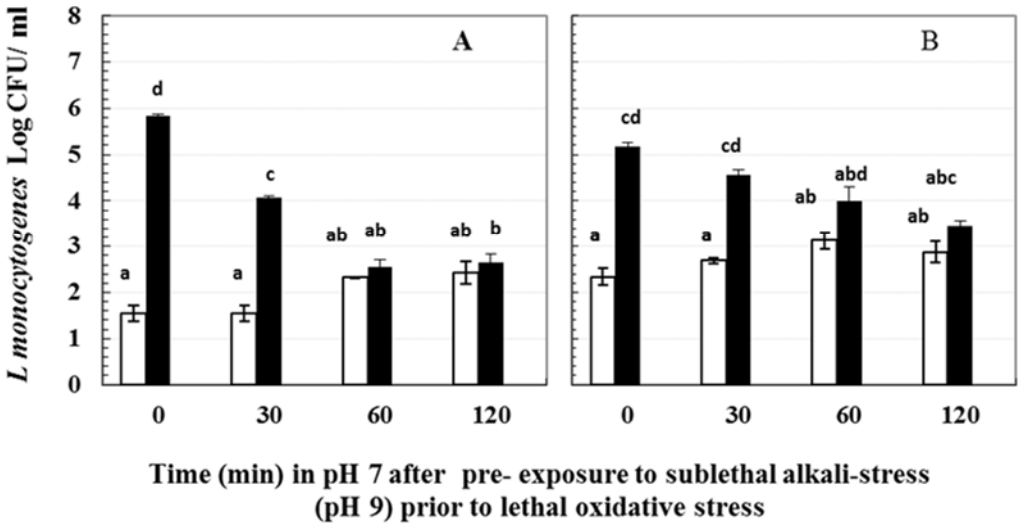

Figure 3.13 Stability of oxidative stress adaptation induced by sublethal alkali-stress (pH 9 for 30 min) (■) after 30-120 min of non-stress (pH 7) at 37°C prior to lethal oxidative stress challenge (1000 ppm of H_2O_2 at 37°C) when compared to non-adapted control cells (□) for two serotypes: (A) 1/2a (Bug 600); and (B) 4b (F1057) .

Note: Bars with different lowercase letters indicate mean significant differences based on Tukey ANOVA test ($P < 0.05$).

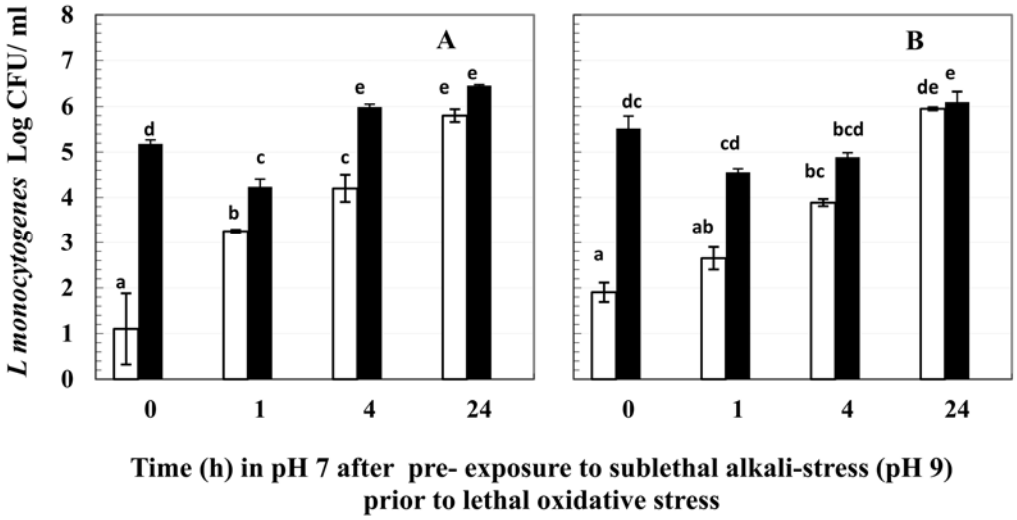

Figure 3.14 Stability of oxidative stress adaptation induced by sublethal alkali -stress (pH 9 for 30 min) (■) after 1-24 h of non-stress (pH 7) at 4°C prior to lethal oxidative stress challenge (1000 ppm of H_2O_2 at 37 °C) compared to non-adapted control cells (□) for two serotypes: (A) 1/2a (Bug 600) ; and (B) 4b (F1057) .

Note: Bars with different lowercase letters indicate mean significant differences based on Tukey ANOVA test ($P < 0.05$).

3.5 Summary and Conclusion

L. monocytogenes is capable of producing stress-hardened cells in which cells when exposed to mild stress conditions that develop increased resistance to lethal stress challenges. In this study, we have examined the induction of an oxidative-stress adaptation for *L. monocytogenes* Bug600 (serotype 1/2a) and F1057 (serotype 4b) when cells were pre-exposed to sublethal H_2O_2 and alkali-stress either singly or sequentially. Our findings indicate that the oxidative-stress adaptation in *L. monocytogenes* Bug600 and F1057 was induced by pre-exposure to 50 ppm H_2O_2 for 30 min at a cell density of 7

log CFU/ml. This optimum concentration of H_2O_2 required for inducing oxidative-stress adaptation was lower at a cell density of 5 log CFU/ml which was 30 ppm for 30 min. In addition to sublethal H_2O_2, *L. monocytogenes* Bug600 and F1057 were also capable of inducing an oxidative stress adaptation under sublethal alkali-stress ranging from pH 8 to pH 10 while a pre-exposure to pH 9 for 60 min was optimum. The pH 9 induced oxidative-stress adaptation in *L. monocytogenes* was almost instantaneous and was induced by several alkaline agents including, NaOH, KOH and NH_4OH. Also, our findings show that the sequential pre-exposure of cells to pH 9 for 30 min followed by 50 ppm H_2O_2 for 30 min at 37°C yielded the highest oxidative-stress resistant phenotypes of *L. monocytogenes* Bug600 and F1057. Both sublethal H_2O_2 and sublethal alkali-stress induced oxidative-stress adaptations were completely reversible within 60 min at 37°C in the absence of such sublethal stress. However, oxidative-stress adaptations induced at 37°C were stable at 4°C in a 24 h test period in both *L. monocytogenes* Bug600 and F1057. Also, cold stress alone for 24 h at 4°C was capable of inducing resistance to lethal oxidative-stress in both *L. monocytogenes* Bug600 and F1057. Our future studies will focus on the potential cross-resistance of oxidative-stress adapted *L. monocytogenes* serotypes 1/2a and 4b to commonly used disinfectants and GRAS antimicrobials.

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