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UNDERSTANDING STRESS ADAPTIVE RESPONSE IN *LISTERIA MONOCYTOGENES*

A Dissertation by

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”Discere ne cessa, cura sapientia crescit,
Rara datur longo prudentia temporis usu.”
(Cato)

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ABSTRACT

The food industry utilizes heat processing as one of the most common food processing methods. Heat processing is used alone, or in combination with other hurdle technologies. The stress response of foodborne pathogens to these food processing hurdles needs careful attention. Non-thermal food processing technologies such as γ irradiation are being intensively studied due to the increasing interest of consumers in foods that are either fresh, or appear to be fresh. My dissertation research attempted to understand the response of *Listeria monocytogenes* to low-dose γ irradiation and heat exposure. As the first part of my work, the applicability of combining low-dose γ irradiation with Modified Atmosphere Packaging (MAP) of sprouts to eliminate *L. monocytogenes* in alfalfa and radish sprouts was studied. The latter half of my work was aimed at understanding the physiological, genomic, and proteomic responses of *L. monocytogenes* when exposed to heat stress and sub-lethal heat exposure.

Two different gas compositions were used in the MAP conditions, (i) 2% O_2, 4% CO_2, 94% N_2, and (ii) 3-5% O_2, 10-15% CO_2 balanced with N_2. These gases were used in combination with irradiation (1 and 2 kGy) on raw and inoculated alfalfa and radish sprouts. For the inoculation studies, the survival of *L. monocytogenes* on alfalfa sprouts was examined. Microbiological and gas composition analysis were carried out periodically. In order to determine the survival of *L. monocytogenes* after ionizing radiation treatment, Thin Agar Layer (TAL) plating was performed in parallel with selective media plating to promote the recovery of the sub-lethally damage/injured cells. Experiments were also performed to develop a *L. monocytogenes* detection tool using rapid impedimetric method to screen treated sprouts. The combination of MAP and 2 kGy γ irradiation was able to reduce the natural microflora to low levels, and no further population increases were detected up to 10 days storage at 5°C. The D_{10}-value of *L. monocytogenes* 4ab strain on alfalfa sprouts was estimated to be 0.46 kGy when packaged in air, 0.58 kGy when packaged in a gas mixture containing 2% O_2, 4% CO_2, 94% N_2 gas and 0.45 kGy in a gas mixture containing 3-5% O_2, 10-15% CO_2 balanced with N_2 gas. The study also demonstrated that the impedimetric method can be used to detect and enumerate *L. monocytogenes* within 24 hours if they are present in numbers higher than 10^3 CFU/g.

Previous studies have shown the increased thermo-tolerance of pathogenic bacteria if pre-exposed to temperatures above their optimal levels prior to a particular heat treatment. It was unclear, however, whether there was a direct relationship between the synthesis of heat shock proteins and the induced thermo-tolerance. In my initial studies, I examined the effect of the sub-

lethal temperature (46°C, 48°C and 50°C for 30 and 60 min) exposure and thermo-tolerance of *Listeria monocytogenes* after a 60°C heat treatment. For further studies, 48°C for 30 min sub-lethal temperature treatment was chosen. Fluorescence spectroscopy, microscopy, genomic, and proteomic approaches were used to investigate the sub-lethal heat stress response in *L. monocytogenes*. I studied the physiological, genomic, and proteomic response of *L. monocytogenes* when exposed to 60°C heat treatment with and without prior exposure to the sub-lethal temperature conditions. Pre-exposure to sub-lethal temperature of 48°C for 30 min increased the D-values at 60°C of a virulent strain of *L. monocytogenes* from 3.7 minutes to 4.6 minutes. There was a significant difference in the viability estimates using culture media as compared to direct viability estimates using the Live/Dead BacLight™ viability stain. When the cells were exposed to 60°C for 9 minutes, only 1% of the cells were viable based on culture counts. However, when the cells were examined for viability using the BacLight™ viability assay, almost 100% of the cells were found to be still viable. When the cells were pre-exposed to 48°C for 30 minutes prior to 60°C heat treatment for 9 minutes, slightly more than 1% of the cells were still culturable, and almost 100% of the cells were still viable per the BacLight™ viability assay. The results suggest that *L. monocytogenes* cells can enter into a viable but non-culturable (VBNC) state when exposed to sub-lethal temperature stress conditions.

Microarray analysis was performed to identify the differentially expressed genes during heat stress by comparing the transcriptome of *L. monocytogenes* under optimal temperature (37°C), heat shock (60°C for 0 minute), prolonged heat shock (60°C for 9 minutes), and thermo-tolerance inducing (48°C for 30 minutes prior to exposure to 60°C for 9 minutes) conditions. A majority of the differentially expressed genes were up-regulated at heat shock as compared to those that were down-regulated when the cells were exposed to prolonged heat exposure, and thermo-tolerance inducing conditions. Only 10 genes were commonly expressed across the three different temperature treatments. Though many of the differentially expressed genes could be tentatively classified based on the current functional classification of genes (COG) per the NCBI database, many of the gene loci could not been attributed to a specific function due to the current limited knowledge on the functional genomics of *L. monocytogenes*.

Two dimensional gel electrophoresis (2DGE) coupled with MALDI-TOF analysis were performed to study the differential expression of *Listeria monocytogenes* (ATCC 43256) soluble proteins at heat shock (60 °C for 0 minute) conditions and prolonged heat shock (60 °C for 9 minutes) conditions and thermo-tolerance inducing (48°C for 30 minutes followed by 60°C for 9 minutes) conditions. The proteome was compared under these conditions to the proteome at 37°C. Eighteen different proteins were differentially expressed at 60°C for 0 minute (6 up-regulated and

12 down-regulated), 21 proteins were differentially expressed (12 up-regulated and 9 down-regulated) when the cells were exposed to 60°C for 9 minutes, and 20 proteins were differentially expressed (10 up-regulated and 10 down-regulated) when cells were initially exposed to 48°C for 30 minutes prior to 60°C for 9 minutes. There was one protein (which could not be identified) with observed MW of 50 kDa which was differentially expressed across the three temperature treatments.

CHAPTER 1
INTRODUCTION

Thousands of people around the world die each year from pathogen and toxin contaminated foods. The pathogenic organisms that are of concern to food safety originate in the environment, in farm animals, and in humans. The contamination can occur at all points ”from the farm to the fork”. The pathogens can be transmitted between humans, animals, the environment, and foods through air, water, soil, and also via contaminated or improperly cleaned equipment. There is an increasing consumer demand for foods that are fresh, natural, foods that “look fresh”, foods that are crisp, and foods with high nutrient content. The food industry utilizes a variety of “hurdles” (hurdle technology) to prevent or eliminate pathogens from foods. These techniques include reduction or increase of temperature (freezing and heat treatment), use of ionizing radiation (food irradiation), reduction of water activity (by drying or by addition of salt or sugar), reduction of pH (by addition of acids or by fermentation), removal of oxygen (vacuum packaging) or modifying the packaging atmosphere (Modified Atmosphere Packaging or MAP) by the addition of carbon dioxide, the addition of bacteriocins (eg., nisin) or organic and inorganic preservatives. A majority of these preservative techniques are used alone, or in combination to preserve foods and ensure their safety by inhibiting microbial growth. Heat treatment is still the most commonly used preservation techniques and if it is properly applied, heat can successfully eliminate the biological agents in the food. Alternative technologies such as food irradiation, high hydrostatic pressure (HHP), pulsed electric field technology (PEF) are being developed for producing safe food with high quality.

The response of foodborne bacterial pathogens to stresses caused by these hurdle technologies or “stressors” is a concern. Some of these stress responses can result in enhanced survival, enhanced virulence, and even cross protection against multiple stressors. Microorganisms can also become more heat resistant. Sub-lethal heat stress (heat shock) or prior exposure temperatures above optimal growth temperatures can render the organism to become more resistant to subsequent heat treatment which, under normal conditions would have been lethal. This stress response is also termed induced thermo-tolerance. The practical importance of thermo-tolerance to the food industry relates primarily to foods that are exposed to temperatures below 65°C. Thermo-tolerance can become a concern for meat products kept on warming trays before a final heating or reheating step, or when there is an interruption in the cooking cycle during food processing (due to equipment failure). The heat shock response and increased thermo-tolerance has been previously reported for *L. monocytogenes*. *L. monocytogenes* is of particular concern to the Ready–To-Eat (RTE) food

industry because it has a variety of genetically encoded survival mechanisms to withstand environmental stressors such as heat, cold, salt, and acidic conditions. Moreover, this organism is ubiquitous and can be found in soil, water, and on food processing equipments. More importantly, this pathogen has a very high case-fatality rate. *L. monocytogenes* is a growing issue in sprouts. Contamination of sprouts can occur through seeds, contaminated equipment, contaminated water, or poor hygienic handling. Modified Atmosphere Packaging (MAP) is commonly utilized for a variety of fresh produce to extend the shelf-life, and also to maintain the high quality of minimally processed fruits and vegetables. Low-dose irradiation with doses ranging from 0.5 to 2 kGy has value in eliminating this pathogen from vulnerable foods such as sprouts.

The overall focus of my dissertation research was to better understand the stress adaptive response in *L. monocytogenes* using sub-lethal temperature as the stress factor. As part of this study I also investigated the applicability of combining low-dose irradiation with modified atmosphere packaging to control *L. monocytogenes* in fresh produce. The underlying hypothesis of my research was that *Listeria monocytogenes* elicits unique physiological, genomic and proteomic responses as part of its overall stress adaptation in response to sub-lethal temperature stress. The specific objectives of my research were,

1. To understand the physiological responses of *L. monocytogenes* to sub-lethal temperature stress conditions.
2. To understand the transcriptomic response of *L. monocytogenes* to sub-lethal temperature stress conditions.
3. To understand the proteomic response of *L. monocytogenes* to sub-lethal temperature stress conditions.
4. To evaluate the applicability of combining low-dose γ irradiation with Modified Atmosphere Packaging (MAP) to control *L. monocytogenes* in packaged fresh produce.

I utilized a variety of conventional microbiology, microscopy, fluorescent spectroscopy, genomic, and proteomic approaches to address the underlying research questions.

CHAPTER 2
LITERATURE REVIEW

2.1 Background and Occurrence

Listeria spp. are Gram-positive, non spore-forming, non-capsulated, facultative anaerobic, catalase positive, oxidase negative bacteria. Occasionally, catalase negative strains have also been isolated from clinical specimens (Bubert et al., 1997; Swartz et al., 1991). The genus *Listeria* currently contains six species namely, *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi.* This specification is based on DNA:DNA homology values, 16S rRNA and DNA sequencing information, chemotaxonomic properties, and multilocus enzyme analysis (Khelef et al., 2006; Rocourt and Buchrieser, 2007). Two of the species (*L. monocytogenes* and *L. ivanovii*) are pathogens. *Listeria monocytogenes* is a food-borne human pathogen responsible for listeriosis, while *Listeria ivanovii* is an animal pathogen mainly in sheep and cattle. *L. monocytogenes* can be harbored within the human gastrointestinal tract asymptomatically (Seeliger and Jonesy, 1986). *Listeria* spp. can also colonize various inert surfaces and can form biofilms on food-processing surfaces (Wong, 1998; Taormina and Beuchat, 2002). *L. monocytogenes* has an alarmingly high case-fatality rate, especially in immuno-compromised and pregnant women (Goulet and Marchetti, 1996; Mead et al., 1999). In European Union countries, the mortality rate in 2006 was reported to be around 14% (Denny et al., 2007).

L. monocytogenes is capable of surviving unfavorable environmental conditions both in the natural environment such as soils, streams, and within food-processing environments i.e. on food processing equipments (Sauders and Wiedmann, 2007). *L. monocytogenes* is found in a variety of raw foods, such as uncooked meats, vegetables, and processed foods that become contaminated after processing, such as soft cheeses and cold cuts at deli counters (Fleming et al., 1985; Linnan et al., 1988; Schwartz et al., 1989; Riedo et al., 1994; Bula et al., 1995; Salamina et al., 1996; Dalton et al., 1997; Goulet et al., 1998; Aureli et al., 2000; Ooi and Lorber, 2005). Unpasteurized milk or foods made from unpasteurized milk may also contain the bacterium. *L. monocytogenes* can be eliminated by pasteurization and cooking, but in some cases Ready-To-Eat (RTE) foods such as hot dogs and deli meats can get contaminated between cooking and final packaging (Khelef et al., 2006). In Europe, the incidences of *L. monocytogenes* in cheeses from various countries were: Italy 17.4%, Germany 9.2%, Austria 10%, and France 3.3% (Rudolf and Scherer, 2001). *Listeria* spp. were found most frequently in soft and semi-soft cheese. Greenwood et al. (1991) report finding

eight samples containing more than 100 *L. monocytogenes* CFU/cm^2 cheese surface, 2 samples with counts above 10^4 CFU/cm^2 cheese surface. Surprisingly, a higher incidence of *L. monocytogenes* was observed in cheeses made from pasteurized milk (8.0%) than in cheeses manufactured from raw milk (4.8%). In the U.K. a study of pre-packaged ready-to-eat (RTE) mixed salads containing meat or seafood ingredients from retail premises was undertaken to determine the frequency and level of *L. monocytogenes* (Little et al., 2007). The overall contamination of *Listeria* spp. and *L. monocytogenes* in mixed salads was 10.8% and 4.8%, respectively. Salad samples with meat ingredients were twice likely to be contaminated with *Listeria* spp. and *L. monocytogenes* (14.7% and 6.0%, respectively) compared to samples with seafood ingredients (7.4% and 3.8%, respectively). Pre-packaged mixed salads were contaminated with *Listeria spp*. and *L. monocytogenes* more frequently when obtained from sandwich shops that were not packaged on the premises and stored or displayed above 8°C. Harvey and Gilmour (1992) have reported an overall incidence of *Listeria* spp. in raw milk samples in Northern Ireland to be 25.0% with *L. monocytogenes* to be around 15%. The incidence in samples from processing centres was found to be 54.0% with *L. monocytogenes* to be around 33%. This occurrence level was much higher than that found in dairy farm samples which had occurrence levels ranging only around 9% with *L. monocytogenes* at around 5%. In the European Union, the number of cases of listeriosis increased from 1,427 in 2005 to 1,583 in 2006 which followed a similar increasing trend that was observed in the preceding years. In 2006, *Listeria* spp. were most commonly reported above the legal safety limit from cheeses, RTE fish products, and other RTE products (Denny et al., 2007). Ralovich and Domján-Kovács (1996) in an early study reported that listeriosis is a rare human disease in Hungary. However, it can be argued that this could be due to poor tracking and reporting. Kiss et al. (2006), however, in 2004 tested a variety of food samples and *L. monocytogenes* was most commonly detected (72%) in milk and dairy products. Of the different serotypes, 45% of the serotypes were 1/2a and 27% were 4b. In 2004, they reported that there were only 3 perinatal and 14 nonperinatal human listeriosis cases. A majority of the cases (53%) were caused by serotype 4b and 24% by serotype 1/2a. A number of listeriosis outbreaks have been reported, most human listeriosis cases likely represent sporadic cases (and possibly small outbreaks) caused by a wide variety of *L. monocytogenes* strains (McLauchlin, 1996; Sauders et al., 2003). In a survey of about 31,700 RTE foods in two U.S. states, Gombas and co-workers detected this pathogen in about 1.8% of the samples tested (Gombas et al., 2003). Ready-to-eat foods were sampled over of a three year time frame by Holah et al. (2004) to screen for *L. monocytogenes* among other organisms. Though the prevalence was low (0.08-0.35%), the detection of these organisms in foods and food processing environment suggest that they were capable of withstanding low temperatures, wide pH ranges,

fluctuating nutrient supplies, varying moisture levels, and withstanding industrial cleaning and disinfection practices.

In Europe, the European Union Commission Regulation (EC) No 2073/2005 established microbiological criteria in foods (Carrasco et al., 2007). For *L. monocytogenes* in the category of RTE foods able to support its growth, (other than those intended for infants and for special medical purposes), two different microbiological criteria have been proposed namely (i) *L. monocytogenes* levels should be <100 CFU/g throughout the shelf-life of the product, (ii) absence in 25 g of the product at the stage before the food has left the immediate control of the food business operator, who has produced it. The application of either the first or the second of these criteria depends on whether or not the manufacturer is able to demonstrate that the level of *L. monocytogenes* in the food product will not exceed 100 CFU/g throughout it's shelf-life. This demonstration has to be based on physico-chemical characteristics of the target product and consultation of scientific literature, and, when necessary, on quantitative models and/or challenge tests. According to Article 3 of Regulation (EC) No 2073/2005, it indicates that Food Business Operators (FBO) shall ensure that foodstuffs comply with the relevant microbiological criteria and limits set out in the Regulation. Furthermore, Article 3 refers to the shelf-life studies (listed in Annex II of the Regulation), that the FBO shall conduct in order to investigate compliance with the criteria throughout the shelf-life. In particular, this applies to RTE foods that are able to support the growth of *L. monocytogenes* and that may pose a *L. monocytogenes* risk for public health. The regulation has identified some of the limits and growth factors for *L. monocytogenes* that are meant to assist the FBO in identifying the factors controlling the pathogen's survival and growth in foods (Table 2.1).

In the late 1980's, in the United States, *L. monocytogenes* emerged as a problem in deli meats and other processed products. The USDA-FSIS (Food Safety Inspection Service) and the U.S. FDA worked with food processing plants to improve their procedures and emphasized the "zero" tolerance (no detectable level permitted) for the pathogen in RTE products. Based on the known characteristics of this microorganism and the disease, the U.S. FDA maintains a policy of "zero-tolerance" for *Listeria monocytogenes* in RTE foods (i.e., products that may be consumed without any further cooking or reheating). This means that the detection of any *Listeria monocytogenes* in either of two 25-gram samples of a food renders the food "adulterated" as defined by the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 342(a)(1) (Shank et al., 1996). It needs to be emphasized, however, that there is no epidemiological evidence that demonstrates whether a zero or non-zero tolerance policy leads to a greater rate of listeriosis. Estimates of disease rates between different countries are difficult to compare due to different surveillance methods and public health reporting

systems. The overall rates of listeriosis is thought to range between 0.1 to 11.3 cases per million persons per year in Europe, 3.4 to 4.4 cases per million people per year in the United States, and 3 cases per million per year in Australia (Rocourt et al., 2003).

Table 2.1. Factors identified to have an impact on the growth and survival of *L. monocytogenes* (adapted from SANCO, 2008).

Factor	**Can Grow**[a]			**Can Survive**[c] **(No Growth)**
	Lower Growth Limit	**Optimum**[b]	**Upper Growth Limit**	
Temperature(°C)	-1.5 to +3.0	30.0 to 37.0	45.0	-18.0
pH[d]	4.2 to 4.3	7.0	9.4 to 9.5	3.3 to 4.2
Water Activity (a_w)	0.90 to 0.93	0.99	> 0.99	< 0.90
Salt Concentration[e] **(%)**	< 0.5	0.7	12 – 16	≥20
Atmosphere	Facultative anaerobe (it can grow in the presence or absence of oxygen, e.g. in a vacuum or modified atmosphere package)			
Heat Treatment during Food Processing	A temperature/time combination e.g. of 70°C and 2 min is required for a D_6 (i.e. 10^6 or 6 decimal) reduction in numbers of *L. monocytogenes* cells. Other temperature/time combinations may also provide the same reduction.			

[a]Based on experimental data and hence provide only a rough estimate; [b]Optimum indicates when the growth of *L. monocytogenes* is fastest; [c]Survival period will vary depending on nature of food and other factors; [d] Inhibition of *L. monocytogenes* is dependent on type of acid present;[e] Based on percent sodium chloride, water phase.

There are multiple sources for the occurence of *L. monocytogenes* in food products. This pathogen could enter the food through the ingredients such as raw meat, poultry, and seafood. They could also be present in produce and in milk and milk products. *L. monocytogenes* could also contaminate foods via food processing products such as compressed air, ice and brine solutions which used for chilling and refrigeration. This pathogen could also contaminate food products through contact (eg. filling and packaging equipment, slicers, dicers, shredders, utensils, gloves) and also non-contact surfaces (in-floor weighing equipment, conveyor belt rollers, cracked hoses, equipment bearings, condensate drip pans, vacuum cleaners, on/off switches, etc). Food products could also get contaminated via the plant environment: floors, walls, drains, wet insulation, and door seals.

Listeria spp. are capable of growing in a wide range of temperatures (1-45°C) (Chavant et al., 2002; George et al., 1988). This endows the organism with a unique capacity to survive food processing and food storage conditions. However, optimal growth of the organism occurs between 30–37°C (Petran and Zottola, 1989). *Listeria* spp. are motile at low temperatures (20°C), although some *Listeria* strains are nonmotile at 37°C because they lack the expression of the flagellin

proteins at this temperature (Way et al., 2004). *Listeria* spp. are generally inactivated at 60°C, making pasteurization a suitable food processing technique to eliminate this bacterium from dairy products (Seeliger and Jonesy, 1986). Though the optimal pH condition for growth is 7.0, *Listeria spp.* are capable of growing between pH 4.5 and pH 9.2 in broth media (George et al., 1992; Parish and Higgins, 1989, Petran and Zottola, 1989). It can multiply in 10% (w/v) NaCl, and can survive at even higher concentrations (Seeliger and Jonesy, 1986; Shahamat et al., 1980). Survival at low pH and high salt concentration has been reported to depend strongly on temperature (Cole et al., 1990). The minimum pH that allowed survival at 30°C was around pH 4.7. Low salt concentration (4-6%) improved survival, however, high salt concentration reduced survival especially at pH ranges that were restrictive to *L. monocytogenes*. *Listeria* is one of the few foodborne pathogens that can multiply at low water activity. Studies have shown that this organism can multiply at a_w below 0.93 (Farber et al., 1992; Petran and Zottola, 1989).

2.2 Microbial Stressors and Stress Adaptation

Yousef and Courtney (2003) define microbial stress as any deleterious physical, chemical, or biological factor that adversely affects microbial growth or survival. The biological stressors could include competition, metabolites produced by other bacteria, and microbial antagonism (Abee and Wouters, 1999). Hence, traditional or novel food preservation techniques such as use of brine, high hydrostatic pressure, ionizing radiation, pulsed electric fields, and UV irradiation can be considered as microbial stressors. These "hurdles" can introduce varying degrees of "stress" in different bacteria. These stressors will influence the physiology, function, and activity of microorganisms (benign or pathogenic or spoilage) that are found in foods. Based on the magnitude of the stress involved, stress can be differentiated as either "sub-lethal" or "lethal or severe". Sub-lethal stress modifies the metabolic activities of the cells. It can result in microbial "injury" and can be manifested as either retarded growth or complete prevention of growth (Donnelly, 2002). Lethal or severe stress causes irreversible damage to the microbial cells. When microorganisms are exposed to sub-lethal stress, it is generally thought that this exposure can induce adaptation to subsequent lethal levels of the same type of stress. This microbial adaptation is considered as "stress adaptation" (Lou and Yousef, 1997). Stress adaptation can also be described as the general principle in which a bacterium that is exposed to a sub-lethal stress can become more resistant to subsequent applications of the same stress or at times to a different stress (Hill et al., 2002). During food processing, microorganisms adapt to the "hurdles" or stressors and can survive under conditions which would have normally inactivated them (Beales, 2004). The Acid Tolerance Response (ATR) observed in *L. monocytogenes* is an example of a typical stress response. Pre-stressed or acid-adapted cells are much more tolerant to normally lethal pH levels. The ATR in *L. monocytogenes*

could be induced in acidic foods, when exposed to gastric juices, or within the macrophage phagosome (Gahan and Hill, 1999). Marron et al. (1997) have shown that the ATR is required by *L. monocytogenes* for successful murine infection. The optimal growth temperature for *L. monocytogenes* is between 30°C and 37°C, and any temperature above this optimal range is expected to exert a stress (Petran and Zottola, 1989). When microbial cells are exposed to temperatures above optimal growth temperatures even for short periods of time, unique physiological responses are triggered within the cells including the synthesis of heat shock proteins (Lindquist, 1986; Knabel et al., 1990). Pagàn et al. (1997) have reported a 7-fold increase in thermo-tolerance of *L. monocytogenes* when the cells were exposed to 45°C for 180 minutes. The extent of exposure to temperature above optimal levels, and the matrix in which the cells are exposed are reported to influence the extent of the observed thermo-tolerance (Linton et al., 1990; Sergelidis and Abrahim, 2009).

Microbial adaptation to stress also causes extended tolerance to multiple other lethal stressors. This has been termed "cross-protection" (Begley et al., 2002). Microorganisms are thought employ cross protection as a defense strategy against the lethality of various food preservation techniques (Rodriguez-Romo and Yousef, 2005b). In addition to acid tolerance, ATR has also been shown to cross-protect against thermal and osmotic stresses. Foster (2000) has reported that the induction of acid shock proteins protects microbial cells against lethal acid proteins or other stresses such as high temperature, oxidative damage and high osmolarity. ATR is also known to cross-protect against nisin, ethanol, and crystal violet (O'Driscoll et al., 1996). Leyer and Johnson (1993) observed that *Salmonella* Typhimurium became more resistant against heat, salt, or activated lactoperoxidase system when this organism was exposed to mild acid stress (pH 5.8) previously. Duffy et al. (2000) showed that *Escherichia coli* O157:H7 when stored at pH 4.8 for 96 h under stimulated meat fermentation condition (followed by pH 5.6 or 7.4 growing condition), showed enhanced tolerance against the subsequent heat treatment at 55°C. The heat resistance of *E. coli* O157:H7, *Salmonella enterica* and *L. monocytogenes* increased significantly after acid adaptation when exposed to single-strength apple, orange, and white grape juices adjusted to pH 3.9 (Mazzotta, 2001). Hsing-Yi and Chou (2001) found that the survival of acid-adapted *E. coli* O157:H7 was longer than the non-adapted bacteria in mango juice and asparagus extracts but not in selected fermented milk products under refrigeration (7°C) temperatures. Bacon et al. (2003) examined the stress adaptation of *Salmonella* spp. under gradual exposure to acidic conditions (1% glucose) and showed that it caused cross protection against lethal heat treatments of 55°C, 57°C, 59°C, and 61°C. Koutsoumanis et al. (2003) examined stationary-phase *L. monocytogenes* cells grown in glucose-free and glucose-containing media when exposed to various stressors such acid (pH 4.0, 7.0),

osmotic (10.5-20.5% NaCl) and temperature (-5°C to 50°C) and when further exposed to pH 3.5. The growth of *L. monocytogenes* in the presence of glucose resulted in enhanced survival of the pathogen at pH 3.5. Sub-lethal stresses other than acidic stresses, i.e., osmotic, heat, and low-temperature stresses, did not appear to affect the acid resistance of *L. monocytogenes.* Acid-adapted *L. monocytogenes* (pH 5.5, 2 h) had an increased resistance against heat shock (52°C), osmotic shock (25–30% NaCl) and alcohol stress (15%). Acid adaptation thus appeared to provide a general cross-protection against other stresses. Moreover, heat-adapted *Listeria* (50°C for 45 min) also displayed an increased resistance to acid shock (Phan-Thanh et al., 2000). The cross resistance of acid adapted cells to other stresses has an importance for the food industry, especially because foods commonly encounter sub-lethal acidic treatments during processing (Van Schaik et al., 1999). Yousef and Courtney (2003) include the production of protective proteins (eg. for damage repair, cell maintenance), transformation in the physiology and morphology of cells (spore-formation, viable but non-culturable state), increased resistance or tolerance to lethal factors, evasion of host organism defenses, and adaptive mutation as microbial stress responses. Proteins which are expressed in *L. monocytogenes* specifically when heat shocked have been previously reported (Phan-Thanh and Gormon, 1995; Hu et al., 2007a, b). De Angelis and Gobbetti (2004) identify two classes of adaptive response namely, “limited” and “multiple” response. In case of the “limited” response, microbial exposure to sub-lethal dose of a physical, chemical or biological stress protects the cells against subsequent lethal treatment of the same stress (Sanders et al., 1999). In case of the “multiple” response, microorganisms are able to adapt to stressors that they had not previously encountered (De Angelis and Gobbetti, 2004; Hecker et al.,1996; Juneja and Novak, 2003). This type of cross-protection is generated in response to nutrient starvation, exposure to high or low temperatures, high osmolarity, and low pH (Hengge-Aronis, 1999; Pichereau et al., 2000). The influence of incubation temperature on thermo-tolerance before and after a heat shock at 58°C in *L. monocytogenes*, and the relationship between cell morphology and thermo-tolerance have also been reported (Rowan and Anderson, 1998; Jørgensen et al., 1996).

Reduced growth rate or induced entry into stationary phase is indicative of general stress response (Hill et al., 2002). Stress response is genetically regulated. Activation of the general stress response results in the expression of stress adaptive genes. Abee and Wouters (1999) have mentioned stress adaptive genes such *bolA* which play a role in controlling cell morphology, *cfa* which is involved in cyclopropane fatty acid synthesis, and *uspB* which is important in ethanol resistance as examples of stress adaptive genes. The regulation of general stress response has been studied in several microorganisms; especially in *E. coli* where the stress response mechanism is quite well understood. The regulation of general stress response is under the control of alternative

sigma factors such σ^S in *E. coli* and other Gram-negative bacteria. The regulation of general stress response is mediated by the *rpoS* gene, which encodes the σ^S RNA-polymerase subunit in *E. coli*, and also in *Shigella flexneri*, and *Salmonella enterica* serovar Typhimurium (Abee and Wouters, 1999; Hengge-Aronis, 2000; Komitopoulou et al., 2004). The regulation of *rpoS* includes differential levels of transcription, translation, and post-translational modification depending on the type of stress that is involved (Abee and Wouters, 1999; Hengge-Aronis, 2000; Venturi, 2003). The general stress response in Gram-positive bacteria such as *Bacillus* spp. is regulated by the alternative sigma factor σ^B (Hengge-Aronis, 1999; Price, 2000).

2.3 Heat Stress

Thermal processing is one of the oldest and most common techniques employed to control pathogens in food. Microorganisms are exposed to heat stress in the environment and during food processing. Identification of the appropriate heat treatment is a critical issue that dictates whether a microorganism is inactivated or becomes resistant to the temperature stress. Sub-lethal heat stress can be defined as a stress when cells are exposed to above-optimal (but below lethal) heat stress (Rodriguez-Romo and Yousef, 2005a,b). When microbial cells are exposed to temperatures above optimal growth temperatures even for short periods of time, unique physiological responses are triggered within the cells (Lindquist, 1986; Knabel et al., 1990). Heat resistance of *L. monocytogenes* in foods is highly varying (Casadei et al., 1998; Kenney and Beuchat, 2004; Mackey et al., 1990). Doyle at al. (2001) have comprehensively reviewed heat resistance in *L. monocytogenes* in culture media and foods. The data strongly suggests that heat resistance of *L. monocytogenes* depends on the age of the culture, growth conditions, recovery media, and characteristics of foods (salt content, a_w, acidity, presence of inhibitors). Microorganisms are known to increase their thermo-tolerance when they are exposed to a variety of environmental stressors such as sub-lethal heat shock, osmotic stress, starvation, acid exposure, alkaline treatment, ethanol or hydrogen peroxide (Farber and Brown, 1990; Jørgensen et al., 1995; Lou and Yousef, 1996, 2007; Mazzotta and Gombas, 2001). The thermo-tolerance response of bacteria is also strongly influenced by other factors such as previous growth condition (Jørgensen et al., 1999; Teixeria et al., 1994) strain variation (Mackey et al., 1990; Sörqvist, 1994), and the heating menstruum in terms of its pH and the presence of other compounds (Smith and Marmer, 1991; Jørgensen, 1999; Pagàn et al., 1997). Foods that require long heating periods at lower temperature (to retain flavor, texture etc.) are particularly susceptible to harboring microorganisms that exhibit increased thermo-tolerance. Sergelidis and Abrahim (2009) summarized the different studies which demonstrate that bacteria increase their thermo-tolerance when they are exposed to moderately elevated temperatures (ie., above their optimal growth temperature) before the real heat treatment (Table 2.2).

Table 2.2. Thermotolerance due to heat shock in case of *Listeria monocytogenes* and other bacteria

Strain	**Sub-lethal heat stress or adaptation**	**Heating menstruum**	**Increase in D-value to prior heat stress or adaptation**	**Reference**
Salmonella Typhimurium	42-48°C/5-60 min	TSYEB	1.1-3.0-fold in $D_{57.8}$	Bunning et al. (1990)
Escherichia. coli O157:H7	42°C/5 min	TSB	1.5-fold in D_{55}	Murano and Pierson (1992)
Enterococcus faecalis	45 or 50°C/30 min	BHI broth	increased D_{60} and D_{62}*	Boutibonnes et al. (1993)
Salmonella Thompson	48°C/30 min	minced beef	2.4-fold in D_{54} 2.7-fold in D_{60}	Mackey and Derrick (1987)
Escherichia. coli	46°C/60 min	NB	1.33-fold in D_{56}	Gadzella and Ingham (1994)
Streptococcus thermophilus	48°C, 50°C, 52°C/30 min	M17 L broth	increased D_{58}*	Auffray et al. (1995)
Escherichia. coli O157:H7	45°C/30 min	TSB	1.37-fold in D_{54} 1.68-fold in D_{58} 1.5-fold in D_{62}	Williams and Ingham (1997)
Salmonella Enteritidis	42°C/60 min	CASO-YE	3.2 $(1.18)_{an}$-fold in D_{52} 1.67 $(1.36)_{an}$-fold in D_{54} 1.92 $(1.28)_{an}$-fold in D_{56} 1.44 $(1.46)_{an}$-fold in D_{58}	Xavier and Ingham (1997)
Lactobacillus delbrueckii subsp. *bulgaricus*	52°C/20 min	skim milk	increased D_{64}*	Teixeira et al. (1994)
Yersinia enterocolitica	45°C/60 min	ground pork	2.4-fold in D_{55}*	Shenoy and Murano (1996)
L. monocytogenes	48°C/60 min	TSBYE	increased D_{60}*	Fedio and Jackson (1989)
L. monocytogenes	42-48°C/5-60 min	TSYEB	1.1-1.4 in $D_{57.8}$	Bunning et al. (1990)
L. monocytogenes	48°C/5-60 min	TSBYE	1-3-fold in $D_{62.8}$	Knabel et al. (1990)
L. monocytogenes	48°C/10 min	TSYE	2.1$(2.2)_{an}$-fold in D_{55}	Linton et al. (1992)
L. monocytogenes	46°C/30 min	TPB	2.2-fold in D_{58}	Stephens and Jones (1993)
L. monocytogenes	46°C/30 min	TPB	2.2-fold in D_{58}	Jørgensen et al. (1996)
L. monocytogenes	47.5°C/180 min	TSYE	4-fold in D_{65}	Pagàn et al. (1997)
L. monocytogenes	46°C/30 min	TSB+8g/l lactic acid	5-7-fold in D_{60}	Jørgensen et al. (1999)
L. monocytogenes	46°C/30 min	TPB+ 8 g/l lactic acid	1-8-fold in D_{60}	Jørgensen et al. (1999)

L. monocytogenes	40°C/24 h	BHI	3-4-fold in $D_{62.5}$	Sergelidis et al.. (2001)
L. monocytogenes	48°C/60 min	milk	increased D_{60}*	Fedio and Jackson (1989)
L. monocytogenes	48°C/30 min	pork meat	4-fold in D_{58}	Quintavalla and Barbuti (1989)
L. monocytogenes	48°C/120 min	pork (66%) and beef (33%) curing mixture	2.4-fold in D_{64}	Farber and Brown (1990)
L. monocytogenes	43°C/18 h	milk	6-fold in $D_{62.8}$	Knabel et al. (1990)
L. monocytogenes	48°C/15 min	sterile whole bovine milk	1.5-fold in $D_{71.7}$	Bunning et al. (1992)
L. monocytogenes	42°C/60 min	ham	1.4-fold in D_{60}	Carlier et al. (1996)
L. monocytogenes	42.8°C/24 h	pasteurized whole milk	2.5-3-fold in D_{56}, D_{60} and D_{63}	Rowan and Anderson (1998)
L. monocytogenes	46°C/30 min	minced beef	1-7-fold in D_{60}	Jørgensen et al. (1999)
L. monocytogenes	48°C/10 min	potato slices	1.4-2.4-fold in D_{55}	Walsh et al. (2001)
L. monocytogenes	46°C/60 min	ground beef	2-fold in D_{60}	Novak and Juneja (2003)

CASO-YE: casein-peptone-soymeal peptone broth supplemented with 0.5% yeast extract; NB: nutrient broth; PB: phosphate buffer; TPB: tryptic phosphate broth; TSYE: trypticase soy yeast extract broth; TSBYE: Trypticase soy broth-0.6% yeast extract; *exact data has not been calculated; **$(..)_{an}$: anaerobic incubation

The heat shock response of bacteria is a universal protective response against heat stress. This response results in a temporary induction of heat shock proteins (HSPs) which protect the cells against heat damage or other stressors. The appearance of proteins that are expressed in *L. monocytogenes* when heat shocked has been previously reported (Phan-Thanh and Gormon, 1995; Hu et al., 2007a,b). All organisms examined produce proteins encoded by the hsp70 and hsp90 gene families in response to elevated temperatures. These proteins are highly conserved among prokaryotic and eukaryotic organisms and increase the protection of bacteria against subsequent stressors (Lindquist, 1986; Lindquist and Craig, 1988). Pagàn et al. (1997) have reported a 7-fold increase in thermo-tolerance of *L. monocytogenes* when the cells were exposed to 45°C for 180 minutes. The extent of exposure to temperature above optimal levels, and the matrix in which the cells are exposed are reported to influence the extent of the observed thermo-tolerance (Linton et al., 1990; Sergelidis and Abrahim, 2009). The influence of incubation temperature on thermo-tolerance before and after a heat shock at 58°C in *L. monocytogenes*, and the relationship between cell morphology and thermo-tolerance have also been reported (Rowan and Anderson, 1998; Jørgensen et al., 1996) as mentioned earlier. In 1988, Lindquist and Craig categorized heat shock proteins (Hsp) in general in biological systems into the following groups namely, Hsp110, Hsp90, Hsp70, GroE-Hsp 58, and small Hsp. Hsp110 proteins are greater than 100 kDa observed in

eukaryotes in response to high temperature and seen primarily in mammalian cells. The Hsp90 group of proteins is highly conserved, is abundant at normal temperatures, and is induced further by heat. The Hsp70 proteins are found in high abundance associated with other proteins, and are associated with a variety of cellular processes. The DnaK, DnaJ, DnaG, DnaB, Ssb (single stranded binding protein) proteins are known members of the Hsp 70 family. They bind with high affinity with ATP suggesting their involvement in metabolism. The GroE-Hsp 58 proteins made up of proteins such as *GroEL* a 65 kDa Mr protein and *GroES* a 15 kDa Mr protein are thought to be essential for growth (Tilly et al., 1981; Wada and Itikawa, 1984; Wada et al., 1987). The small Hsp are a very diverse group of proteins with varying molecular weights. Their role in thermo-tolerance is still debated. Burdon (1986), Katchinski (2004) and Schlesinger (1994) have described Hsp protein families such as Hsp40, Hsp60, Hsp70, Hsp100 and small heat shock proteins (sHsp).

Even though heat shock proteins are observed during heat stress, it is still not clear whether there is a direct cause and effect relationship between the synthesis of heat shock proteins and the induction of higher thermo-tolerance (Lindquist, 1986; Parsell and Lindquist, 1993). The heat shock proteins are thought to play an important role in the repair of heat-injured cells and are involved as molecular chaperones in the re-folding of denatured proteins. Examples of such heat shock associated chaperones are DnaK, GroEL, and GroES (Rosen and Ron, 2002). Chaperones are thought to prevent undesirable interactions between complementary surfaces of proteins (Vorob'eva, 2004). Other heat shock proteins such as CplC and CplP have an ATP-dependent protease activity, and their induction is thought to ensure stress tolerance and degradation of heat-damaged proteins (Krüger et al., 2001).

Some of the Hsp are known to be expressed at low levels even under non stress conditions in all prokaryotic and eukaryotic cells. These heat shock proteins are thought to play an essential role in protein maintenance (Ellis and Van der Vies, 1991; Georgopoulos and Welch, 1993). The alternative sigma factor σ^{32}, plays a role in transcription of the majority of heat shock proteins in *E. coli* (Rosen and Ron, 2002). The alternative sigma factor σ^{E}, is involved in the regulation of heat induced genes in the periplasmic space (Alba and Gross, 2004; Raivio and Silhavy, 2000). The heat shock response is regulated by several regulons in bacteria. *Bacillus subtilis* is generally used as a model organism for studying heat shock response in Gram-positive bacteria (Hill et al., 2002). The regulatory systems such as σ^{B}, HrcA-CIRCE, and Clp protease system are known to be associated with the heat shock response in *B. subtilis*. Sigma factors such as σ^{B} are transcriptional activators that recognize specific heat shock promoters upstream of heat shock genes (Kazmierczak et al., 2003). Two types of signals can elicit *sigB* the gene which encodes σ^{B}. These signals can be extracellular that result in a drop of ATP levels or it could be physical signals/stressors such as heat,

salt, or acid stress (Maul et al., 1995; Hecker and Volker, 1998). The non-growing cells of *B. subtilis* have an intricate network for adaptation to various stressors. Stressors such as heat shock, salt stress, ethanol, starvation for oxygen or nutrients etc. induce the same set of proteins, called general stress proteins (Hecker et al., 1996). These general stress proteins are thought to provide either general or specific protection under these different adverse conditions. In addition to these non-specific general stress proteins, all extracellular signals induce a set of specific stress proteins that may confer specific protection against a particular stress factor.

Hecker et al. (1996) identified at least three different classes of heat-inducible genes in *B. subtilis* based on their common regulatory characteristics: (i) Class I genes (eg., dnaK and groE operons which are induced by heat stress, involves a σ^A-dependent promoter, an inverted repeat (called the CIRCE element), and probably a repressor interacting with the CIRCE element, (ii) Class II genes (the majority of general stress genes which total over 40 genes) are induced at σ^B-dependent promoters by different growth-inhibiting conditions. The activation of σ^B by stress or starvation is the crucial event in the induction of this large stress regulon, (iii) Class III genes (which comprise only a few such as Ion, clpC, clpP, and ftsH) can respond to different stress factors independently of σ^B or CIRCE. Stress induction of these genes is thought to occur at promoters recognized by σ^A and involves additional regulatory elements (Hecker et al., 1996). Hecker and Volker (1998) assigned the identified GSPs proteins to five main groups. The HrcA-CIRCE (Control Inverted Repeat of Chaperone Expression) system is a repression system first described in *Bacillus subtilis* by Zuber and Schumann (1994). The system comprises of an inverted repeat cis element and a trans protein-repressor encoded by the *hrcA* gene. In *Bacillus subtilis* the operons (*groE* and *dnaK*) are regulated by this system during heat shock (Yuan and Wong, 1995). The genes encoding for the Clp protease system are under the control of CtsR (Rosen and Ron, 2002; Yousef and Courtney, 2003). Other stressors such as pH, osmolarity, presence of ethanol, antibiotics, aromatic compounds, heavy metals, etc. are also able to indicate the synthesis of heat shock proteins and cross protection as mentioned earlier (Ramos et al., 2001). Gandhi and Chikindas (2007) have published a comprehensive review of the effects of acid and osmotic stress in *L. monocytogenes.*

2.4 Acid Stress

Acidification of foods is another common food preservation method that is used worldwide. It is achieved either via fermentation or the addition of specific food preservatives such as acetic acid, propionic acid and lactic acids. The weak acid in non-dissociated form can diffuse into microbial cells, and inside the cytoplasm its dissociated form decreases the intracellular pH which disrupts the metabolic activities. The acid tolerance response (ATR) is an induced protective response in microorganisms against acid stress (Gahan et al., 1996). The microbial response to acid stress is

thought to include changes in membrane composition, increase in proton efflux, increase in amino acid catabolism, and induction of DNA repair enzymes (Beales, 2004; Yousef and Courtney, 2003). Differences in ATR among different bacteria and between exponential and stationary phase cells have been reported (Hartke et al., 1996; Jordan et al., 1999). Intracellular or extracellular pH fluctuations can be a signal for induction of acid shock or stress adaptation proteins. External or periplasmic proteins may also be sensed by membrane bound proteins (Foster, 1999). Internal pH fluctuations may also affect gene expression or modulate a regulatory element that controls gene expression.

L. monocytogenes responds to, and survives in low pH environments utilizing a number of stress adaptation mechanisms. Exposure to mild acidic stress (pH 5.5) induces the acid tolerance response (ATR) (O'Driscoll et al., 1996). Phan-Thanh and Mahouin (1999) examined *L. monocytogenes* exposing cells to a lethal acidic pH (acid stress) and an intermediary non-lethal acidic pH (acid adaptation) and the expression of proteins were studied. The majority of these induced proteins were common to the two pHs and the lethal acidic pH induced more proteins than the mildly acidic pH. The presence of groEL, ATP synthase, thioredoxin reductase and diverse transcriptional regulators and ferric uptake regulator were noted. *L. monocytogenes* is thought to employ processes such as homeostasis (Shabala et al., 2002). The active transport of H^+ is coupled with electron transport in respiratory chains. The F_0F_1-ATPase is a multisubunit enzyme, is a channel for proton translocation across the cell membrane via ATP utilization. The enzyme is highly conserved, the F_1 portion of it includes five subunit α_3, β_3, γ, δ, ε and F_0 contains three kind of trans-membrane subunit a, b, $c_{10\text{-}14}$. The F_0 rotation caused by proton gradient and followed by rotation of the $\gamma\varepsilon$-subunits of F_1 lead to ATP synthesis. In the reverse reaction, ATP hydrolysis in F_1 induces the rotation of γ and, hence, of the F_o rotor in the reverse direction. This then is thought to drive proton pumping (Yoshida et al., 2001). Cotter et al. (2000) observed 3 log reduction under acid stress in the presence of a proton inhibitor (DCCD-N,N'-dicyclohexylcarbodiimide) and they concluded that F_0F_1-ATPase has a role in ATR in *Listeria*.

L. monocytogenes also utilizes the glutamate decarboxylase (GAD) system to survive acid stress. The GAD system is composed of three genes *gadA*, *gadB* and *gadC*. The *gadA* and *gadB* genes encode two glutamate decarboxylases and the *gadC* genes encode a glutamate-γ-aminobutyrate (GABA) antiporter (Cotter et al., 2001). A specific transporter takes glutamate up from the cell then its decarboxylaton happens in the cytoplasm. In the process γ-aminobutyrate is produced with intracellular proton utilization and it is exported from the cell via an antiporter located in the cell membrane. Due to the proton loss increased cytoplasmatic pH can be observed (Small and Waterman, 1998). The role of GAD system in the acid resistance of *L. monocytogenes*

during gastric transit has been studied by Cotter et al. (2001) and they found that the addition of glutamate increased the survival of the wild strain in gastric fluid. Deletion of *gadA*, *gadB* and *gadC* genes resulted in an increased sensitivity to low pH. These results show the importance of GAD system in the acid resistance.

Wiedmann et al. (1998) studied the role of general stress transcription factor σ^B on the acid resistance of *L. monocytogenes*. The acid resistance of *sigB* mutant was weaker in stationary phase than in the wild type. They concluded that expression of σ^B–dependent proteins has an important role in acid resistance and ATR in *L. monocytogenes*. Kazmierczak et al. (2003) observed that the alternative σ^B factor regulates the expression of the *gadB* gene and also regulates the virulence gene expression in *L. monocytogenes*. The acidic pH of foods helps to prevent the growth of foodborne pathogens. Several studies show that acid adapted *Listeria* survives better in foods (Gahan et al., 1996; O'Driscoll et al., 1996). The ATR is noteworthy during food processing because the exposure of the pathogen to mild acidic conditions could result in better resistance to more severe acidic conditions.

2.5 Osmotic Stress

In the food industry, salting is a food preservation method designed primarily to obtain lower water activity. However, *L. monocytogenes* is able to survive high concentration of salt and is thus not an easy pathogen to control by osmotic stress alone. Osmo-adaptation in bacteria can involve both physiological changes and as well as regulation at the gene expression level (Hill et al., 2002). Duché et al. (2002a) used 2D gel-electrophoresis to understand the differential protein expression patterns that occurs in *L. monocytogenes* under salt stress. Forty different proteins out of a total of 400 to 500 proteins were differentially expressed (either repressed or induced at a higher rate) during salt stress. Twelve proteins showed high induction after salt stress. The general stress proteins (Ctc and DnaK), transporter proteins (GbuA and mannose-specific phosphotransferase system enzyme IIAB) and general metabolism proteins (alanin dehydrogenase, Ccp, CysK, EF-Tu, Gap, GuaB, PdhA and PdhD) were differentially expressed. Gardan et al. (2003) suggested that Ctc protein of *L. monocytogenes* is involved in osmotic stress tolerance in the absence of any osmoprotectant (glycine betaine, carnitin) in the medium. Bayles and Wilkinson (2000) observed the osmoprotectant function of glycine betaine, proline betaine, acetyl carnitine, carnitine, γ-butyrobetaine and 3-dimethylsulphoniopropionate in *L. monocytogenes*.

In *L. monocytogenes*, the general σ^B factor has an important role in the utilization of betaine and carnitine as osmoprotectants (Becker et al., 1998). Kazmiercak et al. (2003) identified the genes regulated by σ^B. Using a combination of bio-informatics and microarray experiments they showed

that the σ^B- dependent *L. monocytogenes* genes included both stress response genes (e.g., *gadB*, *ctc*, and the glutathione reductase gene *lmo1433*) and virulence genes (e.g., *inlA*, *inlB*, and *bsh*). Overall, the data demonstrated that in addition to regulating expression of genes important for survival under environmental stress conditions, σ^B also contributes to regulation of virulence gene expression in *L. monocytogenes*. Gardan et al. (2003) showed that the expression of *ctc* gene is dependent on σ^B factor in *L. monocytogenes.* Kallipolitis and Ingmer (2001) also identified 7 response regulators that are part of the two-component signal transduction system and are involved in the osmotic stress response linked virulence mechanism in *L. monocytogenes*.

2.6 Applications of Genomics and Proteomics in Microbiology

Understanding the physiology and metabolism of microorganisms at the transcriptome and proteome levels are becoming increasingly possible due to significant technological improvements in laboratory instrumentation and reagents as well as the growing amount of scientific databases that can be used in these analyses. Analysis of gene expression using microarrays, real-time PCR assays and analysis of protein expression patterns using 2D gel electrophoresis and MALDI-TOF-TOF have enabled significant improvements in understanding of cellular processes, microbial physiology and function. Fleischmann et al. (1995) published the first complete genomic sequencing of a human pathogen *Haemophilus influenzae*. Presently, the complete genome sequences of about a hundred different organisms are known. *Campylobacter jejuni* was the first foodborne pathogen that was completely sequenced (Parkhill et al., 2000).

For understanding biological processes, genomic information alone is not enough. It is critical to link genetic information or genomics with functionality (ie., functional genomics) to develop a clear picture of microbial function under different conditions. The ultimate goal is to link functional genomics with metabolomics (systems biology) in order to obtain a system-level understanding of microbial function. Transcriptome analysis helps in understanding microbial gene expression. Microarrays are now routinely employed to understand global gene expression patterns (Hu et al., 2007 a,b; Call et al., 2003). Significant improvements have taken place in microarray technologies (Stears et al., 2003; Kuo et al., 2002). These arrays are produced by the robotic deposition of polymerase chain reaction (PCR) products, plasmids or oligonucleotides or cDNA onto a glass slide. They can also be created using *in situ* synthesis of oligonucleotides using photolithography (Stoughton, 2005). Array-based approaches are useful in a targeted view of cellular response, especially in situations where one does not have a prior knowledge of which genes or mechanisms are important. However, it must be kept in mind that mRNA is only one step in the conversion of the DNA-encoded genetic information to cellular response and function. Proteins and metabolites are extremely important and one can argue that they should be the center of interest.

Changes in the temporal expression and accumulation patterns of proteins and metabolites can be very useful to understand the phenotypic responses of the cell. Expression profiles at the protein level provide information about potential function other than those at the transcript level. This is because mRNA levels do not necessarily correlate with protein levels (Gygi et al., 1999). Cellular activities are mediated by complex networks of interactions in response to physiological signals, and the cell type and state determines the nature of the response. These interactions can be elucidated by combining information obtained at the transcript level and the proteomic level (Vaidyanathan and Goodacre, 2005).

The proteome is defined as the entire protein complement of a cell, tissue or organ (Kahn, 1995; James, 1997). Proteome analysis involves the assessment of the global protein expression profiles (Soni et al., 2007). The ultimate objective or goal of proteomics should be to define the identities, quantities, structure, and function of all proteins produced in a cell under all different conditions and states. However, this is rarely achieved. Presently, proteomics is still limited to single parameters studies (Soni et al., 2007). Some of the major analytical tools involved in proteomics include (i) two-dimensional sodium dodecyl polyacrilamyde gel electrophoresis (2D-SDS PAGE)-based separation followed by mass spectrometric (MS) identification of separated proteins, (ii) (multidimensional) liquid chromatography (LC) or capillary electrophoresis (CE)-based separation of proteins/digested peptides, followed by MS-based identification, and (iii) analytical microarray technology. 2D-SDS PAGE is one of most widely used expression proteomics tool. The first dimension is a charge (pI)-based separation using isoelectric focusing (IEF) and size (Mr)-based separation in the second dimension. This analysis is usually carried out in slab gel, and the technique must be capable of separating and resolving the different expressed proteins ideally with minimal sample preparation. Mass spectrometry (in particular, matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), and improvements in mass resolution, their sensitivity and accuracy, have significantly enabled identification and quantification of proteomic expression (Merchant and Weinberger, 2000).

A majority of the strategies and techniques in expression proteomics are still dependent on mass spectrometry. For the analysis and identification of the excised proteins which were separated with 2D-PAGE two main approaches can be followed. Henzel et al. (1993) described the “peptide mass fingerprinting” where the protein spots (bands) are subjected to in-gel digestion by a sequence-specific protease, usually trypsin, after destaining, reduction, alkylation and washing steps. This is followed by analysis of the eluted peptides by MALDI-MS. The set of masses from the MS analysis is then compared with theoretically expected tryptic peptide masses in a database to identify the protein. The outcome of the analysis that require attention because there are issues

related to differences in ionization efficiencies of different peptides (Zhu et al., 1995) and the influence of sample preparation conditions (Padliya and Wood, 2004).

The widespread use of 2D-PAGE technology has resulted in the availability of 2D-PAGE reference maps for expressed proteomes of several microbes, including pathogens, under different experimental and physiological conditions. Ramnath et al. (2003) presented a partially annotated proteome reference map of *Listeria monocytogenes* by partial fractionation of membrane and cytosolic proteins, in which 261 spots were detected, and 33 were identified. Planktonic and biofilm modes of growth were examined by 2D-PAGE in *Campylobacter jejuni* (Dykes et al., 2003), *Pseudomonas aeruginosa* (Arevalo-Ferro et al., 2003), *Brucella melitensis* (Eschenbrenner et al., 2002) and *Actinobacillus actinomycetemcomitans* (Fletcher et al., 2001) and differences in protein expression has been observed. In general biofilm-associated bacterial cells are more resistant to stress conditions than their planktonic counterparts. The transcriptional down-regulation of flagellar genes (using 2D-PAGE analysis) was observed under conditions of low pH in *Salmonella* (Adams et al., 2001). This analysis provided the information that a signal transduction system (implicated in virulence) controls motility of the pathogen at low pH. These studies showed that *Salmonella* cells may be non-motile in very low pH environments in the host such as the stomach, perhaps conserving ATP for survival of the pathogen (Adams et al., 2001). Hommais et al. (2002) examined the response to mild acidic pH in *Vibrio cholerae* (again by 2D-PAGE analysis) and decreased accumulation levels of several proteins known to be involved in the organization and functioning of membranes, including lipopolysaccharide, has been observed. The result was similar in the case of *E. coli*. Mild acidic pH could constitute a signal for the outbreak of the acid tolerance response in cells, which is known to protect cells at extreme pH for several hours. Oxidative stress-related differential expression of proteins in *Helicobacter pylori* has also been reported (Baek et al., 2004).

Several methods have been developed to detect and quantify stress proteins using 2D-PAGE analysis (Browne and Dowds, 2001; Cash, 1998; Phan-Than and Gormon, 1995, 1997; Santos et al., 2004; Villarreal et al., 2000) and using proteome analysis to study microbial stress response as is mentioned above (Duché et al., 2002b; Leverrier et al., 2004; Phan-Thanh and Mahouin, 1999; Rosen and Ron, 2002; Santos et al., 2004; Vanbogelen, 2003; Van Schaik et al., 2004). In addition to gel-based proteomic analysis, Yates et al. (1993) have developed a “shotgun” based approach of proteomics. In this technique termed MudPIT (Multidimensional Protein Identification Technology), whole cell protein extracts are immediately cleaved and the peptide mixture is subjected to separation before mass spectrometry to generate the peptide sequence data. Multidimensional chromatography is an integral part of this procedure to enhance fractionation of the complex peptide mixture of the whole cell protein extract.

2.7 Ionizing Irradiation of Fresh Cut and Pre-Packaged Chilled Produce

Ionizing irradiation is approved for use in over 40 different countries, and has been approved by Codex Alimentarius. It has been proven effective through over 50 years of research. Yet, it unfortunately continues to generate controversy. Pathogens enter the food supply through fecally-contaminated irrigation water supplies, aerosols, poor hygienic handling during food processing and via grazing animals. The use of HACCP (Hazard Analysis of Critical Control Points) has proven to greatly reduce the chance of contamination and the prevalence of pathogens in foods (USDA, 1999). However, improved food processing technologies, such as irradiation used as a critical control point within HACCP programs, can further improve post-harvest food safety. There is an increasing consumer demand for producing minimally processed vegetables and fruits without preservatives. This type of food is perceived as fresh, healthy and convenient. Minimal processing (MP) covers a wide range of technologies that aim to preserve food during transport from farm to fork, with minimal changes to the inherent fresh-like attributes (Nicholl et al., 2004). Sales of MP (minimally processed) ready-to-eat fruits and vegetables have grown rapidly in developed countries in the last decade.

Minimally processed, chilled vegetables and fruits usually carry pseudomonads, enterobacteria, lactic acid bacteria and yeasts and molds as natural microbiota. The high moisture content and damaged plant tissues surfaces provide excellent conditions for the growth of microorganisms in these pre-cut/prepared products. There are several reports of outbreaks of enteric pathogens due to consumption of fresh fruits and vegetables (Table 2.3.) (Beuchat, 1996; Buck et al., 2003). The potential sources of pathogenic bacteria include the raw produce, plant workers, and processing environment (Odumeru et al., 1997). Sprouted seeds are also increasingly consumed as a part of health diets. Sprouts represent a specific issue because the sprouting procedure (conducted under high humidity at higher/elevated temperatures) is extremely favorable to growth of bacterial pathogens. The first reported outbreak of human illness associated with seed sprouts was in 1973 (Portnoy et al., 1976). Vegetable sprouts produced using a home sprouting kit contained large numbers of *Bacillus cereus*. Raw alfalfa and clover sprouts have emerged as recognized sources of foodborne illness in the United States. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1999) reviewed the literature of sprout-associated outbreaks and identified the organisms and production practices of greatest public health concern. *Salmonella* or *Escherichia coli* O157:H7 infections are the most common illnesses associated with sprout consumption. Some publications reported, however, the presence of *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Aeromonas hydrophila* in sprouts (Beuchat, 1996).

Table 2.3. Examples of fresh produce and juice from which bacterial pathogens have been isolated (Buck et al., 2003).

Pathogen	**Product**
Aeromonas	alfalfa sprouts, asparagus, broccoli, cauliflower, celery, lettuce, pepper, spinach
Bacillus cereus	alfalfa sprouts, cress sprouts, cucumbers, mustard sprouts, soybean sprouts
Campylobacter jejuni	green onions, lettuce, mushroom, potato, parsley, pepper, spinach
Clostridium botulinum	cabbage, mushrooms, pepper
E. coli O157:H7	alfalfa sprouts, apple juice, cabbage, celery, cilantro, coriander, cress sprouts, lettuce
Listeria monocytogenes	bean sprouts, cabbage, chicory, cucumber, eggplant, lettuce, mushrooms, potatoes, radish, salad vegetables, tomato
Salmonella	alfalfa sprouts, artichokes, beet leaves, celery, cabbage, cantaloupe, cauliflower, chili, cilantro, eggplant, endive, fennel, green onions, lettuce, mungbean sprouts, mustard cress, orange juice, parsley, pepper, salad greens, spinach, strawberries, tomato, watermelon
Shigella	celery, cantaloupe, lettuce, parsley, scallions
Staphylococcus	alfalfa sprouts, carrot, lettuce, onions sprouts, parsley, radish
Vibrio cholera	cabbage, coconut milk, lettuce

Sprout-associated outbreaks have become a world-wide problem (NACMCF, 1999). Although contamination of the sprouts can occur from seeds, contaminated equipment, contaminated water, or poor hygienic handling, seeds appear to be the most likely source of contamination in sprouts associated outbreaks (NACMCF, 1999). Several chemical methods to decontaminate alfalfa seed have been investigated. These include rinsing with calcium hypochlorite, acidified sodium chlorite, acidified chlorine dioxide, trisodium phosphate, peracetic acid and ethanol (Beuchat, 1997; Taormina and Beuchat, 1999a,b). The combination of a hot water treatment and a rinse in various chlorine-containing compound solutions has also been investigated (Jaquette et al., 1996). Nevertheless, until now, no treatment has been found which is capable of completely eliminating *E. coli* O157:H7 or *Salmonella* spp. from alfalfa seeds that are destined for sprouting. The surface of fresh sprouts is difficult to clean. Additionally, the pathogen can be present not only on outer surfaces but also in inner tissues and in stomata as demonstrated when radish sprouts were raised from *E. coli* O157:H7-contaminated seeds (Itoh et al., 1998).

Ionizing irradiation is more efficient in reduction of bacterial contamination than sanitizers (Nguyen-the and Carlin, 2000). Literature reviews (Brackett, 1992; Nguyen-the and Carlin, 1994; Farkas, 2001) show that ionizing irradiation with doses ranging from 0.5 to 2 kGy had no adverse effect on fresh produce stored a few days under refrigeration as minimally processed fresh fruits

and vegetables. At 2 kGy, number of bacteria was usually reduced by 3 to 4 log cycles and yeasts by 1 or 2 log cycles. Limiting factors in irradiation of horticultural products are, however, sensorial changes, particularly softening of fruit and vegetable tissues, and vitamin losses.

Modified Atmosphere Packaging (MAP) is commonly applied to various fresh products to extend the shelf-life and maintain high quality of minimally processed fruits and vegetables. These conditions reduce deterioration by limiting product respiration and maturation as well as by slowing down the proliferation of aerobic spoilage organisms. MAP may be passive, in which packages are sealed in air, or active, in which a defined mixture of gases are used to flush the package, typically with reduced O_2 (2-3%) and increased CO_2 (5-20%), with the balance composed of N_2. For vegetables packaged under either system, there is no single ideal or standard gas mixture; the mixture of gases within the package changes over time in response to the respiration of the produce and the gas permeability of the packaging material, and the specific vegetable under consideration (Al-Ati and Hotchkiss, 2002).

CHAPTER 3
IMPROVING THE MICROBIOLOGICAL SAFETY OF FRESH PRE-CUT AND PRE-PACKAGED CHILLED PRODUCE BY LOW-DOSE GAMMA (γ) IRRADIATION AND MAP

3.1 Introduction

There is an increasing global consumer demand for produce that is minimally processed. Sprouted seeds are being increasingly consumed as a part of health diets. Produce that is minimally processed is perceived to be fresh and healthy. Fresh-cut fruits and vegetables by virtue of its cultivation, handling, and consumption practices are, however, prone to pathogen contamination and therefore become a vehicle for widespread food-borne outbreaks (Odumeru et al., 1997; Beuchat, 1996; Buck et al., 2003). Even in highly developed countries such as the United States, there continues to be a number of large outbreaks associated with fresh produce. As many as 200 people were affected by the *E. coli* O157:H7 outbreak due to contamination of spinach was traced back to animal feces in the field (California Department of Public Health, 2007). These contamination events, outbreaks, and recalls economically devastate the fresh produce industry and retail food franchises. The reality today is that fresh produce is a food item that is unfortunately viewed as highly vulnerable to pathogen contamination. There is a high risk of contamination because produce are grown under natural conditions, packed, and consumed without extensive post-harvest treatments or cooking.

Minimally processed fruits and vegetables harbor a variety of microorganisms including pseudomonads, enterobacteria, lactic acid bacteria and yeasts and moulds as natural microbiota. The high moisture content, bruised plant tissues surfaces provide excellent environments for microbial growth in these products. Sprouts represent a high risk commodity because the sprouting procedure (conducted under high humidity at higher/elevated temperatures) is favorable to pathogen growth. *Salmonella* or *Escherichia coli* O157:H7 infections are the most common illnesses associated with sprout consumption. Some publications reported the presence of *L. monocytogenes, S. aureus, B. cereus, K. pneumoniae* and *A. hydrophila* in sprouts (Beuchat, 1996). Although contamination of the sprouts could occur from seeds, contaminated equipment, contaminated water, and/or poor hygienic handling of seeds appear to be the most likely sources of contamination (NACMCF, 1999).

Modified Atmosphere Packaging (MAP) is used to extend the shelf-life and quality of minimally processed fruits and vegetables. These conditions reduce deterioration by limiting product respiration and maturation as well as by slowing down the proliferation of aerobic spoilage organisms. MAP may be passive, in which packages are sealed in air, or active, in which a defined mixture of gases are used to flush the package, typically with reduced O_2 (2-3%) and increased CO_2 (5-20%), with the balance composed of N_2. For vegetables packaged under either system, there is no single ideal or standard gas mixture; the mixture of gases within the package changes over time in response to the respiration of the produce and the gas permeability of the packaging material, and the specific vegetable under consideration (Al-Ati and Hotchkiss, 2002). Given these challenges, there is a need for a "pathogen-kill" step in the production, processing, and packaging of fresh-cut produce. Ionizing irradiation is one such "pathogen-kill" technology.

Ionizing radiation is one of the most widely studied food processing technologies. However, there is unfortunately significant amount of misperceptions and consumers generally lack a thorough understanding of the technology. The use of irradiation can avoid the use of chemical sanitizers (Nguyen-the and Carlin, 2000). A number of studies have shown that irradiation doses ranging between 0.5 and 2 kGy had no adverse sensory or organoleptic effect on fresh produce (Brackett, 1992; Nguyen-the and Carlin, 1994; Farkas, 2001). Ionizing radiation at doses as low as 2 kGy reduced bacterial populations by as much as 3 logs and yeasts by about 2 logs. In the United States, the U.S. Food and Drug Administration has approved the use of 4 kGy of ionizing radiation for fresh-cut spinach and lettuce. In order to validate pathogen-kill steps such as ionizing radiation, the fresh produce industry need robust, easy-to-perform microbiological analysis.

Impedance microbiology is a rapid method that enables qualitative and quantitative tracing of microorganisms by measuring the change in the electrical conductivity. With direct impedance technology, the change in the conductivity of a liquid culture medium serves as a measuring parameter, whereas with indirect impedimetry, the change in the electrical conductivity of a reaction solution, which occurs through the absorption of gases from the inoculated bacterial culture, is measured. Most investigations concerning the applicability of impedimetry in food microbiology deal with the impedimetric detection or enumeration of *Enterobacteriaceae,* especially the detection of *Salmonella.* Furthermore, a great number of published findings concern the impedimetric determination of the total bacterial count.

The aim of these studies was to study the effect of low dose gamma (γ) radiation on *L. monocytogenes* when inoculated onto alfalfa and radish sprouts and identify those doses that do not diminish the sensory quality parameters. The value of combining γ irradiation with MAP for alfalfa and radish sprouts was also evaluated. Since traditional methods to detect pathogenic

organisms are very labor- and time-consuming, the application of impedimetric methods for the detection of *Listeria monocytogenes* was a secondary objective. This part of my dissertation research was performed as part of an International Atomic Energy Agency (IAEA) funded project Coordinated Research Project titled, "Use of irradiation to ensure the hygienic quality of fresh, pre-cut fruits and vegetables and other minimally processed food of plant origin" (Contract No 11619/RBF).

3.2 Materials and Methods

3.2.1 Bacterial strain

L. monocytogenes 4ab No. 10, an avirulent strain (originally isolated from meat) obtained from Dr. B. Ralovich, Hungarian Meat Research Institute was used in these studies.

3.2.2 Seed sprouts and Inoculation of Samples

Fresh alfalfa and radish sprouts were obtained from a local "Bio" shop in Budapest. The shelf-life of these products as stated by the supplier was 10 days at 5°C. The avirulent *L. monocytogenes* strain was shake-incubated in BHI broth (Oxoid, DSMZ, Braunschweig, Germany) for 24 hours at 30°C. The culture was diluted with sterile water to yield approximately 10^7 CFU/mL. Alfalfa sprouts (150 g) were dipped in 500 mL volume of the *L. monocytogenes* cell suspension for 1 minute with constant gentle agitation. The solution was then decanted, and the sprouts placed on sterile filter paper. After drying, the inoculated samples were packaged and γ irradiated.

3.2.3 Modified Atmosphere Packaging

Alfalfa and radish sprouts (5 g) were placed in CombiTherm 80 bags, and flushed with 2 different gas mixtures namely Gas Mixture # 1 and Gas mixture # 2. The composition of Gas Mixture # 1 was oxygen-carbon dioxide-nitrogen (2%-4%-94%) while that of Gas Mixture # 2 was 3-5 % oxygen, 10-15% carbon dioxide balanced with nitrogen. The bags were sealed using the MULTIVAC packaging equipment. The head-space gas composition was analysed periodically in triplicate sample bags by CombiCheck 9800-1 apparatus (PBI Dansensor, Denmark).

3.2.4 Low dose Irradiation

The experimental samples were irradiated at room temperature to defined target doses (1 kGy and 2 kGy) using a NORATOM Co^{60} gamma irradiator of the Institute for Radiobiology, Budapest. (The 1 kGy dose was jointly decided upon for the storage study among the IAEA study

participants). The dose rate during γ irradiation was 6.47 kGy/h. The 0 kGy samples were considered as control.

3.2.5 Microbiological Analysis

At periodic intervals for up to 10 days, the alfalfa and radish sprout samples were analyzed for specific target microbial groups. Triplicate samples were used during each sampling time frame. The alfalfa and radish sprout samples were diluted in peptone saline and homogenized using a stomacher. The homogenized samples were analyzed for total aerobic plate counts using Plate Count Agar (Merck, Darmstadt, Germany) by spread-plating of 0.1 mL aliquots. The plates were incubated at 30°C for 48 h. *Enterobacteriaceae* were enumerated using double layers of Violet-Red-Bile-Dextrose agar (Merck, Darmstadt, Germany) and incubation at 37°C for 24 hours. Lactic acid bacteria were enumerated using media overlays of de Man-Rogosa-Sharp medium, (Merck, Darmstadt, Germany) and aerobic incubaton at 30°C for 3 days. Yeast and molds were enumerated using Rose Bengal Chloramphenicol agar (Merck, Darmstadt, Germany) and incubation at 25°C for 3 to 5 days.

3.2.6 Survivors and growth of *L. monocytogenes* in MAP packaged alfalfa sprouts after γ irradiation

Survival curves were estimated from radiation doses of 0, 0.5, 1.0, 1.5, and 2.0 kGy for *L. monocytogenes* strain 4ab. The irradiated samples were also stored at 5°C for 10 days and microbiologically analysed periodically. To determine the number of ionizing radiation survivors, the Thin Agar Layer (TAL) method (Kang and Fung, 1999) in addition to plating on Palcam Agar (Merck, Darmstadt, Germany) containing Palcam Listeria selective supplement (Merck, Darmstadt, Germany) was also performed to estimate the number of sublethally injured cells. The plates were incubated at 37°C for 24 h. For the TAL method, double thin layer of Plate Count Agar was poured on the surface of Listeria Selective Agar, and 0.1 mL of the bacterial suspensions were spreaded. The plates were incubated for 24 h at 30°C. The D_{10}-value was determined by calculating the reciprocal of the slope provided by the log CFU/g versus irradiation dose.

3.2.7 Impedimetric estimation of *L. monocytogenes* survival and growth

The RABIT impedimetric instrument (Don Whitley Scientific, U.K.) was used in conjunction with a method developed in our laboratory to estimate *L. monocytogenes* growth and survival (Kiskó et al., 2004). Aliquots (0.5 mL) of the diluted suspensions were placed in 4.5 mL selective impedance broth (Whitley Impedance Broth + glucose (2 g/L) + lithium-cloride (15 g/L) + aesculin

(1 g/L) + Fe(III)-ammonium-citrate (1 g/L) + FRASER Listeria Selective Supplement broth (Merck, Darmstadt, Germany) in triplicate and incubated at 30°C in the RABIT equipment for 24 h. The "indirect measurement" was carried out as previously described (Bolton and Gibson, 1994) and the TTD-values (Time to Detection) were recorded.

3.3 Results and discussion

3.3.1 Changes in the Head-Space gas composition of γ irradiated MAP samples during refrigerated storage

Changes in head-space gas composition in the Gas Mixture # 1 and Gas Mixture # 2 MAP radish and alfalfa sprout samples during storage at 5°C after γ irradiation are shown in Figures 3.1-3.3.

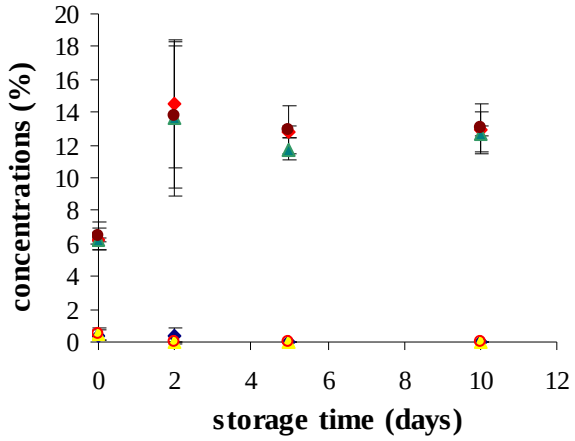


Figure 3.1. Changes in the oxygen and carbon dioxide concentrations in the MAP radish sprouts during storage at 5°C after γ irradiation. The MAP contained oxygen-carbon dioxide-nitrogen (2%-4%-94%) (Gas Mixture # 1).

◆ radish sprouts, O_2-0 kGy; ◆ radish sprouts, CO_2-0 kGy; ▲ radish sprouts, O_2-1 kGy;
▲ radish sprouts, CO_2-1 kGy; ○ radish sprouts, O_2-2 kGy; ● radish sprouts, CO_2-2 kGy

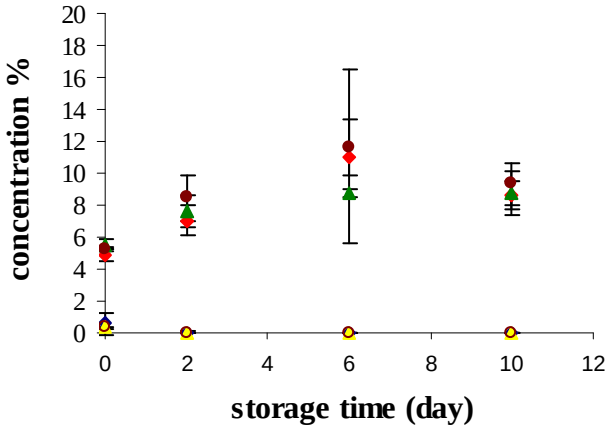


Figure 3.2. Changes in the oxygen and carbon dioxide concentrations in the MAP alfalfa sprouts during storage at 5°C after γ irradiation**.** The MAP contained oxygen-carbon dioxide-nitrogen (2%-4%-94%) (Gas Mixture # 1).

◆ alfalfa sprouts, O_2-0 kGy; ◆ alfalfa sprouts, CO_2-0 kGy; ▲ alfalfa sprouts, O_2-1 kGy;
▲ alfalfa sprouts, CO_2-1 kGy; ○ alfalfa sprouts, O_2-2 kGy; ● alfalfa sprouts, CO_2-2 kGy

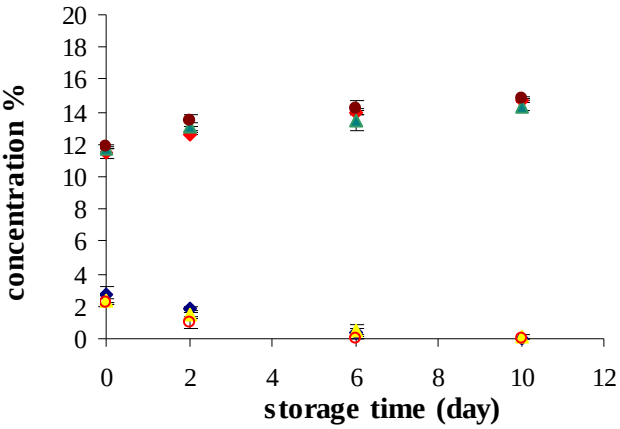


Figure 3.3. Changes in the oxygen and carbon dioxide concentrations in the MAP alfalfa sprouts during storage at 5°C after γ irradiation. The MAP contained 3-5 % oxygen, 10-15% carbon dioxide balanced with nitrogen (Gas Mixture # 2).

◆ alfalfa sprouts, O_2-0 kGy; ◆ alfalfa sprouts, CO_2-0 kGy; ▲ alfalfa sprouts, O_2-1 kGy;
▲ alfalfa sprouts, CO_2-1 kGy; ○ alfalfa sprouts, O_2-2 kGy; ● alfalfa sprouts, CO_2-2 kGy

The radish samples respired more than that of alfalfa sprouts in Gas Mixture # 1. The head-space CO_2 content reached an equilibrium (15 and 10%, respectively) after about 7 days of refrigerated storage. In Gas Mixture # 2, the CO_2 content reached an equilibrium (approx. 15%) on the 6th day when the O_2 concentration were not detectable.

3.3.2 Effect of MAP and γ irradiation on the microbiological shelf-life of alfalfa and radish sprouts

The effect of MAP using Gas Mixture # 1 and γ irradiation on the natural microbiota of radish sprouts is shown in Figure 3.4.

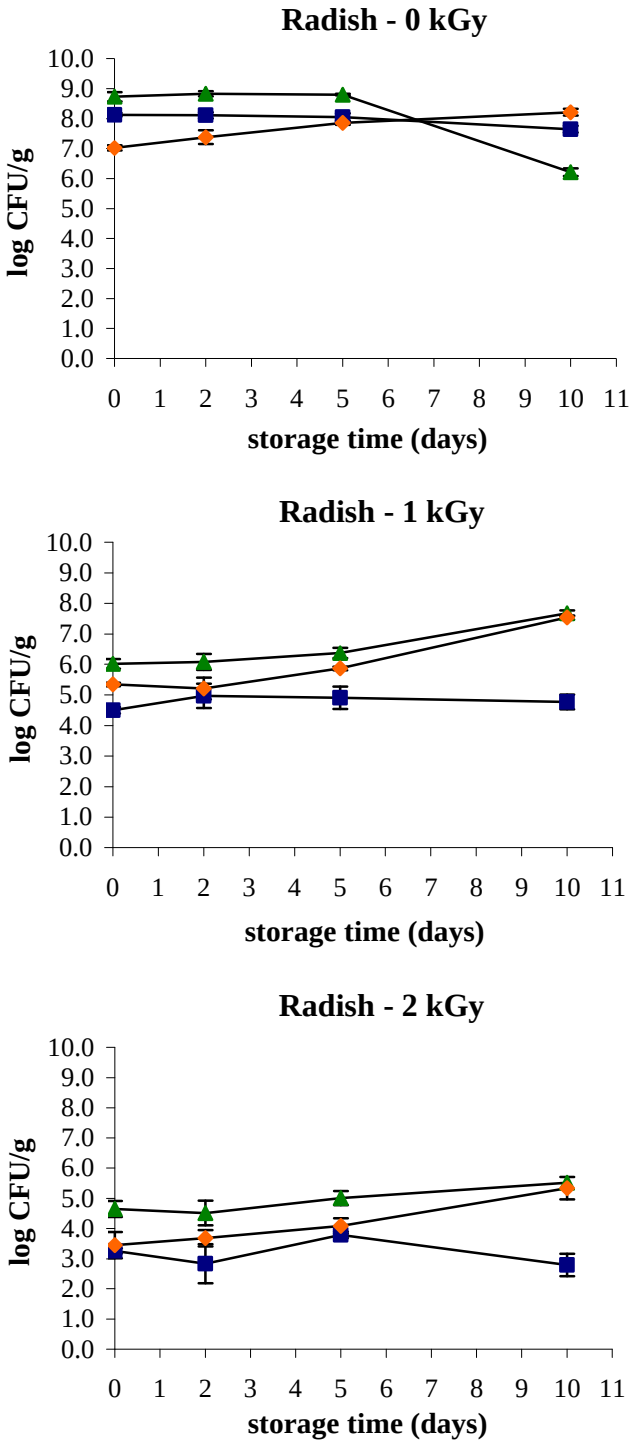


Figure 3.4. The effect of packaging with Gas Mixture # 1 (2% O_2, 4% CO_2, 94% N_2) and γ irradiation on the natural microbiota of radish sprouts

▲ TPC (Total Plate Count); ■ *Enterobacteriaceae*; ◆ LAB (Lactic Acid Bacteria)

The effect of MAP using Gas Mixture # 1 and γ irradiation on the natural microbiota of alfalfa sprouts is shown below in Figure 3.5. The numbers of the LAB were lower in the alfalfa sprouts to begin with as compared to the radish sprout samples.

When these samples were stored for 10 days (without any exposure to γ irradiation), the numbers held steady except for the LAB which increased by about 1 log unit. The exposure to 1 kGy did reduce the populations of the target microorganisms by about 2 -3 log units. During storage of these irradiated samples, the numbers remained relatively stable for up to 10 days except for LAB which increased by about 1 log unit after 5 days at 5°C. Exposure to 2 kGy further reduced the bioburden by about 4 log units for the TPC, 4 log units of the *Enterobacteriaceae* and about 2.5 log units of the LAB. These numbers remained stable during the 10 days of refrigerated storage.

The studies with MAP with Gas Mixture # 2 and γ irradiation were performed with only alfalfa sprouts (Figure 3.6).

In the non-irradiated samples, the TPC, LAB, yeasts and molds, and *Enterobacteriaceae* remained stable over 10 days of storage. When these samples were irradiated with 1 kGy, there was about 3-log decline in the TPC and LAB. The *Enterobacteriaceae* and the yeast and molds only declined by about 1-log unit. These numbers, however, remained steady over the course of the 10-day refrigerated storage conditions. When the alfalfa sprout samples were exposed to 2 kGy of γ irradiation, there was a further 1-log decline in the target microbial populations. The numbers of all the target organisms except LAB remained stable over the 10-day storage conditions. The LAB appears to have increased by about 1.5 log units over the 10-day storage.

It is clear that that gamma (γ) irradiation of MAP sprouts with 1 and 2 kGy reduced the numbers of both the total aerobic bacteria and *Enterobacteriaceae* by 3 and 4 log-units respectively. Importantly, however, the results suggest that during MAP storage (under both Gas Mixture # 1 and Gas Mixture # 2) and especially after exposure to 2 kGy, LAB has the potential to increase in numbers by about 1 to 1.5 log units over 10 days at refrigerated storage. The increase in LAB appears to be related to the levels of CO_2 in the head space of the MAP samples (Figures 3.1, 3.2 and 3.3). Zagory (1999) reported in studies using salted Chinese cabbage that an elevated CO_2 condition extends the lag phase of bacterial growth and slowed bacterial multiplication. Lactic acid bacteria increased substantially during 3-weeks of storage, coinciding with the higher levels of the CO_2 packaging conditions. After 3 weeks of storage, though coliform bacteria in the Chinese cabbage treated with both irradiation and MAP were not detected, the samples stored under aerobic conditions showed between 2 to 4 log CFU/g of coliform bacteria. The irradiation effects (which

reduced aerobic bacterial counts) were maintained, irrespective of its packaging condition, for up to 3 weeks at 4°C. Aerobic bacteria and coliforms were not detected in samples irradiated at 2 kGy.

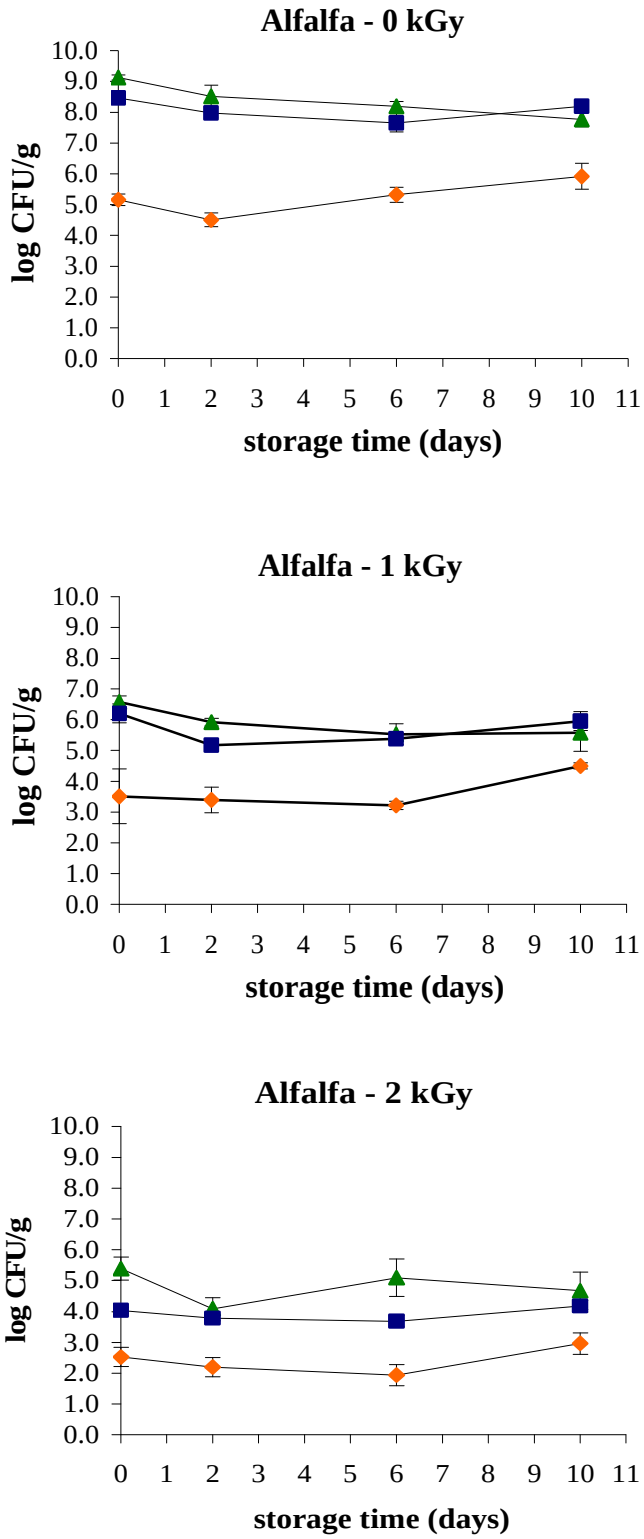


Figure 3.5. The effect of packaging with Gas Mixture # 1 (2% O_2, 4% CO_2, 94% N_2) and γ irradiation on the natural microbiota of alfalfa sprouts

▲ TPC (Total Plate Count); ■ *Enterobacteriaceae*; ◆ LAB (Lactic Acid Bacteria)

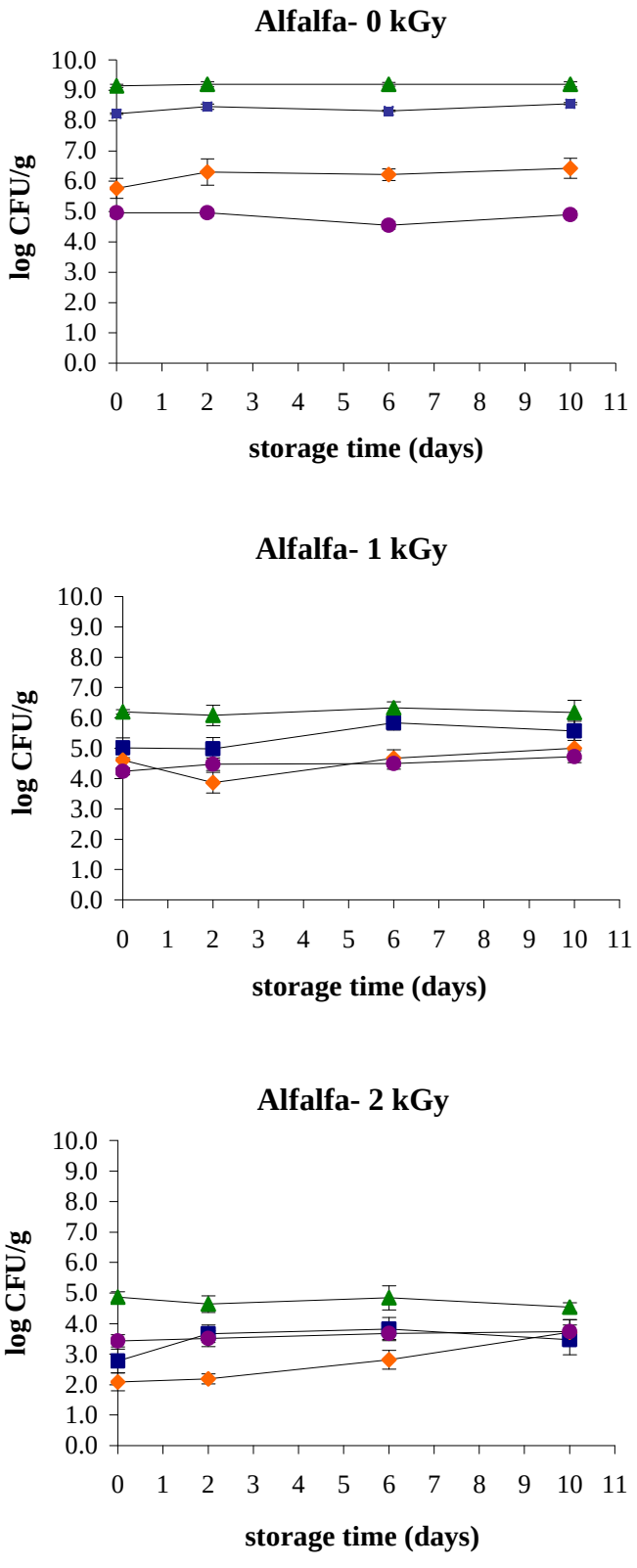


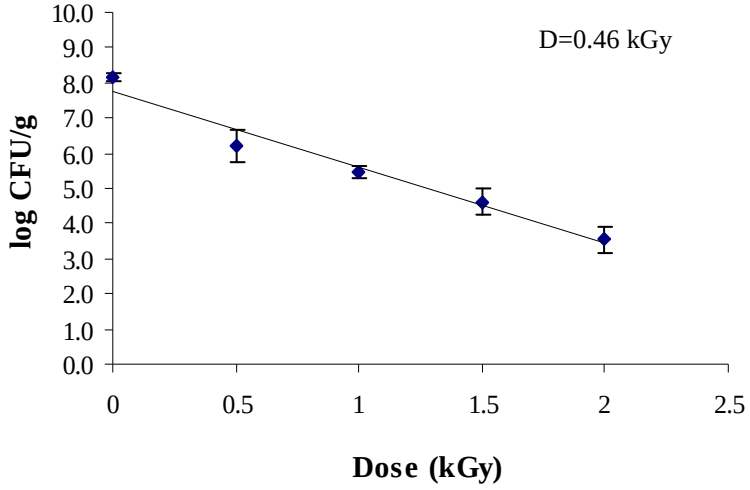
Figure 3.6. The effect of packaging with Gas Mixture # 2 (3-5% O_2, 10-15% CO_2, balanced with N_2) and γ irradiation on the natural microbiota of alfalfa sprouts

▲ TPC (total plate count); ■ *Enterobacteriaceae*; ◆ LAB (Lactic Acid Bacteria); ● yeasts and moulds

3.3.3 Survival and growth of pathogenic bacteria on MAP alfalfa after γ irradiation

The survival of *L. monocytogenes* after irradiation with 0.5 to 2 kGy is shown in Figures 3.7, 3.8 and 3.9. The estimated D_{10}-value for the avirulent *L. monocytogenes* strain 4ab is 0.58 kGy (R^2= 0.98) under Gas Mixture # 1, and the D_{10}-value is 0,45 kGy (R^2=0.95) under Gas Mixture # 2 conditions. The test strain on these sprout samples appears to be more resistant to these irradiation conditions in the MAP samples (Gas Mixture # 1) compared to air packaged samples (D_{10}=0.46 kGy, R^2=0.97).

Figure 3.7. Survival of *L. monocytogenes* 4ab on alfalfa sprouts after γ irradiation in air packaging (adapted from IAEA, 2006)



(y=-2.1613 x + 7.768, R^2=0.97)

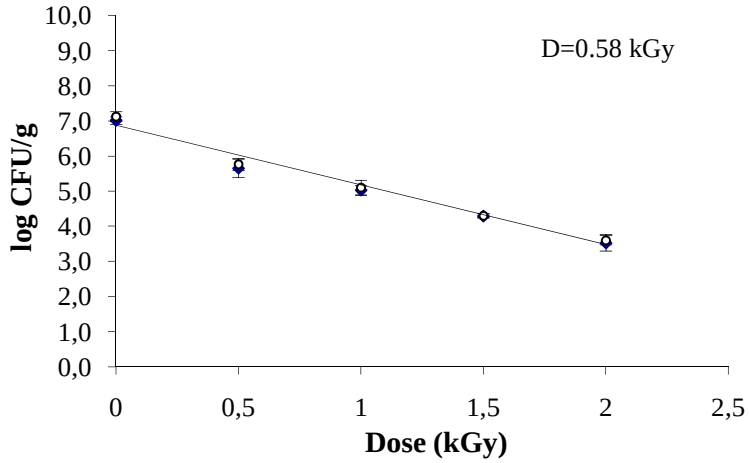


Figure 3.8. Survival of *L. monocytogenes* 4ab on alfalfa sprouts after γ irradiation in packaging containing Gas Mixture # 1 **(**2% O_2, 4% CO_2, 94% N_2)

(y=-1.7001 x + 6.8787, R^2=0.98); ◆ plating onto Palcam agar; ○ TAL method

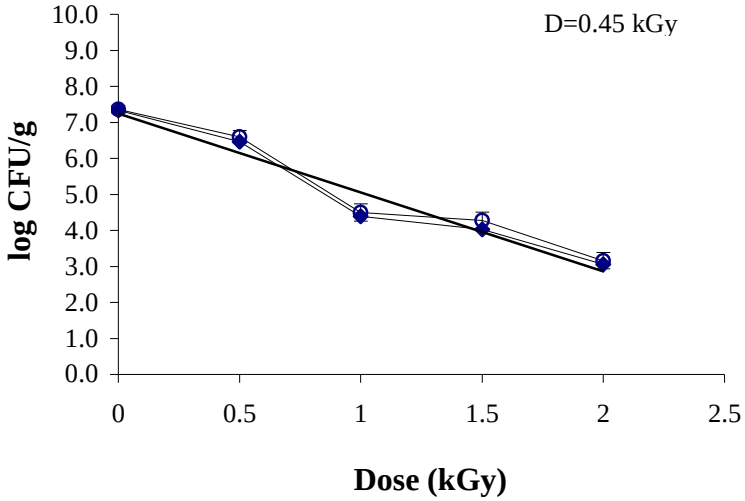


Figure 3.9. γ irradiation survival of *L. monocytogenes* 4ab on alfalfa sprouts in packaging containing Gas Mixture # 2 (3-5% O_2, 10-15% CO_2, balanced with N_2)

(y=-2.194 x + 7.248, R^2=0.95); ◆ plating onto Palcam agar; ○TAL method

Table 3.1 is a compilation of the D_{10}-values of *Listeria* spp. on fresh produce when exposed to ionizing radiation. These results were obtained from the IAEA sponsored Coordinated Research Project, "Use of irradiation to ensure the hygienic quality of fresh, pre-cut fruits and vegetables and other minimally processed food of plant origin." (IAEA, 2006). From this compilation, it is evident that the virulent *L. monocytogenes* strain (ATCC15313) examined by the investigators in Argentina showed a D_{10}-value of 0.37 kGy on alfalfa sprouts. The investigators from India (who examined the same avirulent strain as used in this study ie., 4ab No. 10) on different produce items (such as carrot, cucumber, and seeds and sprouts of green gram, dew gram, chick pea and garden pea) observed the D_{10}-value to range between 0.31-0.58 kGy. The D_{10}-value of virulent strains used by the investigators from other countries ranged between 0.1-0.37 kGy. The resistance of *Listeria innocua* was examined by the Portugese and U.K. investigators. Their results showed D_{10}-values to range between 0.16-0.45 kGy.

The investigators from Canada examined the effect of irradiation both under aerobic condition and MAP condition (60% O_2, 30% CO_2, 10% N_2). The radiosensitization of *L. monocytogenes* was significantly higher ($p \leq 0.05$) under MAP conditions, regardless of the presence or absence of antimicrobial compounds (Table 3.1). Interestingly, in their study, the elevated O_2 (60%) concentration decreased the D_{10}-value of *Listeria monocytogenes* HPB 2812 from 0.36 kGy to 0.17 kGy (aerobic condition). In my study, however, the D_{10}-value for the avirulent *L. monocytogenes* strain was 0.58 kGy (Gas Mixture # 1), and 0.45 kGy (Gas Mixture # 2) as compared to 0.46 kGy under oxygen-rich conditions.

Table 3.1. Summarized D_{10}-values of inoculated *Listeria* spp. on fresh produce items studied by different investigators as part of the IAEA study (adapted from IAEA, 2006)

Participant country	Produce studied	Packaging conditions	D_{10}-value	Inoculated pathogens/Surrogates
Argentina	Chicory	Aerobic	0.24 kGy	*L.monocytogenes* ATCC15313
	Soy sprouts		0.4 kGy	
	Alfalfa sprouts		0.37 kGy	
	Mix salad (cherry tomatoes, carrots, lettuce and cabbage)		0.23 kGy	
	Organic chicory		0.26 kGy	
	Organic rugola		0.28 kGy	
Canada	Mini carrot	Aerobic	0.36 kGy	*L. monocytogenes* HPB 2812 serovar 1/2a
		MAP (60%O_2, 30% CO_2, 10% N_2)	0.17 kGy	
	Mini carrot coated with *trans*-cinnamaldehyde	Aerobic	0.10 kGy	
		MAP (60% O_2, 30% CO_2, 10% N_2)	0.09 kGy	
	Mini carrot coated with Spanish oregano essential oil	Aerobic	0.13 kGy	
		MAP (60% O_2, 30% CO_2, 10% N_2)	0.12 kGy	
	Mini carrot coated with winter savory essential oil	Aerobic	0.14 kGy	
		MAP (60% O_2, 30% CO_2, 10% N_2)	0.10 kGy	
	Mini carrot coated with Chinese cinnamon essential oil	Aerobic	0.12 kGy	
		MAP (60% O_2, 30% CO_2, 10% N_2)	0.09 kGy	
India	Carrot	Aerobic	0.31 kGy	*L. monocytogenes* 4ab
	Cucumber		0.35 kGy	
	Green gram sprouts		0.58 kGy	
	Green gram seeds		0.30 kGy	
	Dew gram sprouts		0.53 kGy	
	Dew gram seeds		0.32 kGy	
	Chick pea sprouts		0.54 kGy	
	Chick pea seeds		0.34 kGy	
	Garden pea sprouts		0.54 kGy	
	Garden pea seeds		0.32 kGy	
Portugal	Coriander	Aerobic	0.27 kGy	*L. innocua* ATCC 33090
	Lettuce		0.19 kG	
	Mint		0.29 kGy	
	Parsley		0.23 kGy	
	Turnip		0.25 kGy	
	Watercress		0.16 kGy	
	Melon		0.26 kGy	
U.K.	Alfalfa seeds	Aerobic	0.45 kGy	*L. innocua* MP 2418
USA	Endive (cut leaf)	Aerobic	0.21 kGy	*L. monocytogenes* ATCC 49594
			0.22 kGy	*L. innocua* ATCC 51742

The results from these studies make it very clear that *Listeria* spp. exhibit wide variation in resistance to ionizing radiation when present on fresh produce. These differences could be due to the inherent genetic differences in some of the strains, the produce that the organisms were tested with as well as the irradiation conditions such as the dose rate and other treatment conditions.

In my study, the *L. monocytogenes* strain was able to grow after MAP irradiation of 1 kGy and during refrigerated storage (Figures 3.10 and 3.11). The numbers increased by 2 log units. There was no difference in numbers obtained using the selective plating and the TAL method.

The potential for combining MAP with low-dose irradiation has been explored in a variety of foods including lettuce (Hagenmaier and Baker, 1997; Prakash et al., 2000). The extension of the lag phase and reduction of the growth rate are often considered to be a major effect of CO_2 (Farber, 1991). Bennik et al. (1995) have, however, shown that this effect is evident only at very high CO_2 concentrations. Thus, CO_2-enriched MAP conditions may not be a reliable approach to control the fate of *L. monocytogenes* in vegetable products. Carlin et al. (1996) have reported that psychrotrophic pathogens such as *L. monocytogenes* and psychrotrophic strains of *B. cereus* are not suppressed under MAP conditions considered optimal for respiring produce. The extent to which headspace gas composition influences the re-growth of irradiated *L. monocytogenes* on vegetables is poorly understood, particularly with regard to the bacteriostatic effects of elevated CO_2 levels on spoilage and pathogenic bacteria (Yuan, 2003). Niemira and co-workers (2005) inoculated cut pieces of endive with *L. monocytogenes*, packaged in gas-impermeable bags in air, 5/5/90% or 10/10/80% CO_2, O_2 and N_2 (‘‘Air-0’’, ‘‘5/5’’ and ‘‘10/10’’, respectively) and irradiated to 0.0 (control), 0.3 or 0.6 kGy. Irradiation significantly reduced initial levels of *L. monocytogenes* and total microbiota under each of the three atmospheric conditions examined (Air-0, 5/5 and 10/10 O_2/CO_2). During storage, *L. monocytogenes* and total microbiota multiplied on the irradiated Air-0 samples. In contrast, the *L. monocytogenes* and the total microbial bioburden on the irradiated 5/5 and 10/10 samples remained at or near the initial reduced levels. In each of the three atmospheres, O_2 declined and CO_2 increased, irrespective of radiation dose.

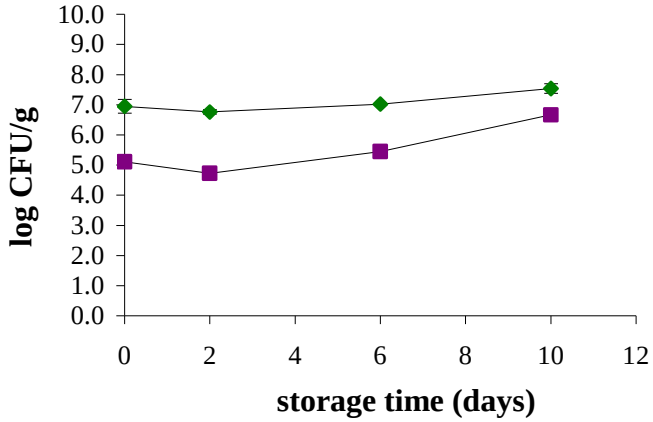


Figure 3.10. Growth of *L. monocytogenes* 4ab on γ irradiated alfalfa sprouts in packages with Gas Mixture # 1 (2% O_2, 4% CO_2, 94% N_2) stored at 5°C

◆ 0 kGy, ■ 1 kGy

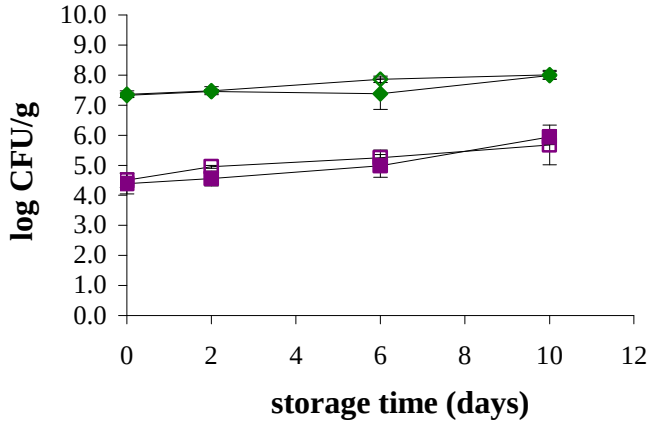


Figure 3.11. Growth of *L. monocytogenes* 4ab on γ irradiated alfalfa sprouts in packages with Gas Mixture # 2 (3-5% O_2, 10-15% CO_2, balanced with N_2) stored at 5°C

◆ 0 kGy; ◇ 0 kGy-TAL method; ■ 1 kGy; □ 1 kGy-TAL method

3.3.4 Survival and growth of *Listeria monocytogenes* on alfalfa sprouts under MAP conditions after irradiation as determined by RABIT impedimetry

A strong correlation (R^2=0.94) was found between the impedimetric TTD and the log CFU/g. This correlation was very evident between log CFU/g of *L. monocytogenes* in the range of log 3 to log 7 CFU/g (Figure 3.12). In samples having lower numbers of *Listeria*, an additional 24 hours enrichment step was required before impedimetric investigation. Samples showing positive electrical response within 20 hours require additional steps to verify the presence of *L. monocytogenes*.

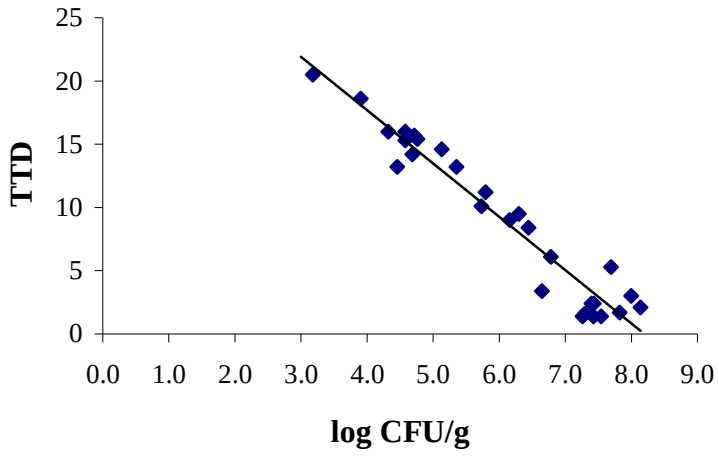


Figure 3.12. Correlation between log CFU/g and TTD in detection of *L. monocytogenes*

$y = -4.2197 x + 34.583$, $R^2 = 0.9381$

3.3.5 Conclusions

The combination of low-dose gamma irradiation with MAP and refrigerated storage improves the microbiological safety and shelf-life of alfalfa and radish sprouts. Combination of MAP with 2 kGy gamma (γ) irradiation was able to reduce the natural bioburden to relatively low levels. Though the total plate count (TPC) and the *Enterobacteriaceae* did not exhibit any further increase in numbers, the LAB did exhibit the potential to increase by about 1.5 log units during 10-day storage at refrigerated (5°C) conditions. This increase appears to coincide with head-space CO_2 concentrations which also reaches equilibrium around 5-7 days.

The D_{10}-values of *L. monocytogenes* 4ab strain on alfalfa sprouts was 0.46 kGy when packaged in air, 0.58 kGy when packaged in gas mixture containing 2% O_2, 4% CO_2 and 94% N_2, and 0.45 kGy when packaged in gas mixture containing 3-5% O_2, 10-15% CO_2 balanced with N_2. This result implies that it is critically important to understand the effect of a particular MAP and ionizing irradiation on the D_{10}-value of the target pathogen. This study also demonstrated that the impedimetric method can be used to detect and enumerate *L. monocytogenes* within 24 hours if present in numbers higher than 3 log CFU/g. According to the European Commission Regulation No 2073/2005 for RTE foods, *L. monocytogenes* cannot exceed 100 CFU/g throughout the food products' shelf-life. This threshold is currently lower than the 1000 CFU/g of the impedimetric method. In such situations, where numbers less than 3 log CFU/g *Listeria* are encountered, however, further enrichment step and confirmation are needed. Additional research is needed to optimize the head-space composition in MAP-packaged produce to prevent the re-growth of surviving pathogens such as *Listeria monocytogenes* during chilled storage.

CHAPTER 4
EXPOSURE TO SUB-LETHAL TEMPERATURES INDUCES ENHANCED HEAT RESISTANCE IN *LISTERIA MONOCYTOGENES*

4.1 Introduction

Listeria monocytogenes is a particular concern for the food industry due to its high case fatality, widespread distribution, ability to survive a wide variety of food processing conditions, and the severity of illness associated with foods contaminated with this pathogen (Goulet and Marchetti, 1996; Mead et al., 1999). This pathogen can be deadly in immuno-compromised patients and pregnant women. It is found in a variety of raw foods, such as uncooked meats, vegetables, and processed foods that become contaminated after processing, such as soft cheeses and cold cuts at deli counters (Fleming et al., 1985; Salamina et al., 1996; Aureli et al., 2000). Unpasteurized milk or foods made from unpasteurized milk may also contain the bacterium. *L. monocytogenes* can be eliminated by pasteurization and cooking, but in some cases Ready-To-Eat (RTE) foods such as hot dogs and deli meats can get contaminated between cooking and final packaging (Khelef et al., 2006). In Europe, the incidence of *L. monocytogenes* in cheeses from various countries were: Italy 17.4%, Germany 9.2%, Austria 10%, and France 3.3% (Rudolf and Scherer, 2001). In Europe, the European Union Commission Regulation (EC) No 2073/2005 has established microbiological criteria in foods (Carrasco et al., 2007). This regulation has identified some of the limits and growth factors for *L. monocytogenes* that are meant to assist Food Business Operators (FBO) in identifying the factors controlling the pathogen's survival and growth in foods. Heat treatment during food processing is one of these factors (SANCO, 2008).

The food industry employs a variety of stressors including elevated temperatures, cold, pH, and osmotic stress as "hurdles" to inactivate or prevent the multiplication of *L. monocytogenes* and other pathogens in foods. The optimal growth temperature for *L. monocytogenes* is between 30°C and 37°C and any temperature above this optimal range is expected to exert a temperature stress (Petran and Zottola, 1989). Heat resistance of *L. monocytogenes* is influenced by many factors such as strain variation, previous growth condition, prior exposure to heat shock, acid stress or other stresses (Golden et al., 1988; Mackey et al., 1990; Sörqvist, 1994; Jørgensen et al., 1999; Doyle et al., 2001). When microbial cells are exposed to temperatures above optimal growth temperatures even for short periods of time, unique physiological responses such as thermo-tolerance are triggered (Lindquist, 1986; Knabel et al., 1990; Farber and Brown, 1990; Linton et al., 1990; Pagàn

et al., 1997). The extent of exposure to temperature above optimal levels, and the matrix in which the cells are exposed are reported to influence the extent of the observed thermo-tolerance (Linton et al., 1990; Sergelidis and Abrahim, 2009). The objective of this study was to identify the extent of enhanced heat resistance of *L. monocytogenes* at 60°C when the cells were pre-exposed to sub-lethal temperature of 46°C, 48°C and 50°C for 30 min and 60 min.

4.2 Materials and Methods

4.2.1 Bacterial strain

The avirulent strain of *L. monocytogenes* (4 ab No 10) (a meat isolate) was kindly provided by Prof. B. Ralovich of the Hungarian Meat Research Institute in Budapest. The culture was grown on Brain Heart Infusion (BHI, Merck, Darmstadt, Germany) broth (pH 7.4) at 37°C.

4.2.2 Determination of D-value

Overnight cultures (25 mL) were harvested by centrifugation (4000 rpm for 5 min at 4 °C) and washed twice with 25 mL of phosphate buffer (pH 6.8) to remove unspent media and possible metabolic by-products (Koutsoumanis and Sofos, 2004). The washed cell suspension was inoculated into Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany) (pH 7.3) to yield a cell population of approximately 10^8 CFU/mL. The TSB medium was the test matrix. Portions (1 mL) of the culture were transferred into 1.5 mL Eppendorf tubes (Molecular BioProducts, San Diego, CA) in triplicates. The samples were placed in a thermostatically controlled circulating water bath (Haake, Germany). The water level in the bath was adjusted above the level of the sample in the tubes. The tubes were manually agitated underwater throughout the duration of the experiment. The samples were exposed to 55°C (for 10, 20, 30, 40, 50, and 60 min), 60°C (for 3, 6, 9, 12, 15, and 18 min) and 65°C (for 1, 2, 3, 4, 5, and 6 min). The heat treatments were performed using temperature probes that facilitated temperature monitoring within the sample in the microfuge tubes.

4.2.3 Identification of Enhanced Heat Resistance

The time-temperature combinations used for sub-lethal heat stress was 46°C, 48°C and 50 °C for 30 and 60 min. Based on the D-value, 60°C heat treatment was chosen for identifying the enhanced heat resistance. Portions (1 mL) of the washed culture were placed in TSB (pH 7.3) and exposed to the sub-lethal heat stress under conditions mentioned above. After exposure to the sub-lethal heat stress, the samples were immediately transferred to a water bath set appropriately, so that the samples were exposed to temperatures of 60°C for 3, 6, 9, 12, and 15 min. The heat treated samples were immediately placed in an ice-bath prior to serial dilution in tubes containing 9 mL of

peptone-NaCl (0.85 %) buffer. The diluted samples were spread plated on Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany) and on TSA plates supplemented with 5% NaCl. Aliquots were plated on the TSA+NaCl since it was previously shown that sub-lethally heat injured cells were sensitive to NaCl (Golden et al., 1988; Smith and Archer, 1988). The assumption was that the difference in counts obtained on TSA and TSA+NaCl would provide information on the percentage of cells that were heat-injured. The plates were incubated for 48 h at 37°C prior to enumeration.

4.2.4 Data Analysis

The D-value (the time required to achieve a 90% reduction) of the *L. monocytogenes* strain (4 ab No 10) at 55°C, 60°C and 65°C was calculated (linear portion of the curve) as previously described (Farber and Brown, 1990). Linear regressions were performed using the linear regression function of the Excel software (Microsoft Corp. WA). The D_{60}-value of the strain (D-value at 60°C) after the sub-lethal heat exposure of 30 min and 60 min at 46°C, 48°C and 50°C was also calculated to determine whether the sub-lethal heat exposure increased the D-value. An increase in the D_{60}-value would be indicative of enhanced heat resistance after sub-lethal heat exposure. The D_{60}-values are based on the TSA counts.

4.3 Results

The reduction in the populations of the *L. monocytogenes* strain at 55°C, 60°C and 65°C is shown in Fig. 4.1, and D-values were calculated to be 15.19 min (R^2=0.93), 3.03 min (R^2=0.98) and 1.29 min (R^2=0.947), respectively. Since 60°C provided a reasonable number of survivors even after 15 min of exposure, this temperature was chosen for determining the enhanced heat resistance. The bacterial response to 60°C was used as a control for comparing the responses to the sub-lethal temperature exposure. The bacterial response of this *L. monocytogenes* strain to 60°C after prior exposure to sub-lethal temperatures of 46°C, 48°C and 50°C for 30 min and 60 min is shown in Figure 4.2 to Figure 4.7.

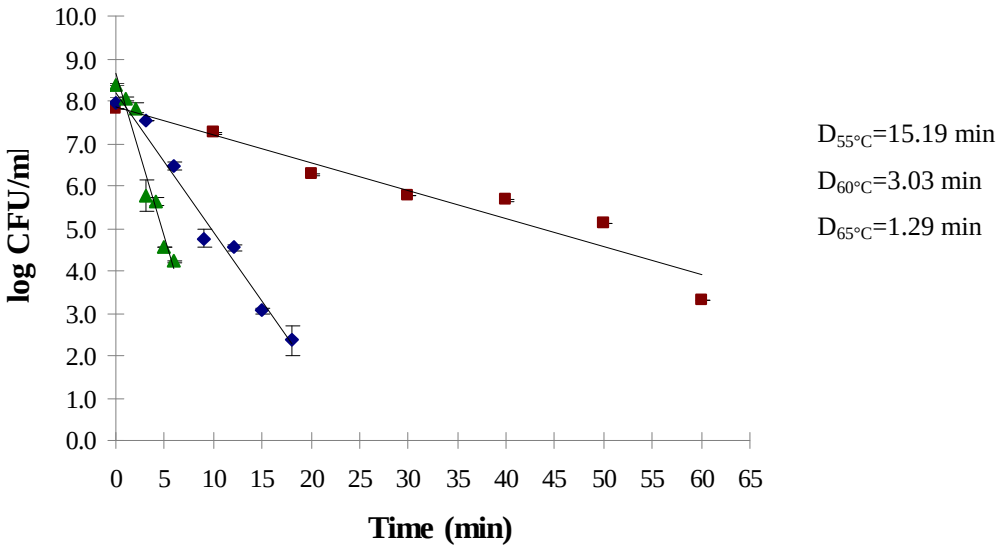


Figure 4.1. Inactivation kinetics of *L. monocytogenes* (strain 4 ab No 10) at 55°C, 60°C, and 65°C

■ 55°C heat treatment; ◆ 60°C heat treatment; ▲ 65°C heat treatment

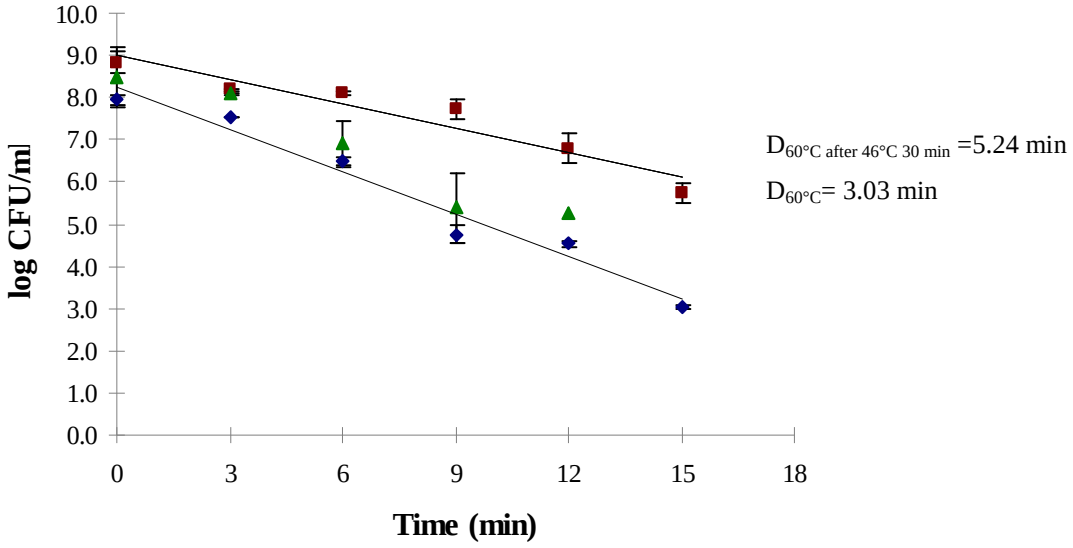


Figure 4.2. Enhanced heat resistance of *L. monocytogenes* (strain 4ab No 10) at 60°C when pre-exposed for 30 minutes at 46°C

◆ 60°C heat treatment; ■ 46°C for 30 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA agar; ▲ 46°C for 30 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA+NaCl agar

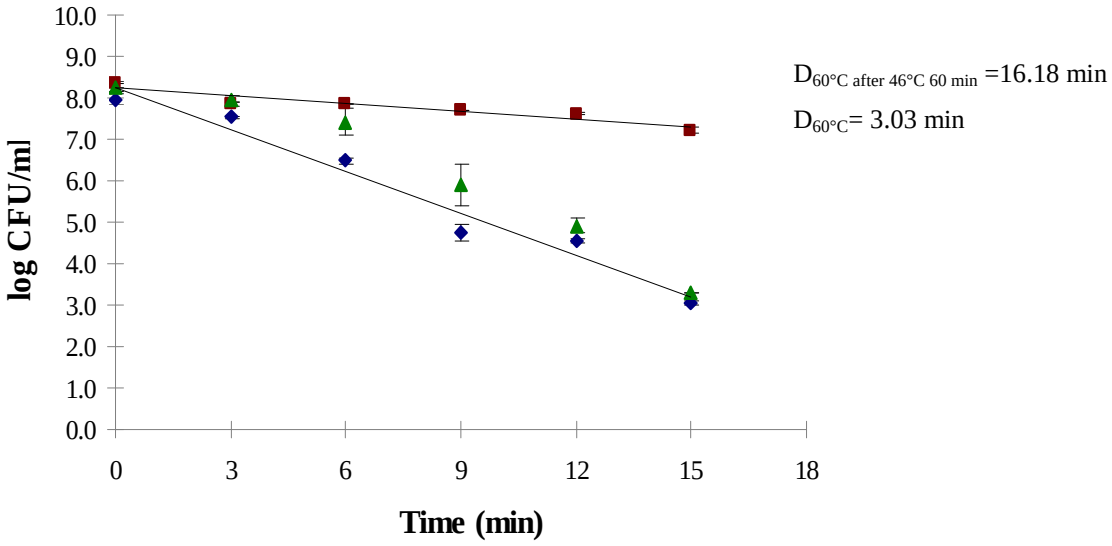


Figure 4.3. Enhanced heat resistance of *L. monocytogenes* (strain 4ab No 10) at 60°C when pre-exposed for 60 minutes at 46°C

◆ 60°C heat treatment; ■ 46°C for 60 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA agar; ▲ 46°C for 60 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA+NaCl agar

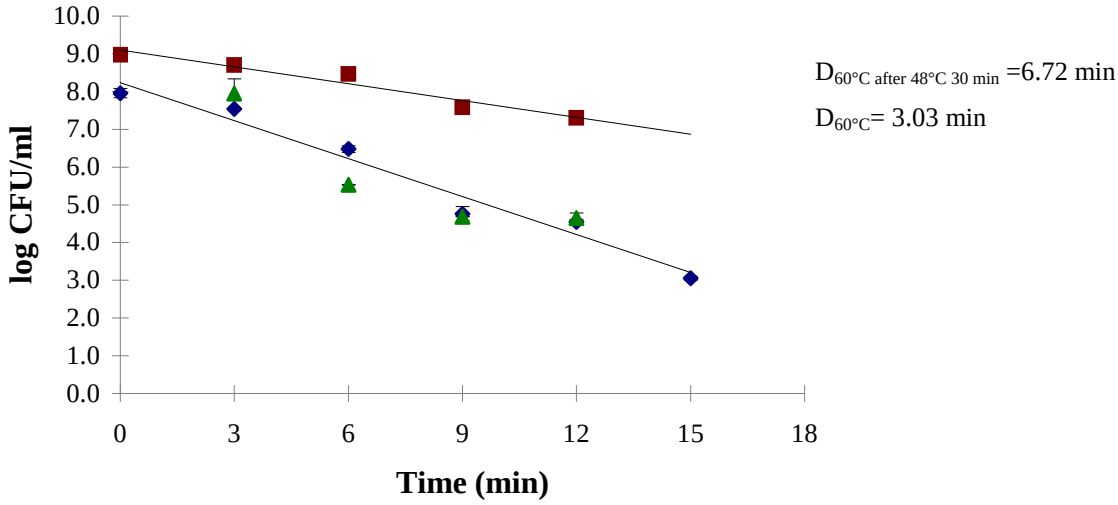


Figure 4.4. Enhanced heat resistance of *L. monocytogenes* (strain 4ab No 10) at 60°C when pre-exposed for 30 minutes at 48°C

◆ 60°C heat treatment; ■ 48°C for 30 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA agar; ▲ 48°C for 30 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA+NaCl agar

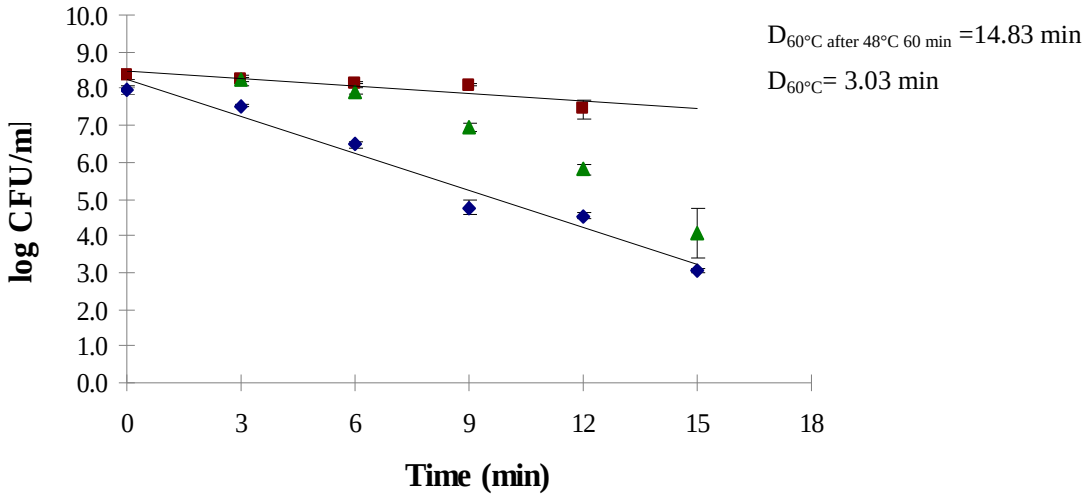


Figure 4.5. Enhanced heat resistance of *L. monocytogenes* (strain 4ab No 10) at 60°C when pre-exposed for 60 minutes at 48°C

◆ 60°C heat treatment; ■ 48°C for 60 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA agar; ▲ 48°C for 60 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA+NaCl agar

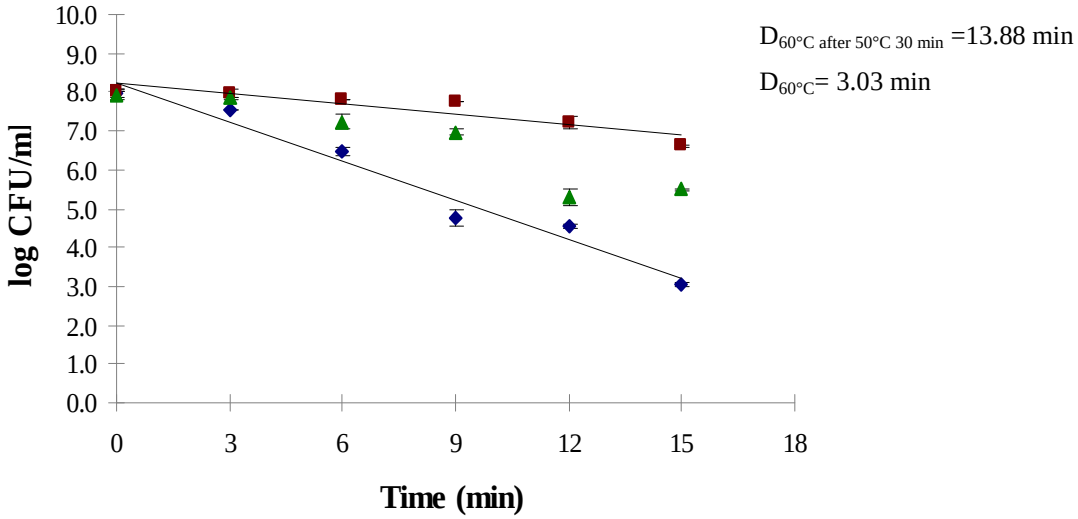


Figure 4.6.: Enhanced heat resistance of *L. monocytogenes* (strain 4ab No 10) at 60°C when pre-exposed for 30 minutes at 50°C

◆ 60°C heat treatment; ■ 50°C for 30 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA agar; ▲ 50°C for 30 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA+NaCl agar

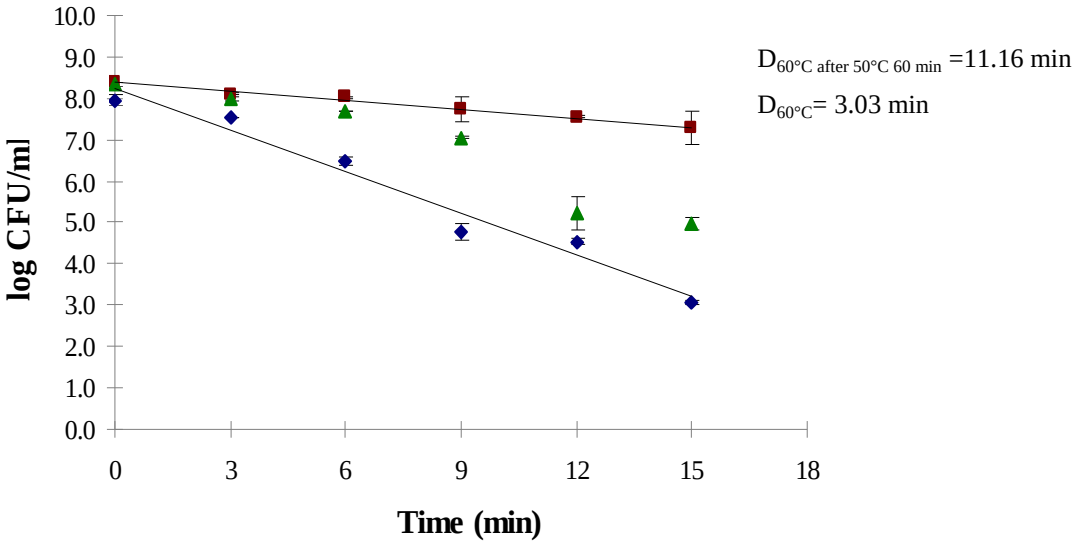


Figure 4.7. Enhanced heat resistance of *L. monocytogenes* (strain 4ab No 10) at 60°C when pre-exposed for 60 minutes at 50°C

◆ 60°C heat treatment; ■ 50°C for 60 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA agar; ▲ 50°C for 60 min pre-exposure followed by 60°C heat treatment and samples plated onto TSA+NaCl agar

Table 4.1. Effect of pre-exposure to sub-lethal temperatures of 46°C, 48°C and 50°C for 30 minutes and 60 minutes on the D_{60}-values for *Listeria monocytogenes* 4ab in Tryptic Soy Broth

	D_{60}-value (minutes)		
mild heat temperature	**time of mild heat treatment**		**without pre-treatment**
	30 min	**60 min**	
46 °C	5.24	16.18	
48 °C	6.72	14.83	3.03
50 °C	13.88	11.16	

The D_{60}-value of the strain after prior exposure to the three sub-lethal temperatures is shown in Table 4.1. Pre-exposing the *L. monocytogenes* strain to three sublethal temperatures (ie. 46°C, 48°C, 50°C) for 30 minutes and 60 minutes enhanced the survival of this strain at 60°C. Compared to direct exposure to 60°C (which resulted in a D_{60}-value of 3.03 min), the D_{60}-value after 30 min pre-exposure at 46°C was 5.24 min and 16.18 min after pre-exposure of 60 minutes at 46°C. Similarly, the D_{60}-value after 30 min and 60 min pre-exposure at 48°C was 6.72 min and 14.83 min, respectively. The D_{60}-value was 13.88 minutes and 11.16 minutes after 30 minutes and 60 minutes pre-exposure at 50°C, respectively (Table 4.1). The increased D_{60}-value is direct evidence for enhanced heat resistance occuring after pre-exposure to sub-lethal temperatures. The enhanced heat resistance is particularly evident when the cells are exposed to 30 minutes at temperatures

increasing from 46°C, to 48°C and then 50°C. The D_{60}-value increased from 5.24 min at 46°C to 13.88 min at 50°C. When the cells were exposed to 60 min at 50°C, the D_{60}-value decreased as compared to the 30 min exposure suggesting that there is an upper limit in terms of sub-lethal heat resistance. It is important to note that these D_{60}-value increases were almost 5-fold when the direct exposure to 60°C is compared to sub-lethal pre-exposure to 46°C and 48°C for 60 minutes (Table 4.1).

Exposure to sub-lethal temperature appears to cause heat injury in this *L. monocytogenes* strain (Figure 4.2- 4.7). This is based on the TSA and TSA+NaCl plate counts after 30 min and 60 min of sub-lethal pre-exposure at 46°C, 48°C and 50°C. There were significant differences in the bacterial numbers obtained on the TSA plates and TSA+NaCl plates. The counts on the TSA+NaCl plates were consistently lower than the TSA plates suggesting that heat injury was occuring. Plating onto TSA supplemented with 5% NaCl was also performed after direct 60°C heat treatment. However, there was no difference between the numbers obtained on TSA as compared to TSA+NaCl plates (data not shown).

4.4 Discussion

A number of studies in the past have shown that *Listeria* spp. exhibit varying survival patterns under different temperature conditions (Bunning et al., 1988; Smith and Archer, 1988). *Listeria* spp. have also been shown to develop thermotolerance if the cells are pre-exposed to varying temperatures (Pagàn et al., 1997, Farber and Brown, 1990, Linton et al., 1990). Many of these early studies were aimed at identifying the safe temperatures for milk pasteurization. Farber and Brown (1990) previously examined the heat resistance of *Listeria monocytogenes* in a sausage mix under prior heat exposure of 48°C for up to 120 min. Their results showed an average 2.4-fold increase in D_{64}-value. In their studies, prior heat exposure at 48°C for 30 or 60 min did not show a significant increase in heat resistance as compared with untreated cells. In this study with this particular *L. monocytogenes* strain, prior heat exposure at 46°C and 48°C for 30 and 60 minutes increased the D_{60}-value (Table 4.1). Fedio and Jackson (1989) studied the effect of prior exposure to *L. monocytogenes* ScottA serotype 4b to sub-lethal temperature exposure in broth and UHT milk. Their studies showed that 48°C for 60 minutes pre-exposure prior to 60°C heat treatment enhanced heat resistance. Pagàn et al. (1997) investigated the effect of growing temperature (37°C and 4°C) of *L. monocytogenes* on the heat shock response. Cells grown at 4°C showed a 7-fold increase in thermo-tolerance as compared to the 4-fold increase that was observed in cells grown at 37°C. They reported D_{65}=0.65 min after a pre-exposure at 47.5°C for 180 min. This was 4-fold higher than that for non-heat shocked cells. Lin and Chou (2004) studied 3 strains of *L. monocytogenes* which were

subjected to heat shock at 45°C for 60 min or 48°C for 10 min. Heat shocked cells at 45°C for 60 min showed an increased survival after 55°C heat treatment for 60 min compared to non heat shocked cells (in two of the strains). However, 48°C for 10 min heat shock resulted in no significant difference, regardless the strain. In the present study, it is noticed that pre-exposure to 50°C for 60 minutes caused reduced survival at 60°C as compared to pre-exposure at 50°C for 30 minutes (Table 4.1). It must be noted that the ”shoulder” and ”tail” portions of the survival curves are also critically important. My research specifically focused on the linear portion of the curve since the focus of my research was aimed at understanding the mechanism of thermo-tolerance. Similar studies are needed to understand processes occuring at the shoulder and tail regions of the curve.

It is well established that the choice of a particular plating medium influences the microbial counts (Olsen and Bakken, 1987; Knabel et al., 1990). Pagàn et al. (1997) observed a ”shoulder” on heat shocked cells when the survival curves were plotted. They report that these ”shoulders” disappeared when 3% NaCl was added to the recovery medium and increased decimal reduction was noticed. Similarly, the exogenous addition of catalase increased the recovery of heat-injured *L. monocytogenes* cells in trypticase soy broth-yeast extract medium (Knabel et al., 1990). In this study the heat treated samples were plated on TSA plates and TSA plates amended with 5% NaCl (Figs. 4.2- 4.7). The assumption was that sub-lethally injured cells were sensitive to NaCl. When the cells were pre-exposed to different temperatures it was an evident that heat injury occured (Figs. 4.2-4.7), based on the difference in the TSA and TSA+NaCl plate counts. Smith and Archer (1988) have previously reported exposing *L. monocytogenes* (Scott A strain) in phosphate buffer (pH 7.2) at 52°C for 1 hour led to injury which was detectable using 5% NaCl amended media. In this study too, pre-exposure of *L. monocytogenes* (strain 4 ab No 10) to 50°C for 30 minutes prior to 60°C exposure resulted in as much as 3-log difference between heat-injured and non-injured cells (Fig. 4.7). However, Table 4.1 highlights the fact that there is an upper limit for the pre-exposure temperature that results in an enhanced D-value as discussed earlier.

In summary, *L. monocytogenes* 4ab exhibits an enhanced heat resistance (increased D-values) after exposure to sub-lethal temperature conditions. Exposing the strain to increasing durations of sub-lethal temperatures enhanced the survival at 60°C of this strain. The data also suggests that heat injury does occur when this organism is exposed to 50°C. Therefore culture media used to enumerate *L. monocytogenes* in heat-treated food samples should be carefully chosen to avoid inadvertent underestimation of the actual numbers of surviving cells, since with increasing exposure to heat stresses there is a greater probability of heat injury.

CHAPTER 5
EXPOSURE TO SUB-LETHAL TEMPERATURE INDUCES THE VIABLE BUT NON-CULTURABLE (VBNC) STATE IN *LISTERIA MONOCYTOGENES* AT 60°C

5.1 Introduction

The viable but non-culturable (VBNC) state is a survival strategy adopted by bacteria when they are exposed to hostile environmental conditions. In this state the bacterial cells supposedly remain viable yet cannot be cultured on culture media (Oliver, 1995, 2000; Kell et al., 1998; Barer and Harwood, 1999). The state is induced by stress from external factors, such as, incubation outside the normal temperature range, elevated salinity, osmotic, and oxygen concentrations, starvation, and processes thought to be bactericidal (Oliver, 1995, 2005; Rice et al., 2000; Grey and Steck, 2001; Kong et al., 2004). This survival state has been recognized in many animal and plant pathogens, and in both Gram-negative and Gram-positive bacteria. Some examples include *E. coli*, *Salmonella* spp., *H. pylori*, *R. leguminosarum*, *Vibrio* spp., *L. monocytogenes*, *K. aerogenes*, *P. putida*, *E. faecalis*, *A. tumefaciens*, *E. amylovora* and *R. solanacearum* (Byrd, 1991; Kondo et al, 1994; Alexander, 1999; Lleo et al., 2003; Kong et al., 2004, Oliver, 2005; Ordax et al., 2006). Besnard et al. (2000b) described a direct microscopic procedure involving the use of the antibiotic ciprofloxacin to detect and count viable but non-culturable *L. monocytogenes* cells. Rudi et al. (2005) have recently reported the detection of VBNC *L. monocytogenes* on gouda cheese. Cappelier et al. (2005) have shown that *L. monocytogenes* does convert into a VBNC state when stored in water at either 20°C or 4°C. However, they report that these cells were not infectious when assayed using the human adenocarcinoma cell line (HT-29) and a mouse model.

Thermal processing is one of the oldest and most common techniques employed to control pathogens in food. Sub-lethal heat stress can occur in bacterial cells when they are exposed to above-optimal, but below lethal levels (Rodriguez-Romo and Yousef, 2005a,b). Exposure to sub-lethal temperatures prior to heating is known to increase the D-values in *L. monocytogenes* (Fedio and Jackson, 1989; Stephens and Jones, 1993; Jørgensen et al., 1999). My previous experiments demonstrated that when *L. monocytogenes* cells are pre-exposed to sub-lethal heat treatments above 48°C prior to exposure to 60°C, the cells are injured. The underlying hypothesis of this study was that when *L. monocytogenes* cells are exposed to sub-lethal heat stress the reduced recovery of the bacterium on culture media is due to the cells entering into a Viable But Non-Culturable (VBNC)

state. The objective of this study was to examine the viability of a virulent strain of *L. monocytogenes* (ATCC 43256) using microscopy and fluorescence spectroscopy (using the Live/Dead BacLight™ fluorescent stain) after the cells were pre-exposed to sub-lethal temperature stress.

5.2 Materials and Methods

5.2.1 Bacterial strain

The virulent *L. monocytogenes* strain (ATCC 43256) was used in this study. It was obtained from the American Type Culture Collection (Manssas, VA). A working culture were prepared in Luria Bertani (LB, Difco) broth (pH 7.0) and incubated at 37°C for 24 h.

5.2.2 Exposure to Sub-lethal Temperature Stress

The sample (25 mL) was harvested by centrifugation (4000 rpm for 5 min at 4°C) and washed twice with 25 mL of PBS (pH 7.4) to remove metabolic end products (Koutsoumanis and Sofos, 2004). The washed cells were resuspended in Luria Bertani broth (LB; pH 7.0) to yield approximately 10^8 CFU/mL. One milliliter aliquots of the sample were placed (in triplicate) in 1.5 mL microfuge tubes. The microfuge tubes were used for the different heat treatments. The heat treatments were performed in a calibrated water-bath (Boekel Grant ORS200, PA. USA) using temperature probes that facilitated temperature monitoring within the sample contained in the microfuge tubes. The water level in the bath was adjusted so that the contents of the tubes were completely submerged throughout the heat treatment. Three experimental conditions were chosen namely, (i) exposure for 60°C for 0 minute (i.e., the cells were exposed until the temperature reached 60°C) , and (ii) 60°C exposure for 9 minutes, and (iii) 48°C for 30 minutes followed by 60°C exposure for 9 minutes. These experimental conditions were based on a previous study which demonstrated that pre exposure to heat stress enhances the heat resistance of the cells.

5.2.3 Live/Dead Fluorescent Dye Staining

The fluorescent dye, LIVE/DEAD BacLight™ (Bacterial Viability Kits, Molecular Probes, Invitrogen, CA) was used to determine the viability of the cells. The protocols suggested by the manufacturer was used for the staining. Briefly, the stains were SYTO® 9 (component A) and propidium iodide (component B). Viability was quantified microscopically and using a fluorometer. For fluorescence spectroscopy, a two-stain solution was prepared. The two stains (6 µL each) were mixed in a microfuge tube and the entire 12 µL mix was added to 2 mL of filter sterilized dH_2O in a

borosilicate glass culture tube, and mixed well. For the microscopic analysis, the dye mixture were prepared using equal volumes of component A and component B in a microfuge tube.

The *L. monocytogenes* cells from the three different heat treatments (as described in Section 5.2.2) were centrifuged (4000 rpm for 5 min at 4°C), washed two times with PBS (pH 7.4) and resuspended in 1 mL of sterile dH_2O. Aliquots (100 µL) of the washed bacterial cell suspension was pipetted into a 96-well flat-bottom microplate in five replicates. The staining solution (100 µL) was added to each well and mixed thoroughly. The samples were incubated in the dark for 15 minutes at room temperature. After incubation, the samples were measured at two wavelength (A_{535} and A_{635}) using the Tecan™ (Tecan US, Durham, NC) fluorescence microplate reader. The excitation wavelength used was 485 nm. The green fluorescence intensity (535 nm, F_{em1}) and red fluorescence intensity (635 nm, F_{em2}) were measured, and the green/red fluorescence ratio ($R_{G/R}$) (A_{535}/A_{635}) were calculated for each experimental sample based on the following formula:

$R_{R/G}=F_{em1}/F_{em2}$.

For the direct microscopic examination, 3.3 µL of the dye mixture was added for each 1 mL of sample. The sample was incubated at room temperature in the dark for 15 minutes. For the microscopic analysis 5.5 µL of the stained bacterial suspension were placed on a glass slide and covered with a cover slip. The stained samples were analysed using fluorescence microscopy (Olympus USA, Center Valley, PA) using a dual emission filter for simultaneous viewing of SYTO 9 and propidium iodide stains. The samples were not diluted to avoid potential errors in detecting live and dead cells. The images were captured using a Spot™ CCD camera (Diagnostic Instruments, Sterling Heights, MI). The percentage of viable cells in each sample was calculated using image analysis and MathCad 14.0 (PTC, Needham, MA) software. Dead cells were considered as those red pixels where the red color component was higher than the green color component. The ratio of red cells were determined on the number of red pixels divided by the total number of red and green pixels. We considered only those pixels where the red + green pixels >100, thereby avoiding the background (Figure 5.1).

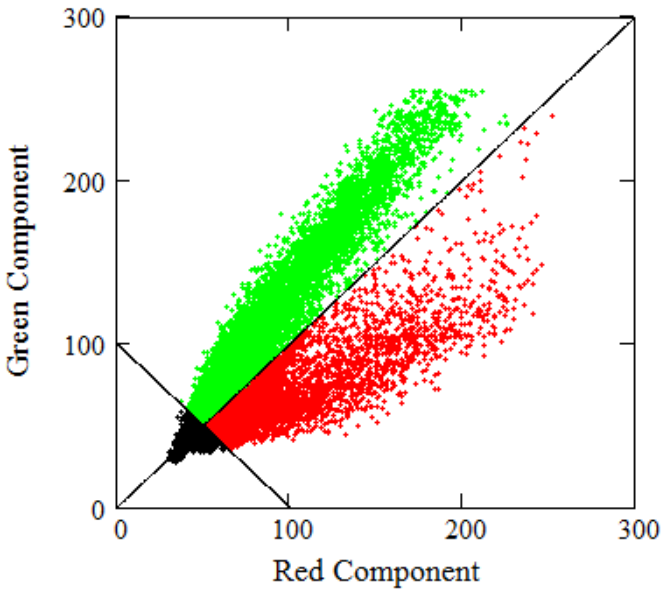


Figure 5.1. Pixel intensities used in the discrimination of live (green) and dead (red) *L. monocytogenes* ATCC 43256 cells

Pixel intensity ≤100 is the cut-off for background. Red and green pixels above pixel intensities of 100 denote dead and live cells respectively. The digital image analysis performed using MathCad 14.0 (PTC, Needham, MA).

5.3 Results

The D-value of *L. monocytogenes* ATCC 43256 strain at 55°C, 60°C and 65°C was 17.39 min (R^2=0.95), 3.74 min (R^2=0.96), 3.15 min (R^2=0.89) respectively (Fig. 5.2.).

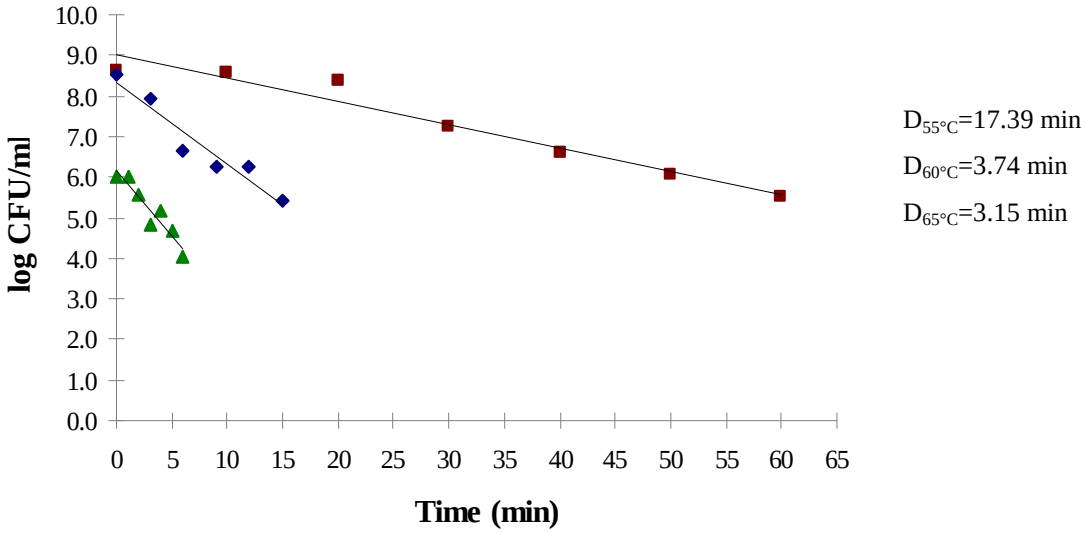


Figure 5.2. Inactivation kinetics of *L. monocytogenes* ATCC 43256 at 55°C, 60°C and 65°C.

■ 55°C heat treatment; ◆ 60°C heat treatment; ▲ 65°C heat treatment

Pre-exposure to sub-lethal temperature of 48°C for 30 min increased the D-values at 60°C. The D_{60}-value (based only on the linear portion of the curve) changed from 3.74 minutes to 4.55 minutes (Fig. 5.3.). According to Figure 4.3, *L. monocytogenes* (ATCC 43256) when exposed to 60°C results in >2 log (~ 99%) reduction of culturable cells at the end of 9 minutes. When the cells were pre-exposed to 48°C for 30 minutes prior to 60°C heat treatment for 9 minutes, there was <2 log (99%) reduction.

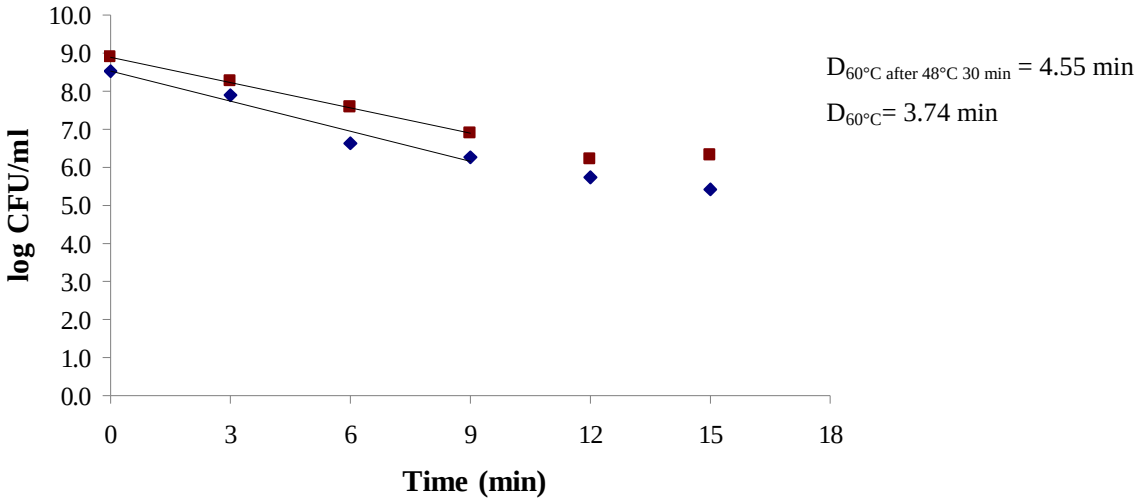


Figure 5.3. Heat resistance of *L. monocytogenes* ATCC 43256 when pre-exposed to 48°C for 30 minutes prior to 60°C exposure as compared to direct exposure to 60°C

◆ 60°C heat treatment; ■ 48°C for 30 min pre-exposure followed by 60°C heat treatment

Standard curves were prepared using live cells and heat-killed cells as per the manufacturer's recommendations (Fig. 5.4). The percentage of live cells after each experimental treatment was estimated based on this standard curve. However, when these same samples were analyzed for % viability using the LIVE/DEAD BacLight™, the results were significantly different (Table 5.1, Fig. 5.4). Based on the fluorescence microplate read-out there was no change in % viability even after exposure to 9 minutes at 60°C (Table 5.1). The % viability remained at 100%. Based on the direct microscopic examination as well, there was only minimal reduction in viability (Table 5.1, Figure 5.4).

Table 5.1. Percentage of live (green) and dead (red) *L. monocytogenes* cells determined using the LIVE/DEAD BacLight™ dye using fluoroscence spectroscopy and direct microscopy

Heat Treatment	**Viability**		
		LIVE/DEAD BacLight™	
	Plate Count*	Fluorescence Spectroscopy	Direct Microscopy
60°C for 0 minute		100 %	99.93 %
60°C for 9 minutes	<1 %	100 %	98.97 %
48°C for 30 min pre-treatment followed by 60°C for 9 minutes	1 %	100 %	99.09 %

*Plate count based on data obtained from Fig. 5.3

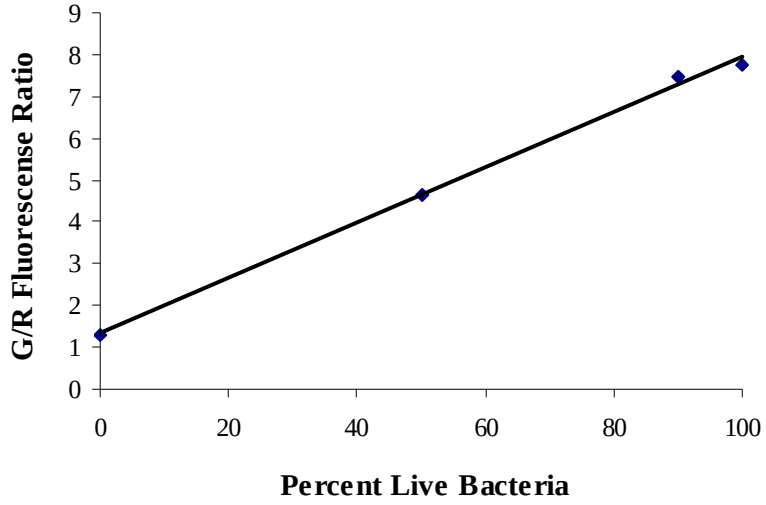


Figure 5.4. Relationship between A_{635} (red)/A_{535} (green) ratio and % viability using the Live/Dead BacLight™ fluorescent dye

$$y=0.0662x + 1.3262, R^2=0.9973$$

When the cells were exposed to 60°C for 9 minutes there was only approximately 1.03% reduction in viability based on the LIVE/DEAD BacLight™ viability assay. However, when the cells were pre-exposed to 48°C for 30 minutes prior to 60°C exposure for 9 minutes, the viability was reduced by only 0.91%. Figure 5.5 shows the majority of cells in the heat treated samples as green indicating their viability.

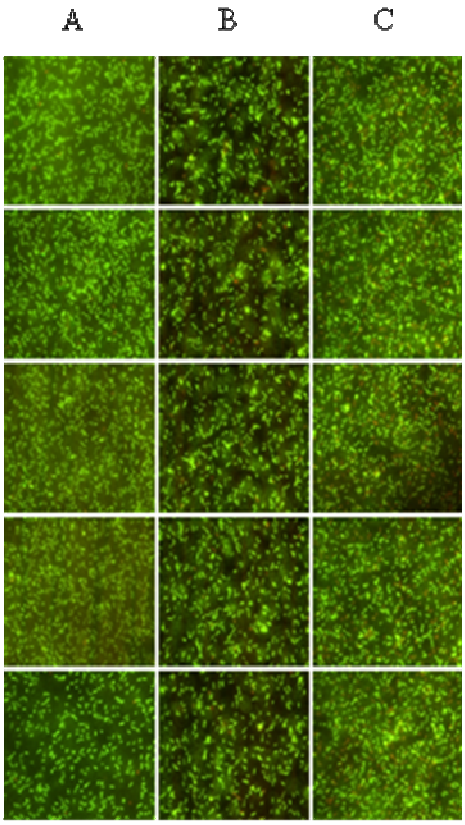


Figure 5.5. Direct microscopic examination (% viability) using the LIVE/DEAD BacLight™ staining of *L. monocytogenes* ATCC 43256 cells

Images in column A represents the unheated control samples, images in column B represent the samples exposed to 60°C for 9 minutes, and images in column C represent samples pre-exposed to 48°C for 30 minutes prior to 60°C exposure

5.4 Discussion

L. monocytogenes ATCC 43256 exhibits enhanced heat resistance when the cells are pre-exposed to sub-lethal temperatures. Pre-exposure to sub-lethal temperature of 48°C for 30 min increased the D-values at 60°C. The D_{60}-value changed from 3.74 minutes to 4.55 minutes (Fig. 5.3). This result is in agreement with a number of other studies including my initial study using an avirulent *L. monocytogenes* strain (4ab no. 10). Fedio and Jackson (1989) studied the effect of prior exposure to *L. monocytogenes* ScottA serotype 4b to sub-lethal temperature exposure in broth and UHT milk. Their studies showed that 48°C for 60 minutes pre-exposure prior to 60°C heat treatment showed enhanced heat resistance. Lin and Chou (2004) studied 3 strains of *L. monocytogenes* which were subjected to heat shock at 45°C for 60 min or 48°C for 10 min. Heat shocked cells at 45°C for 60 min showed an increased survival after 55°C heat treatment for 60 min compared with non heat shocked cells (in two of the strains).

Bacterial cells are known to exist in the viable but non-culturable (VBNC) state and has been reported by a number of investigators (Oliver, 1995, 2005; Grey and Steck, 2001; Kong et al., 2004). This state occurs when a cell is not able to grow on media normally used for growth but remains viable (Oliver, 1995). Normal environmental stressors such as nutrient depletion, cold, high temperature are known to induce this condition in bacterial cells. Rigsbee et al. (2007) have reported that in *E.coli* O157:H7, reduced water temperature rather than salinity was the responsible factor for inducing the VBNC state. The inducing conditions can vary from organism to organism, but all of them appear to be normal environmental stresses. For *Vibrio vulnificus* as well, low temperature (<10°C) was capable of inducing the VBNC state. Other examples of stresses include high temperature and nutrient depletion. Leena et al. (2006) have reported that in *Sinorhizobium arboris*, heat stress reduces the culturability of the cells. However, when they probed the cells with 5-(and 6-)sulfofluorescein diacetate, to determine whether esterase activity was evident, they found that a majority of the cells were metabolically active. Trainor et al. (2006) have also reported possible VBNC state in *Streptococcus pyogenes.* They reported that the bacterium when exposed to oxidative and pH stresses induced the formation of VBNC state when culture counts were compared against rhodamine 123 (dye to measure membrane potential) stained cells. The LIVE/DEAD® BacLight™ Bacterial Viability assay employs two nucleic acid stains—green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain (Invitrogen, CA). These stains differ in their ability to penetrate healthy bacterial cells. The SYTO® 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. A high green: red ratio indicates a higher percentage of live cells. In this study I investigated the VBNC state using two different approaches namely direct microscopy and using a fluorescence microplate reader. The *L. monocytogenes* strain was exposed in Luria Broth to three different temperature treatments, namely 60°C for 0 min, 60°C exposure for 9 min, and 48°C exposure for 30 min prior to 60°C heat treatment for 9 min. Even though there was >2 log reduction of culturable *L. monocytogenes* cells after 60°C exposure for 9 minutes (Fig. 5.3), the results from the live/dead staining assay were completely different. The direct microscopy showed only a 1.03% decline in viability. The fluorescence reading from the microplate assay did not detect any loss of viability (Table 5.1). These results suggest the importance of using more than one method to detect the presence of viable cells of *L. monocytogenes* in foods. This is particularly important for those foods that have undergone a variety of mild temperature treatments since these sub-lethal temperature regimens could induce the VBNC state in *L. monocytogenes*. Besnard et al. (2000a) have previously reported

on the VBNC state in *L. monocytogenes*. However, their study focused on this state in water samples.

Additional research is urgently needed in food samples to better understand the VBNC state in *L. monocytogenes* and determine the prevalence of this state in foods. All of the currently approved *L. monocytogenes* detection protocols involve enrichment in culture media. Even molecular methods rely on an overnight enrichment for *L. monocytogenes*. These methods could underestimate those *L. monocytogenes* cells that are in the VBNC state. Therefore, it is important to investigate the prevalence of VBNC *L. monocytogenes* in RTE foods using a combination of molecular methods, and metabolic assays (Keer and Birch, 2003; Leena et al., 2006).

CHAPTER 6
DIFFERENTIAL EXPRESSION OF GENES IN *LISTERIA MONOCYTOGENES* UNDER THERMO-TOLERANCE INDUCING, HEAT SHOCK, AND PROLONGED HEAT SHOCK CONDITIONS

6.1 Introduction

Listeria monocytogenes is a key foodborne pathogen that is able to grow under a wide variety of environmental conditions. Lethal or severe stress causes irreversible damage to the microbial cells. However, *L. monocytogenes* can withstand a variety of stress conditions and has shown a remarkable ability to adapt to stress conditions (Sauders and Wiedmann, 2007). Yousef and Courtney (2003) define microbial stress as any deleterious physical, chemical, or biological factors that adversely affect microbial growth or survival. When microorganisms are exposed to sub-lethal stress, it is generally thought that this exposure can induce adaption to subsequent lethal levels of the same type of stress. This microbial adaptation is considered as "stress adaptation" (Lou and Yousef, 1997). The microbial responses to stress can be immediate and can result in long-term adaptations if the stress persists (Seeliger and Jonesy, 1986; Farber et al., 1992; Lou and Yousef, 1997).

The optimal growth temperature for *L. monocytogenes* is between 30°C and 37°C and any temperature above this optimal range is expected to exert a stress (Petran and Zottola, 1989, Hill et al. 2002). Pagàn et al. (1997) have reported a 7-fold increase in thermo-tolerance of *L. monocytogenes* when the cells were exposed to 45°C for 180 minutes. The extent of exposure to temperature above optimal levels, and the matrix in which the cells are exposed are reported to influence the extent of the observed thermo-tolerance (Linton et al., 1990; Sergelidis and Abrahim, 2009).

The heat shock response of *L. monocytogenes* using DNA microarray analysis has been reported (Hu et al., 2007a, b; Van der Veen et al., 2007). The enhanced transcription of specific genes coding for proteins such as PrfA (pleiotropic regulatory factor) (which belongs to the Crp/Fnr family of prokaryotic transcriptional activators) has been reported under elevated temperatures (Sokolovic et al., 1990; Bohne et al., 1994; Leimester-Wächter et al., 1992). Nair et al. (2000) studied the role of CtsR regulon controlling heat shock genes in *Listeria monocytogenes*, and they found that CtsR negatively regulates the *clpC*, *clpP* and *clpE* genes. Hu et al. (2007a) identified interactions between two stress response systems, namely σ^B which positively regulates the

transcription of class II stress response genes and CtsR which negatively regulates class III stress response genes. They found that the interaction between the two systems play an important role in *L. monocytogenes* stress resistance and virulence. In further studies, Hu et al. (2007b) found that HrcA (which regulates class I stress response genes negatively) and σ^B as well as CtsR form a regulatory network. The impact of σ^B on the survival of *L. monocytogenes* EGDe has been studied under acid stress, high hydrostatic pressure treatment and during freezing (Wemekamp-Kamphuis et al., 2004).

The food industry employs a variety of stressors including elevated temperatures. cold, pH, and osmotic stress as "hurdles" to inactivate or prevent the multiplication of *L. monocytogenes* and other pathogens in foods. Thus, temperature is one of the key stressors that are commonly employed in the food industry as a "hurdle" to prevent microbial growth or eliminate microbial populations. To the best of my knowledge, the complete heat shock regulon of *L. monocytogenes* in response to a temperature increase has been reported only by Van der Veen al. (2007). In their study, the transcription levels were measured over a 40 min period at 48°C and compared to unexposed cultures at 37°C. The objective of my study, however, was to identify the differentially expressed genes during heat stress by comparing the transcriptome of *L. monocytogenes* under optimal temperature (37°C), heat shock (60°C for 0 minute), prolonged heat shock (60°C for 9 minutes), and thermo-tolerance inducing (48°C for 30 minutes prior to exposure to 60°C for 9 minutes) conditions using microarray analysis.

6.2 Materials and Methods

6.2.1 Bacterial strains and Sample conditions

The bacterial strain used in this study was *L. monocytogenes* ATCC 43256. Overnight cultures of *L. monocytogenes* were inoculated in fresh LB broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey) and the strain was incubated at 37 °C with agitation at 100 rpm until OD_{600} 0.5 (~ 10^8 CFU/mL) was reached. At this point, aliquots were placed into multiple 1.5 mL microfuge tubes for the different heat treatments. The heat treatments were performed in a calibrated water-bath (BOEKEL Grant ORS200, PA, USA) using temperature probes that facilitated temperature monitoring within the sample contained in the microfuge tubes. Four different experimental conditions were studied namely: (i) 37°C (control), (ii) heat shock at 60°C (for 0 minute, as decribed earlier), (iii) prolonged heat shock at 60°C for 9 min, and (iv) thermo-tolerance inducing treatment at 48°C for 30 minutes followed by exposure to 60°C for 9 min. Total microbial RNA was extracted from the samples after the applied heat treatments.

6.2.2 RNA isolation

The total RNA was isolated from the samples using an RNeasy™ midi kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNAprotect™ bacteria reagent (Qiagen, Valencia, CA) was added to the cultures to stabilize RNA before the isolation. The RNase-free DNase set (Qiagen, Valencia, CA) was used for on-column DNase digestion to remove residual genomic DNA. The quantity and quality of RNA was examined using the NanoDrop (ND-1000) spectrophotometer (Thermo Scientific, Wilmington, DE), and the Bioanalyser 2100™ (Agilent Technologies, Santa Clara, CA), respectively.

6.2.3 cDNA synthesis, Labeling and Slide Hybridization

The standard operating protocols (# M007 and M008) of The Institute of Genomic Research (TIGR) were followed with slight modifications for cDNA synthesis, labeling, and hybridization. Total RNA (10 μg) was used to synthesize cDNA using a random primer for reverse transcription (Invitrogen, Carlsbad, CA). Purified cDNAs from the experimental samples were each labeled with Cy-3 mono-Reactive Dye and Cy-5 mono-Reactive Dye (GE Health Care Biosciences Corp, Piscataway, NJ)) and were processed using a dye-swapping design. A total of 4 to 5 microarray slides were used for each treatment condition including dye swap. The labeling mixtures were further purified using a QIAquick™ PCR purification kit (Qiagen, Valencia, CA). Equal amounts of labeled cDNA from the treatment and control were used to hybridize *L. monocytogenes* genome microarrays [version 2. The Institute for Genomic Research (TIGR, Rockville, MD)]. Version 2 arrays were cDNA arrays with 2846 open reading frame each, with 4 replicate spots per ORF. The labeled cDNA was applied to the above arrays. Hybridization was carried out overnight at 42°C in a water bath using Corning hybridization chamber. After hybridization, the slides were washed and scanned using a GenePix 4100A scanner (Molecular Devices, Sunnyvale, CA) at 532 nm (Cy3 channel) and 635 nm (Cy5 channel), and the images were stored for further analysis.

6.2.4 Microarray Data Analysis

The data from four to five individual experiments (4 to 5 slides per experiment including dye swap) were initially filtered for spot quality (signal uniformity. signal to background ratio, threshold intensity) using Genepix pro 5.0 (Molecular Devices Corp, Sunnyvale, CA). Visually flagged spots as well as spots with a median signal value less than the sum of the local background median plus three standard deviations were omitted from analysis (Hegde et al., 2000). Array data were normalized and their statistical significance was evaluated using Acuity 4.0 (Molecular Devices Corp., Sunnyvale, CA). To identify genes differentially expressed between different treatment groups, a Student's *t*-test was performed and the FDR (False Discovery Rate) was calculated using

the Benjamini-Hochberg method in Acuity. Genes with FDR <0.05 were considered as differentially expressed between the control sample and the experimental sample. The microarray data analysis procedures used in this study was fully MIAME (minimum information about a microarray experiment) compliant.

6.3 Results

6.3.1 Transcriptome level Gene Expression

The response of the *L. monocytogenes* ATCC 43256 transcriptome under three different temperature treatments were compared to the response at 37°C. The three temperature treatments were 60°C for 0 minute (heat-shock), 60°C for 9 minutes (prolonged heat shock), and 48°C for 30 minutes prior to exposure to 60°C for 9 minutes (thermo-tolerance inducing treatment). When the cells were exposed to 60°C for 0 minute heat shock conditions, 91 out of 6347 genes (~ 1.4%) were differentially expressed ($p \leq 0.05$) (Table 6.1). When the cells were maintained at 60°C for 9 minutes (prolonged heat shock), 80 out of 6347 (1.2%) were differentially expressed ($p \leq 0.05$) as compared to 37°C (Table 6.2). When the cells were pre-exposed to 48°C for 30 minutes prior to prolonged heat exposure (i.e. thermo-tolerance inducing conditions), 71 genes (1.1%) were differentially expressed (Table 6.3). It needs to be highlighted that some of the differentially expressed genes are, as expected, involved in more than one functional group classification.

Table 6.1. Differentially expressed genes of *L. monocytogenes* ATCC 43256 during 60°C for 0 minute heat shock conditions as compared to 37°C

Gene	Fold-change	Gene designation	Description of product
Amino acid transport and metabolism			
lmo2694	-1.56	-	hypothetical protein lmo2694
Carbohydrate transport and metabolism			
lmo0357	-1.49	-	hypothetical protein lmo0357
lmo2668	-1.08	-	hypothetical protein lmo2668
lmo2458	1.35	*pgk*	phosphoglycerate kinase
lmo0096	1.71	-	hypothetical protein lmo0096
Cell cycle control, mitosis and meiosis			
lmo2506	6.59	*ftsX*	hypothetical protein lmo2506
Cell motility			
lmo0682	1.61	*flgG*	flagellar basal body rod protein FlgG
Cell wall/membrane biogenesis			
lmo2520	-1.43	-	hypothetical protein lmo2520
lmo2522	20.24	-	hypothetical protein lmo2522
Coenzyme transport and metabolism			
lmo2572	-5.97	-	hypothetical protein lmo2572
lmo2641	1.47	-	hypothetical protein lmo2641
Defense mechanisms			
lmo1964	-1.62	-	hypothetical protein lmo1964
Energy production and conversion			
lmo1634	-1.65	-	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
lmo1369	-1.56	-	hypothetical protein lmo1369
lmo2103	2.94	*eutD*	phosphotransacetylase
Unknown (Function unknown 10, Not in COGs 18)			
lmo0720	-99.25	-	hypothetical protein lmo0720
lmo0118	-6.50	*lmaA*	antigen A
lmo0189	-3.11	-	hypothetical protein lmo0189
lmo2120	-2.68	-	hypothetical protein lmo2120
lmo2669	-2.40	-	hypothetical protein lmo2669
lmo1501	-1.68	-	hypothetical protein lmo1501

lmo1776	-1.51	-	hypothetical protein lmo1776
lmo0099	1.29	-	hypothetical protein lmo0099
lmo0077	1.85	-	hypothetical protein lmo0077
lmo2522	20.24	-	hypothetical protein lmo2522
lmo0954	-13.72	-	hypothetical protein lmo0954
lmo0123	-6.52	-	hypothetical protein lmo0123
lmo0117	-6.15	*lmaB*	antigen B
lmo0122	-4.29	-	hypothetical protein lmo0122
lmo0124	-3.72	-	hypothetical protein lmo0124
lmo0726	-1.86	-	hypothetical protein lmo0726
lmo0463	-1.66	-	hypothetical protein lmo0463
lmo0174	-1.49	-	hypothetical protein lmo0174
lmo0585	-1.36	-	putative secreted protein
lmo2395	-1.24	-	hypothetical protein lmo2395
lmo2778	-1.24	-	hypothetical protein lmo2778
lmo0141	-1.11	-	hypothetical protein lmo0141
lmo0477	1.34	-	putative secreted protein
lmo1495	1.45	-	hypothetical protein lmo1495
lmo2204	1.48	-	hypothetical protein lmo2204
lmo0684	1.79	-	hypothetical protein lmo0684
lmo1024	2.15	-	hypothetical protein lmo1024
lmo0731	2.48	-	hypothetical protein lmo0731
General function prediction only			
lmo2520	-1.43	-	hypothetical protein lmo2520
lmo1830	1.13	-	short chain dehydrogenase
lmo1399	1.38	-	phosphodiesterase
lmo1845	1.61	-	hypothetical protein lmo1845
lmo2815	1.68	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase
lmo0272	1.87	-	hypothetical protein lmo0272
lmo1230	1.94	-	hypothetical protein lmo1230
lmo1796	20.71	-	hypothetical protein lmo1796
Inorganic ion transport and metabolism			
lmo2104	-1.36	-	hypothetical protein lmo2104
lmo0524	1.38	-	hypothetical protein lmo0524
lmo2785	2.02	*kat*	catalase

Lipid transport and metabolism			
lmo1830	1.13	-	short chain dehydrogenase
lmo1356	1.20	-	hypothetical protein lmo1356
lmo2815	1.68	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase
Nucleotide transport and metabolism			
lmo1884	2.80	-	hypothetical protein lmo1884
lmo1840	3.75	*pyrR*	pyrimidine regulatory protein PyrR
Posttranslational modification, protein turnover, chaperones			
lmo1474	-19.78	*grpE*	heat shock protein GrpE
lmo2415	2.10	-	hypothetical protein lmo2415
Replication, recombination and repair			
lmo1955	-1.40	-	hypothetical protein lmo1955
lmo0005	-1.31	*recF*	recombination protein F
lmo0045	1.98	*ssb*	hypothetical protein lmo0045
lmo0866	2.81	-	hypothetical protein lmo0866
lmo0001	3.14	*dnaA*	chromosomal replication initiation protein
Secondary metabolites biosynthesis, transport and catabolism			
lmo1830	1.13	-	short chain dehydrogenase
lmo2815	1.68	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase
Signal transduction mechanisms			
lmo0357	-1.49	-	hypothetical protein lmo0357
lmo2668	-1.08	-	hypothetical protein lmo2668
lmo0892	1.46	*rsbU*	hypothetical protein lmo0892
Transcription			
lmo1220	-3.47	-	hypothetical protein lmo1220
lmo1788	-1.70	-	hypothetical protein lmo1788
lmo2827	-1.56	-	hypothetical protein lmo2827
lmo1826	-1.43	-	hypothetical protein lmo1826
lmo1263	-1.22	-	hypothetical protein lmo1263
lmo2668	-1.08	-	hypothetical protein lmo2668
lmo2449	1.03	-	hypothetical protein lmo2449
lmo2241	1.05	-	hypothetical protein lmo2241
lmo0909	1.37	-	hypothetical protein lmo0909
lmo0892	1.46	*rsbU*	hypothetical protein lmo0892

lmo2460	4.58	-	hypothetical protein lmo2460
lmo2016	10.95	*cspB*	hypothetical protein lmo2016
lmo1364	40.51	*cspL*	hypothetical protein lmo1364
Translation			
lmo2623	1.80	*rpsQ*	30S ribosomal protein S17
lmo2622	1.98	*rplN*	50S ribosomal protein L14
lmo0486	2.53	*rpmF*	50S ribosomal protein L32
lmo1784	2.63	*rpmI*	50S ribosomal protein L35
lmo0866	2.81	-	hypothetical protein lmo0866
lmo0250	3.22	*rplJ*	50S ribosomal protein L10
lmo1541	3.83	-	hypothetical protein lmo1541
lmo1658	3.97	*rpsB*	30S ribosomal protein S2
lmo1540	4.07	*rpmA*	50S ribosomal protein L27
lmo1797	5.36	*rpsP*	30S ribosomal protein S16
lmo0251	5.48	*rplL*	50S ribosomal protein L7/L12
lmo1542	8.33	*rplU*	50S ribosomal protein L21
lmo1787	8.47	*rplS*	50S ribosomal protein L19
lmo1657	8.77	*tsf*	elongation factor Ts
lmo1785	8.82	*infC*	translation initiation factor IF-3
lmo2047	10.67	*rpmF*	50S ribosomal protein L32
lmo1816	12.03	*rpmB*	50S ribosomal protein L28
lmo1480	16.54	*rpsT*	30S ribosomal protein S20

Table 6.2. Differentially expressed genes of *L. monocytogenes* ATCC 43256 at 60°C for 9 minutes (prolonged heat shock) conditions as compared to 37°C

Gene	Fold-change	Gene designation	Description of product
Amino acid transport and metabolism			
lmo2818	-7.64	-	hypothetical protein lmo2818
lmo0810	-2.13	-	hypothetical protein lmo0810
Carbohydrate transport and metabolism			
lmo2665	-20.52	-	hypothetical protein lmo2665
lmo2818	-7.65	-	hypothetical protein lmo2818
lmo0075	-5.41	-	hypothetical protein lmo0075
lmo0345	-4.69	-	hypothetical protein lmo0345
lmo2337	-3.93	-	hypothetical protein lmo2337
lmo0401	-2.58	-	alpha-mannosidase
lmo2259	-2.16	-	hypothetical protein lmo2259
lmo0875	-1.50	-	hypothetical protein lmo0875
lmo0874	-1.16	-	hypothetical protein lmo0874
lmo2373	1.49	-	hypothetical protein lmo2373
lmo0298	2.32	-	hypothetical protein lmo0298
lmo0184	5.84	-	hypothetical protein lmo0184
Cell wall/membrane biogenesis			
lmo0197	5.17	-	regulatory protein SpoVG
Coenzyme transport and metabolism			
lmo2212	-2.36	*hemE*	uroporphyrinogen decarboxylase
Energy production and conversion			
lmo1178	-1.26	-	hypothetical protein lmo1178
lmo0619	-1.10	-	hypothetical protein lmo0619
lmo1159	-1.08	-	hypothetical protein lmo1159
lmo2528	6.89	*atpC*	F0F1 ATP synthase subunit epsilon
Unknown (Function unknown 10, Not in COGs 14)			
lmo1164	-3.05	-	ATP:cob(I)alamin adenosyltransferase protein PduO
lmo0189	-2.73	-	hypothetical protein lmo0189
lmo1070	-2.26	-	hypothetical protein lmo1070
lmo0518	-1.96	-	hypothetical protein lmo0518

lmo1338	-1.80	-	hypothetical protein lmo1338
lmo1828	-1.37	-	hypothetical protein lmo1828
lmo1501	-1.37	-	hypothetical protein lmo1501
lmo0387	-1.12	-	hypothetical protein lmo0387
lmo2846	1.27	-	hypothetical protein lmo2846
lmo2223	2.43	-	hypothetical protein lmo2223
lmo0117	-2.94	*lmaB*	antigen B
lmo2255	-2.20	-	hypothetical protein lmo2255
lmo0673	-2.04	-	hypothetical protein lmo0673
lmo0729	-1.48	-	hypothetical protein lmo0729
lmo0174	-1.45	-	hypothetical protein lmo0174
lmo2180	-1.34	-	hypothetical protein lmo2180
lmo1123	-1.25	-	hypothetical protein lmo1123
lmo0378	-1.20	-	hypothetical protein lmo0378
lmo1120	-1.19	-	hypothetical protein lmo1120
lmo2320	-1.13	-	hypothetical protein lmo2320
lmo1841	-1.11	-	hypothetical protein lmo1841
lmo1643	-1.10	-	hypothetical protein lmo1643
lmo0147	-1.07	-	hypothetical protein lmo0147
lmo0461	-1.06	-	hypothetical protein lmo0461
General function prediction only			
lmo2818	-7.65	-	hypothetical protein lmo2818
lmo1050	-3.63	-	hypothetical protein lmo1050
lmo0580	-3.05	-	hypothetical protein lmo0580
lmo1164	-3.05	-	ATP:cob(I)alamin adenosyltransferase protein PduO
lmo0344	-2.85	-	short chain dehydrogenase
lmo1669	-1.21	-	hypothetical protein lmo1669
lmo1129	-1.14	-	hypothetical protein lmo1129
lmo1558	-1.08	-	GTPase EngB
lmo1226	1.43	-	hypothetical protein lmo1226
lmo2217	1.75	-	hypothetical protein lmo2217
lmo0908	2.08	-	hypothetical protein lmo0908
lmo1845	2.20	-	hypothetical protein lmo1845
lmo2254	4.95	-	hypothetical protein lmo2254

Inorganic ion transport and metabolism			
lmo2818	-7.65	-	hypothetical protein lmo2818
lmo2380	1.08	-	putative monovalent cation/H+ antiporter subunit C
Intracellular trafficking and secretion			
lmo2214	1.72	-	hypothetical protein lmo2214
Lipid transport and metabolism			
lmo0344	-2.85	-	short chain dehydrogenase
Posttranslational modification, protein turnover, chaperones			
lmo1961	-2.34	-	hypothetical protein lmo1961
lmo2415	1.20	-	hypothetical protein lmo2415
Replication, recombination and repair			
lmo0313	-1.85	-	hypothetical protein lmo0313
lmo0185	-1.33	-	hypothetical protein lmo0185
lmo1669	-1.21	-	hypothetical protein lmo1669
lmo1574	1.70	*dnaE*	hypothetical protein lmo1574
Secondary metabolites biosynthesis, transport and catabolism			
lmo0344	-2.85	-	short chain dehydrogenase
lmo1178	-1.26	-	hypothetical protein lmo1178
lmo1159	-1.08	-	hypothetical protein lmo1159
Signal transduction mechanisms			
lmo0597	-3.04	-	hypothetical protein lmo0597
lmo1580	-1.36	-	hypothetical protein lmo1580
Transcription			
lmo2337	-3.93	-	hypothetical protein lmo2337
lmo2560	-2.66	-	DNA-directed RNA polymerase subunit delta
lmo1562	-2.50	-	transcriptional regulator NrdR
lmo1367	-2.16	-	arginine repressor
lmo0797	-2.12	-	hypothetical protein lmo0797
lmo1996	-2.03	-	hypothetical protein lmo1996
lmo1850	-1.98	-	hypothetical protein lmo1850
lmo0492	-1.95	-	hypothetical protein lmo0492
lmo1130	-1.19	-	hypothetical protein lmo1130
lmo1263	-1.15	-	hypothetical protein lmo1263
lmo2329	-1.08	-	hypothetical protein lmo2329

lmo0294	-1.08	-	hypothetical protein lmo0294
Translation			
lmo1598	-1.49	*tyrS*	tyrosyl-tRNA synthetase
lmo2511	-1.38	-	hypothetical protein lmo2511
lmo2631	6.29	*rplD*	50S ribosomal protein L4
lmo0248	6.36	*rplK*	50S ribosomal protein L11
lmo1540	8.66	*rpmA*	50S ribosomal protein L27
lmo2633	11.14	*rpsJ*	30S ribosomal protein S10
lmo1787	45.19	*rplS*	50S ribosomal protein L19

Table 6.3. Differentially expressed genes of *L. monocytogenes* ATCC 43256 after 48°C for 30 minutes prior to prolonged heat exposure at 60°C for 9 minutes condition as compared to 37°C.

Gene	Fold-change	Gene designation	Description of product
Amino acid transport and metabolism			
lmo0978	-9.81	-	branched-chain amino acid aminotransferase
lmo0448	-7.16	-	hypothetical protein lmo0448
lmo1437	-6.90	-	hypothetical protein lmo1437
lmo1591	-3.37	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase
lmo0491	1.42	*aroD*	3-dehydroquinate dehydratase
lmo1620	2.05	-	dipeptidase PepV
lmo1907	7.07	*dapB*	dihydrodipicolinate reductase
Carbohydrate transport and metabolism			
lmo0348	-10.82	-	hypothetical protein lmo0348
lmo1031	-7.13	-	hypothetical protein lmo1031
lmo1244	-5.57	-	hypothetical protein lmo1244
lmo2743	-1.96	-	putative translaldolase
lmo0776	3.18	-	hypothetical protein lmo0776
Cell cycle control, mitosis and meiosis			
lmo2427	-17.52	-	hypothetical protein lmo2427
Cell motility			
lmo0680	-9.06	*flhA*	flagellar biosynthesis protein FlhA
lmo0676	-7.37	*fliP*	flagellar biosynthesis protein FliP
Cell wall/membrane biogenesis			
lmo1998	-8.64	-	hypothetical protein lmo1998
lmo0446	-7.27	-	hypothetical protein lmo0446
lmo0582	-5.96	*iap*	P60 extracellular protein. invasion associated protein Iap
lmo0855	-4.33	*ddl*	D-alanyl-alanine synthetase A
Coenzyme transport and metabolism			
lmo0978	-9.81	-	branched-chain amino acid aminotransferase
lmo2710	-7.74	-	hypothetical protein lmo2710
lmo0728	-6.06	-	hypothetical protein lmo0728
lmo1045	2.24	-	hypothetical protein lmo1045
Defense mechanisms			
lmo1651	-8.58	-	hypothetical protein lmo1651

lmo2215	-4.67	-	hypothetical protein lmo2215
Energy production and conversion			
lmo0773	-14.47	-	hypothetical protein lmo0773
lmo0383	-11.78	-	hypothetical protein lmo0383
lmo1166	-3.97	-	hypothetical protein lmo1166
General function prediction only			
lmo0454	-31.14	-	hypothetical protein lmo0454
lmo0773	-14.47	-	hypothetical protein lmo0773
lmo2159	-7.90	-	hypothetical protein lmo2159
lmo2565	-7.84	-	hypothetical protein lmo2565
lmo0869	-6.23	-	hypothetical protein lmo0869
lmo2127	-4.34	-	hypothetical protein lmo2127
lmo0420	-4.23	-	hypothetical protein lmo0420
lmo2031	-2.92	-	hypothetical protein lmo2031
lmo2106	4.96	-	hypothetical protein lmo2106
Inorganic ion transport and metabolism-			
lmo0153	-81.11	-	hypothetical protein lmo0153
lmo1778	-7.41	-	hypothetical protein lmo1778
lmo2430	-5.51	-	hypothetical protein lmo2430
lmo1956	-3.76	*fur*	hypothetical protein lmo1956
lmo2105	-2.26	-	hypothetical protein lmo2105
Intracellular trafficking and secretion			
lmo0680	-9.06	*flhA*	flagellar biosynthesis protein FlhA
lmo0676	-7.37	*fliP*	flagellar biosynthesis protein FliP
lmo1274	-2.52	-	hypothetical protein lmo1274
Lipid transport and metabolism			
lmo1317	-19.97	-	1-deoxy-D-xylulose 5-phosphate reductoisomerase
Unknown (Function unknown 4, Not in COGs7)			
lmo1943	-7.38	-	hypothetical protein lmo1943
lmo1971	-7.00	*ulaA*	ascorbate-specific PTS system enzyme IIC
lmo2487	-2.97	-	hypothetical protein lmo2487
lmo0099	-2.68	-	hypothetical protein lmo0099
lmo2804	-10.06	-	hypothetical protein lmo2804
lmo1265	-9.94	-	hypothetical protein lmo1265

lmo2298	-4.52	-	protein gp4
lmo2326	-1.53	-	hypothetical protein lmo2326
lmo0069	2.12	-	hypothetical protein lmo0069
lmo2180	2.51	-	hypothetical protein lmo2180
lmo0349	3.77	-	hypothetical protein lmo0349
Nucleotide transport and metabolism			
lmo0456	-11.20	-	hypothetical protein lmo0456
lmo1885	1.90	-	xanthine phosphoribosyltransferase
Posttranslational modification, protein turnover. Chaperones			
lmo0961	-9.95	-	hypothetical protein lmo0961
lmo2057	-9.62	*ctaB*	protoheme IX farnesyltransferase
lmo0222	3.69	-	hypothetical protein lmo0222
lmo1472	8.16	*dnaJ*	heat shock protein DnaJ
Replication, recombination and repair			
lmo1582	-28.86	-	hypothetical protein lmo1582
lmo1887	-14.37	-	hypothetical protein lmo1887
lmo0588	-7.09	-	hypothetical protein lmo0588
lmo1274	-2.52	-	hypothetical protein lmo1274
lmo1934	32.28	*hup*	hypothetical protein lmo1934
Signal transduction mechanisms			
lmo0582	-5.96	*iap*	P60 extracellular protein. invasion associated protein Iap
lmo2422	-2.22	-	hypothetical protein lmo2422
Transcription			
lmo1829	-4.62	-	hypothetical protein lmo1829
lmo2422	-2.22	-	hypothetical protein lmo2422
lmo1478	1.66	-	hypothetical protein lmo1478
lmo0776	3.18	-	hypothetical protein lmo0776
Translation			
lmo1949	-12.54	-	hypothetical protein lmo1949
lmo1905	-6.32	*cca*	tRNA CCA-pyrophosphorylase
lmo2605	12.37	*rplQ*	50S ribosomal protein L17
lmo2625	18.77	*rplP*	50S ribosomal protein L16
lmo2548	36.78	*rpmE2*	50S ribosomal protein L31 type B

The maximum level of gene induction was 45.2 fold (*rplS*- translation; when the cells were exposed to 60°C for 9 minutes) while the maximum amount of gene-repression was 99.3-fold (function unknown) during heat shock at 60°C for 0 minute. When the cells were exposed to 60°C for 0 minute, 55 genes were up-regulated and 36 genes were down-regulated. When the cells were maintained at 60°C for 9 minutes, 20 genes were up-regulated and 60 genes were down-regulated. When the cells were exposed to 48°C prior to 60°C heat exposure, 17 genes were up-regulated and 54 genes were down-regulated (Figure 6.1).

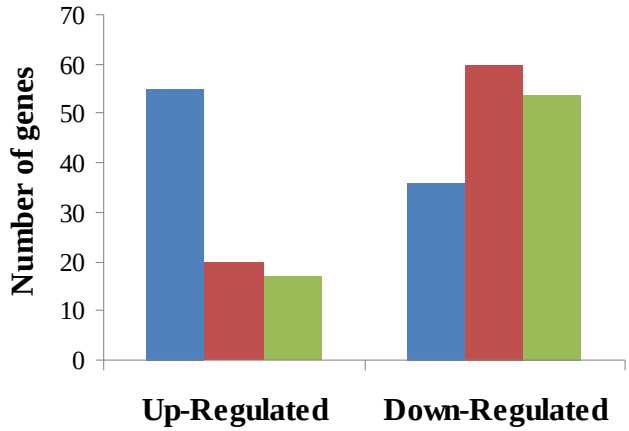


Figure 6.1. Numbers of *L. monocytogenes* ATCC 43256 differentially regulated genes at the different treatment conditions

A majority (60%) of the differentially expressed genes were up-regulated at 60°C for 0 minute as compared to being down-regulated when the cells were exposed to prolonged heat exposure and thermo-tolerance inducing conditions (75% and 76% respectively) (Figure 6.1). In order to understand the differential expression of the genes from a functional stand-point, the differentially expressed genes were grouped into functional gene classes based on the NCBI database (Figure 6.2).

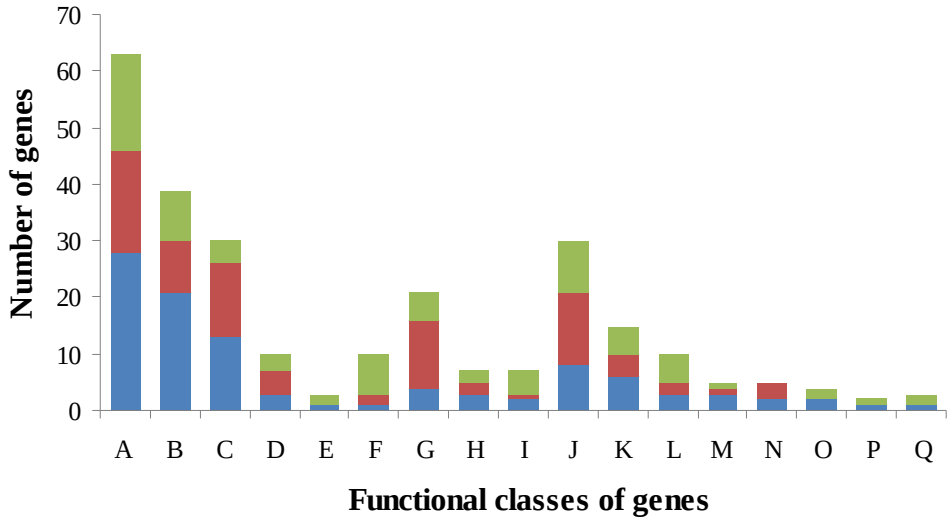


Figure 6.2. Differentially expressed genes (p value ≤0.05) of *L. monocytogenes* ATCC 43256 grouped by functional classification according to the NCBI database (www.ncbi.nlm.nih.gov/COG)

60 °C for 0 minute; 60°C for 9 min; 48°C for 30 min followed by 60°C for 9 min

A Function unknown, Not in COGs; B Translation, Posttranslational modification; C Transcription; D Energy production and conversion; E Defense mechanisms; F Amino acid transport and metabolism; G Carbohydrate transport and metabolism; H Signal transduction mechanisms; I Cell wall/membrane biogenesis; J General function prediction only; K Replication, recombination and repair; L Inorganic ion transport and metabolism; M Lipid transport and metabolism; N Secondary metabolites biosynthesis, transport and catabolism; O Nucleotide transport and metabolism; P Cell cycle control, mitosis and meiosis; Q Cell motility

Those genes which were categorized as "unknown function" and those that were not categorized in the COG database were grouped together. Functions could not be attributed to a large number of the differentially expressed genes. The other classes containing the highest numbers of differentially expressed genes were those that were associated with translation, transcription, amino acid transport, carbohydrate transport and metabolism, general function, replication, recombination and repair, inorganic ion transport, and metabolism respectively. Only 10 genes were commonly expressed across the 3 different temperature treatments (Table 6.4).

There was only one gene, namely lmo2180, (coding for a hypothetical protein with an unknown function) which was commonly expressed between the prolonged heat exposure (60°C for 9 min) condition and the thermotolerance inducing (48°C 30 min followed by 60°C for 9 min) condition. However, the expression pattern of this gene under the two conditions was different. All the other 9 genes had similar expression patterns between the heat shock (60°C for 0 minute) and prolonged heat exposure condition. The two genes (which are involved in translation) were upregulated, and in case of prolonged heat exposure, one of them (lmo1787) was up-regulated 45.2-fold. The genes involved in post-translational modification and associated with general bacterial functions were also up-regulated.

Table 6.4. Commonly differentially expressed genes (up/down regulated) across the 3 treatments (p value ≤ 0.05).

Gene	Fold change			Gene designation	Description of product
	60°C for 0 min	60°C for 9 min	48°C 30 min followed by 60°C 9 min		
Unknown (Function unknown; not in COGs)					
lmo0189	-3.10	-2.73		-	hypothetical protein lmo0189
lmo1501	-1.68	-1.36		-	hypothetical protein lmo1501
lmo0174	-1.48	-1.44		-	hypothetical protein lmo0174
lmo0117	-6.14	-2.94		*lmaB*	antigen B
lmo2180		-1.34	2.50	-	hypothetical protein lmo2180
General function prediction only					
lmo1845	1.60	2.19		-	hypothetical protein lmo1845
Posttranslational modification, protein turnover, chaperones					
lmo2415	2.10	1.19		-	hypothetical protein lmo2415
Transcription					
lmo1263	-1.21	-1.14			hypothetical protein lmo1263
Translation					
lmo1787	8.47	45.18		*rplS*	50S ribosomal protein L19
lmo1540	4.06	8.66		*rpmA*	50S ribosomal protein L27

6.4 Discussion

Based on the microarray analysis, it appears that the expression of this gene under the two temperature conditions was completely different. When the cells were maintained at 60°C for 9 minutes, this gene was down-regulated about 1.3 fold. However, when the cells were exposed to thermo-tolerance inducing conditions (48°C for 30 minutes followed prior to 60°C for 9 minutes), this gene was up-regulated 2.5 fold. Interestingly, when the protein expression patterns in *L. monocytogenes* ATCC 43256 was studied using a proteomic approach, there was no protein that was commonly differentially expressed in the heat shock (60°C for 0 minute) and prolonged heat exposure conditions.

There are studies suggesting a network between transcriptional regulators (Hu et al 2007a, b) in *L. monocytogenes*. As mentioned earlier, only Van der Veen et al. (2007) have studied the heat shock response of *L. monocytogenes* using microarray analysis. However, there are three major differences between this and their studies. The *L. monocytogenes* strains used were different, and it must also be pointed out that in their study they used Brain Heart Infusion Broth as the test matrix. In this study, Luria Broth was the test matrix. In their study, the whole genome expression profiles of the cells that were grown at 37°C (control) and exposed to 48°C were examined using DNA microarrays and the transcription levels were measured over a 40 minutes period after exposure the culture to 48°C. The only similarity between that reported study and the present study is that, in this study 48°C for 30 minutes was one of the temperature treatments. When comparing the results between the two studies, two heat shock associated genes were found to be similar (Table 6.5). The two genes were class I heat shock genes (molecular chaperones), namely lmo1472 (*DnaJ* heat shock protein) and lmo 1474 (*grpE*, heat shock protein). Van der Veen et al. (2007) found both genes to be significantly ($p \leq 0.05$) up-regulated. However, in this study though *DnaJ* was up-regulated in all cases, the statistical significance level (p value) was acceptable ($p \leq 0.05$) only in case of the thermotolerance inducing conditions. The *GrpE* gene was 19.8-fold down-regulated under heat shock (60°C for 0 minute) condition; 1.2-fold down-regulated ($p=0.76$) under thermo-tolerance inducing condition, and 1.5-fold up-regulated ($p=0.57$) under prolonged heat exposure conditions. The class II stress response represent a general stress response mechanism which is regulated by the alternative sigma factor, *sigB*. Two genes namely lmo2511 (*YvyD*) and lmo2572 were similar between this study and that reported by Van der Veen et al. However, the statistical significance of the results in terms of p values was acceptable only under the heat shock conditions (Table 6.5). The response of this gene was different between this study and the previous study. The *ftsX* (lmo2506) gene whose gene product are similar to cell division protein FtsX was down-regulated in Van der Veen's study, but in this study it was up-regulated at heat shock condition (60°C for 0 minute) and thermo-tolerance inducing condition ($p=0.46$). Among the cell wall associated genes, there were 3 genes that were common between the two studies namely, lmo0582 *iap*, lmo1998, and lmo2522. However, only the down-regulation of *iap* was similar between the studies.

Table 6.5. Listing of differentially expressed genes that are in common between this study and that of Van der Veen et al. (2007)

	60°C for 0 min		**60°C 9 min**		**48°C – 60°C**	
	Fold change	**p value**	**Fold change**	**p value**	**Fold change**	**p value**
Group I heat shock						
lmo1474	-19.77	0.00	1.49	0.57	-1.19	0.76
lmo 1472	1.00	0.74	1.00	0.58	8.15	0.02
Class II stress genes (SigB regulated)						
lmo 2572	-5.96	0.01	-2.79	0.27	1.21	0.86
lmo2511	1.00	0.67	-1.38	0.67	1.38	0.42
Cell division						
lmo2506	6.58	0.03	-1.15	0.86	1.29	0.46
Cell wall associated						
lmo 2522	20.23	0.03	1.42	0.35	-3.17	0.28
Cell wall synthesis						
lmo1998	-1.00	0.67	1.07	0.85	-8.63	0.03
Autolysis						
lmo 0582	-1.01	0.47	-1.14	0.86	-5.9	0.02

L. monocytogenes encounters temperature stresses frequently in natural and man-made environments such as during food processing. In this study, microarrays were used to investigate the whole genome expression profiles (i.e., transcriptome) of *L. monocytogenes* in response to heat shock, prolonged heat shock, and thermo-tolerance inducing conditions. These temperature conditions were chosen to best represent some scenarios that this pathogen may encounter during food processing especially in the RTE foods. These results indicate that the pathogen responds to different temperature conditions quite distinctly, and that the transcriptome has very distinct patterns under the three different temperature conditions. While 55 genes were up-regulated at 60°C for 0 minute, only 17 genes were up-regulated when the cells were pre-exposed to 48°C for 30 minutes prior to 60°C exposure (Fig. 6.1). Similar differential gene expression patterns were observed among those genes that were down-regulated under the three different temperature treatments. Thus, temperature not only influences the survival of the pathogen but can also significantly alter the functionality of those surviving the temperature stress. Thus, it should not be surprising that the surviving population is quite distinct in its physiology, and ultimately its

virulence. As mentioned earlier, this study employed LB as the test matrix while the only other reported study similar to this study was performed using BHI broth as the test matrix (Van der Veen et al., 2007). Though the experimental objectives between the two studies were different, the use of 48°C was common between the two studies (the exposure times were, however, different). The difference in the test matrix could be responsible for the differences in the results. Previous studies have also shown that the food matrix (poultry meat as compared to ground beef meat) in which a pathogen is present can alter its functionality (Widmer et al., 2007; Soni et al., 2008).

The probes used in the microarrays employed in this study are grouped or classifed by the functional classification of genes (COG) per the NCBI database. Given the lack of knowledge of the functional genomics of *L. monocytogenes*, many of the gene loci has not been attributed to a specific function or their function is currently unknown. Further research is needed to better understand the functional genomics of the organism so that the differential expression of the genes can be attributed to a specific change in function or phenotype.

CHAPTER 7
DIFFERENTIAL EXPRESSION OF PROTEINS IN *LISTERIA MONOCYTOGENES* UNDER THERMO-TOLERANCE INDUCING, HEAT SHOCK, AND PROLONGED HEAT SHOCK CONDITIONS

7.1 Introduction

Listeria monocytogenes is a facultative intracellular bacterial pathogen with a variety of genetically encoded survival mechanisms to withstand environmental stresses such as heat, cold, salt, and acidic conditions. Given its unique adaptability to survive longer in adverse environmental conditions compared to other non-spore forming bacteria, this pathogen is a serious concern especially within the Ready–To-Eat (RTE) food industry. *L. monocytogenes* is considered an adulterant by the U.S. Food and Drug Administration, and hence from a regulatory perspective, there is a zero tolerance for this organism in foods. However, this organism is ubiquitous and can be found in soil, water, food processing equipment, and other environments. In a survey of about 31,700 RTE foods in two U.S. states, Gombas and co-workers detected this pathogen in about 1.8% of the samples tested (Gombas et al., 2003).

The optimal growth temperature for *L. monocytogenes* is between 30°C and 37°C and any temperature above this optimal range is expected to exert a stress (Petran and Zottola, 1989). Studies have shown that *L. monocytogenes* when heat shocked can induce thermo-tolerance (Bunning et al., 1986, 1990; Pagàn et al., 1997, Farber and Brown, 1990, Linton et al., 1990). Pagàn et al. (1997) have reported a 7-fold increase in thermo-tolerance of *L. monocytogenes* when the cells were exposed to 45°C for 180 minutes. The extent of exposure to temperature above optimal levels, and the matrix in which the cells are exposed are reported to influence the extent of the observed thermo-tolerance (Linton et al., 1990; Sergelidis and Abrahim, 2009). The influence of incubation temperature on thermo-tolerance before and after a heat shock at 58°C in *L. monocytogenes,* and the relationship between cell morphology and thermo-tolerance have also been reported (Rowan and Anderson, 1998; Jørgensen et al., 1996). The heat shock response of *L. monocytogenes* using DNA microarray analysis was recently reported (Hu et al., 2007a,b; Van der Veen, et al., 2007). Transcriptome analysis do not directly correlate with protein expression due to a variety of reasons including, the rapid degradation of abundant mRNA species and post-translational modifications. There are reports detailing the changes in global protein expression under heat shock (49°C for 15 min) conditions using 2D gel electrophoresis analysis (Phan-Thanh and Gormon, 1995).

The induction of 32 proteins was observed in preparative 2-DE gels. However, no biological significance of these proteins could be derived since these heat stress-induced proteins were not identified using mass spectrometry. In *L. monocytogenes*, 2D gel electrophoresis techniques has been previously used to identify the stress proteins involved in salt adaptation (Esvan et al., 2000; Duché et al., 2002a), acid adaptation (Phan-Thanh and Mahouin, 1999), cold adaptation (Phan-Thanh and Gormon, 1995; Bayles et al., 1996) and alkaline stress adaptation (Giotis et al., 2008). There are recent proteomic studies of the different *L. monocytogenes* serotypes (Donaldson et al., 2009; Dumas et al., 2008). Donaldson et al. (2009) studied the proteome of serotype 1/2a strain EGD and the serotype 4b strain F2365 when grown at 37°C. They report differential expression patterns of proteins among the two different serotypes. Similarly, Dumas et al. (2008) report that the extracellular and intracellular protein expression profiles were correlated to the genetic and serological differences amongst the *L. monocytogenes* strains.

The underlying hypothesis of this study was that prior exposure to thermo-tolerance inducing conditions (for example 48°C for 30 minutes) will elicit a unique set of proteins at 60°C as compared to directly exposing the cells to 60°C. Identifying the proteins that are selectively expressed under thermo-tolerance and heat shock conditions can provide an insight into the underlying stress adaptation mechanisms, as well as help to identify potential markers for heat shocked or thermally adapted cells. In order to study the differential expression of proteins under thermo-tolerance and heat shock conditions, a 2D gel electrophoresis-MALDI-TOF-based proteomic study was performed. There has been no previous study where differentially expressed heat shock proteins were identified using MALDI-TOF or other mass spectrometric analysis. In this study the proteins that are differentially expressed under heat stress were identified by comparing the proteome of *L. moncytogenes* under optimal temperature (37°C), heat shock (60°C for 0 minute), prolonged heat shock (60°C for 9 minutes), and thermo-tolerance inducing (48°C for 30 minutes prior to exposure to 60°C for 9 minutes).

7.2 Materials and Methods

7.2.1 Bacterial strain, Growth conditions, and Temperature Stress conditions

Luria Broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey) was used as the culture medium to grow *L. monocytogenes* ATCC 43256 strain (serotype 4b) in this study. ATCC strain was used because the ancestory of the strain was known. This strain was originally isolated from a Mexican-style cheese. The strain was initially grown to OD_{600} 0.5 (~ 10^8 CFU/mL) at 37°C. This strain was not pre-screened for heat resistance because choosing a heat-resistant strain for this

study would have biased the results. Aliquots were placed into multiple 1.5 mL microfuge tubes for the different heat treatments. The heat treatments were performed in a calibrated water-bath (Boekel Grant ORS200, PA. USA) using temperature probes that facilitated temperature monitoring within the sample contained in the microfuge tubes. Preliminary trials were performed to understand the optimal sample volumes and choice of sample container to ensure uniform heating and temperature control. The proteome under four different experimental conditions were studied namely: (i) 37°C (control) , (ii) heat shock at 60°C for 0 minute, (iii) prolonged heat shock at 60°C for 9 min, and (iv) thermo-tolerance inducing treatment at 48°C for 30 minutes followed by exposure to 60°C for 9 min. For the heat shock (60°C for 0 minute) conditions, the cells were exposed to the temperature for no more than 2 seconds which was the minimum time required to remove the samples from the water bath.

7.2.2 Protein extraction

The four experimental treatments were performed in triplicates. The protein fractions were extracted from each sample independently using B-Per® bacterial protein extraction reagent (Pierce, Rockford, IL) in combination with sonication (4x2 min on ice, at power setting 20) in an ultrasonic cell disruptor (Microson, Misonic, Farmingdale, NY). The Ready Prep^{TM} 2-D cleanup-kit (Bio-Rad, Hercules, CA) was used to reduce the ionic contaminants in the protein preparation. The purified proteins were dissolved in 100 µL of rehydration buffer (9.5M urea, 2% w/v, CHAPS, 18mM 1,4-dithio-DL-threitol (DTT), 0.5% ampholytes and one tablet of protease inhibitor (Roche Diagnostic, Mannheim, Germany), and insoluble proteins and cell debris were removed by centrifugation.

7.2.3 Two-dimensional gel electrophoresis

Protein concentrations were measured using the Bradford protein assay kit (Pierce). Two hundred microliters of Bradford reagents were mixed with 1.7 µL of the protein samples and absorbance was measured at 580 nm. Preliminary studies using immobilized pH gradient (IPG) strips in the range of pH 4-7 indicated that the majority of the soluble proteins were detectable in the pH 5.0–6.0 range. Hence for subsequent analysis, IPG strips in the pH 4.7–5.9 range were employed. Protein loads of 35 µg in 125 µL of rehydration buffer and 800 µg in 250 µL of rehydration buffer were used for 7-cm and 11-cm IPG strips (pH 4.7–5.9) respectively. The IPG strips were rehydrated overnight in a rehydration tray (Bio-Rad). For the first dimensional electrophoresis, the isoelectric focusing of 7-cm IPG strips were conducted at a linear voltage gradient with 24,000 final V-h (500 V in 15 minutes, 4000 V in 2 hours, 24,000V in 2.5 hours, and

holding at constant 500 V-h) using Protean IEF cell (Bio-Rad). For 11-cm IPG strips, the isoelectric focusing was conducted in linear mode to achieve 56,000 final V-h (Amersham Bioscience, Piscataway, NJ). After the required V-h was applied, the IPG strips were incubated for 15 minutes in equilibration buffer I (6M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) (w=v), 50mM Tris- HCl (pH 8.8), 1.5% dithiotheritol, and approximately 5 mg of bromophenol blue) followed by 15 minutes in equilibration buffer II (6M urea, 30% glycerol, 2% SDS (w=v), 50mM Tris- HCl (pH 8.8), 3% iodoacetamide, and bromophenol blue as color indicator). Second dimension electrophoresis was performed at 125 constant volts using 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel. The protein spots were visualized using Sypro Ruby fluorescence stain (Molecular Probe, Eugene) for the 7-cm IPG strip gel. GelCode™ blue stain reagent (Pierce, Rockford, IL) was used to stain the 11-cm IPG strip gel and these gels were subsequently used for spot excisions.

7.2.4 Data Analysis

The extracted proteins from the three experimental replicates of each treatment (37°C, 60°C for 0 minute, 60°C for 9 min, 48°C for 30 min followed by 60°C for 9 min) were run on 7-cm IPG strips, resulting in three independent gels for each treatment. Additionally, two dimensional (2D) gels were run from the 11-cm IPG strips to assist in spot excision. The gels were scanned using Gel Doc (Bio-Rad, Hercules, CA) and the raw images were analyzed using PDQuest™ 2-D gel analysis software version-8 (Bio-Rad, Hercules, CA). To analyze the proteome under thermo-tolerance and heat shock conditions, the protein expression pattern observed at 37°C (i) was compared against the other treatments namely: (ii) 60°C for 0 minute, (iii) the 60°C for 9 min treatment, and (iv) the 48°C for 30 min followed by 60°C for 9 min treatment. Only those spots with spot intensities exhibiting ±1.5-fold change difference were picked for MALDI-TOF identification.

7.2.5 Identification of proteins by Mass Spectrometry (MALDI TOF/TOF)

The protein spots of interest were manually excised (approximately 1 mm in size) and placed in a 96-well microtiter plate for in-gel digestion. Proteolytic digestion was performed overnight using trypsin (20 µg/mL) at 37°C. The digested samples were spotted onto matrix-assisted laser desorption/ionization (MALDI) targets using a ProMS™ (Genomic Solutions, Ann Arbor, MI) robot capable of sample clean-up prior to MS analysis. All MALDI-MS experiments were performed using a model 4700 Proteomics Analyzer MALDI-time of flight (TOF)/TOF (Applied Biosystems, Foster City, CA) instrument. The mass spectrometry (MS) data for each gel spot was acquired using the reflection detector and 20 tandem MS spectra per spot were acquired. All MS

and MS/MS data were queried against the Swiss-Prot protein sequence database using GPS Explorer software (Applied Biosystems, Foster City, CA). The parameters for database searching were as follows: taxonomy, *Listeria monocytogenes*, database, Swiss Prot, enzyme, trypsin; maximum missed cleavages, 1; variable modifications, oxidation (Met); peptide tolerance, 85 ppm; and MS/MS fragment tolerance, 0.3 Da. To verify the reproducibility of the MALDI-MS data, 10 spots were reanalyzed.

7.3 Results

The reproducibility of the individual 2D gels was evaluated by employing the correlation coefficient analysis of the PDQuest™ software (Applied Biosystems, Foster City, CA). The average correlation coefficient among individual gel comparisons of different treatment groups was >0.7, suggesting high similarity in the spotting patterns (Bland et al., 2006). When spots exhibiting significant changes on intensity (±1.5-fold) were identified using MALDI-MS, they resulted in protein score confidence interval (CI) between 95% and 100% (Choe et al., 2005). Only those proteins that provided a CI of >95% was assigned an identity. Proteins with a CI <95% were deemed “not identified”. Thus, a high stringency cut-off was used to ensure that the protein identifications were accurate. Using lower CI would have resulted in more protein identifications. However, the reliability of those identifications would be suspect. The proteins that were differentially expressed at heat shock (60°C for 0 minute) (Figure 7.1), 60°C for 9 min (prolonged heat shock) (Figure 7.2), and 48°C for 30 min followed by 60°C for 9 min (thermo-tolerance inducing) (Figure 7.3) conditions as compared to optimal temperature conditions (37°C) (Figure 7.4) are shown in Table 7.1. Out of 47 proteins that were differentially expressed under heat shock, prolonged heat shock and thermo-tolerance inducing conditions, 24 proteins could not be identified by the techniques employed in this study.

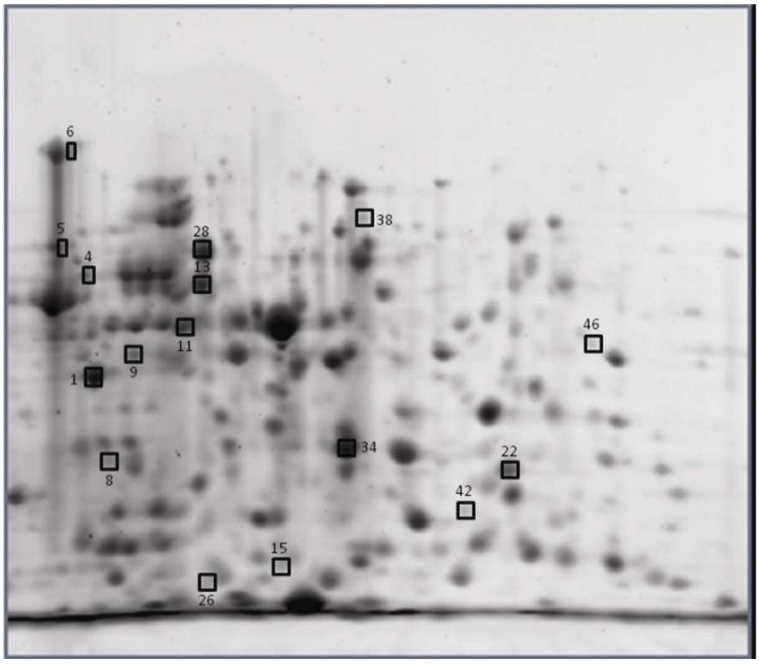


Figure 7.1. 2D gel electrophoresis of soluble proteins fraction of *L. monocytogenes* during heat shock i.e., 60°C for 0 minute heat treatment

The boxes represent proteins that were chosen for MALDI-TOF based identification.

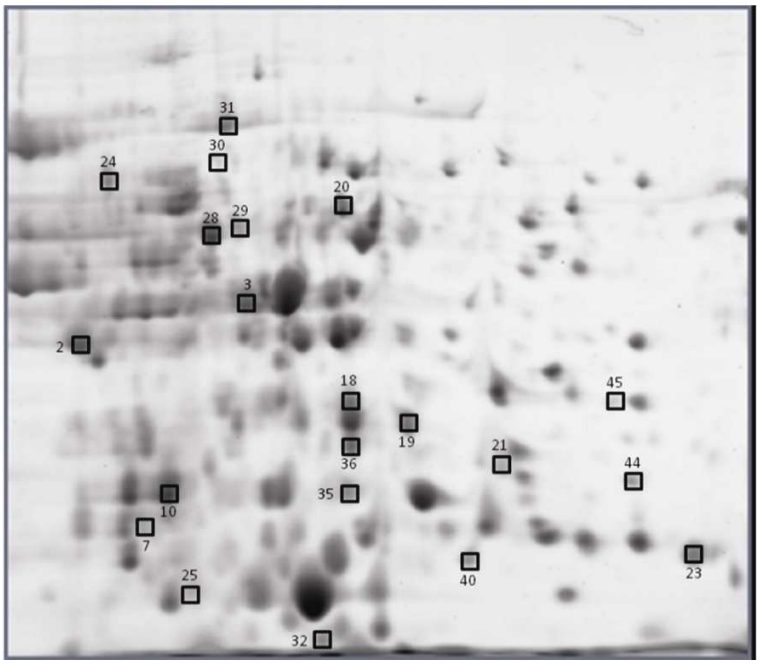


Figure 7.2. 2D gel electrophoresis of soluble proteins fraction of *L. monocytogenes* during prolonged heat treatment i.e., 60°C for 9 minutes

The boxes represent proteins that were chosen for MALDI-TOF based identification.

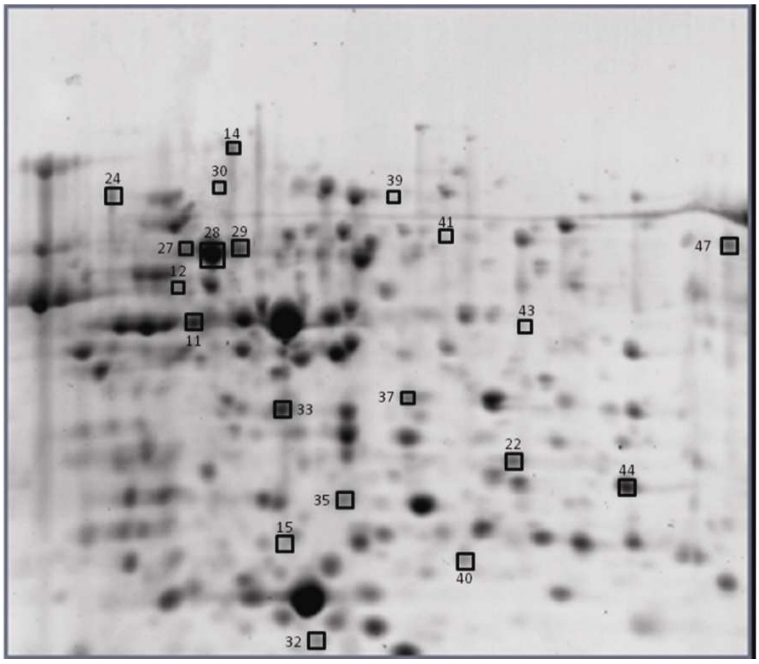


Figure 7.3. 2D gel electrophoresis of the soluble protein fraction in *L. monocytogenes* under thermo-tolerance inducing conditions i.e., exposure to 48°C for 30 minutes followed by 60 °C for 9 minutes

The boxes represent proteins that were chosen for MALDI-TOF based identification.

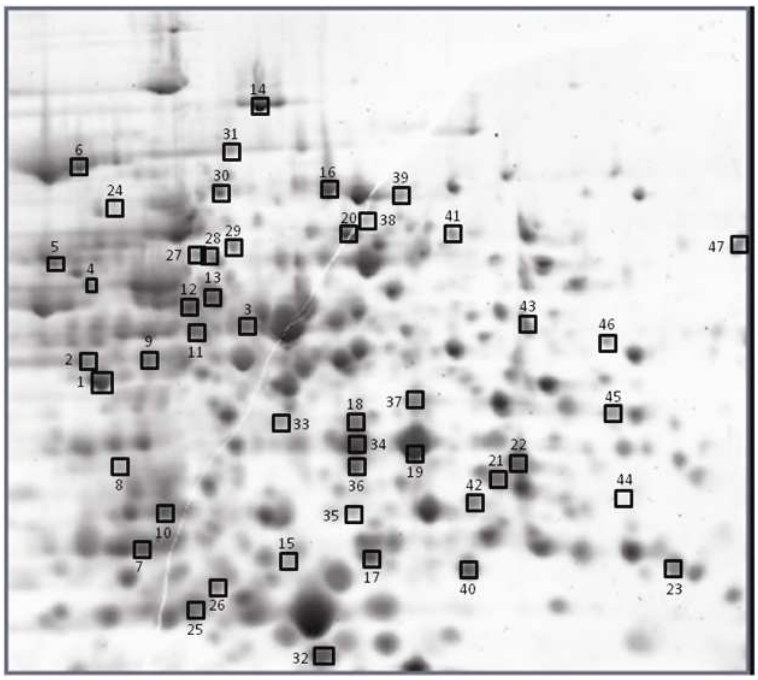


Figure 7.4. 2Dgel electrophoresis of the soluble protein fraction of *L. monocytogenes* at 37°C (control temperature)

The boxes represent proteins that were chosen for MALDI-TOF based identification.

A total of 47 proteins were differentially expressed (>± 1.5-fold difference) across the 4 experimental treatments. Eighteen proteins were differentially expressed (6 proteins up-regulated and 12 proteins down-regulated) when cells were exposed to 60°C for 0 minute as compared to 37°C. Out of the 18 proteins that were differentially expressed, 6 proteins were unidentified. One of the proteins, Chaperonin GroEL was down-regulated by as much as 4 fold. When the cells were exposed to 60°C for 9 minutes as compared to 37°C, 21 proteins (12 were up-regulated and 9 were down-regulated) were differentially expressed. One of the unidentified proteins (Spot ID # 24, MW of 60.9) was up-regulated by as much as 6.8-fold. Under thermo-tolerance-inducing conditions, out of the 20 proteins were differentially expressed, 10 proteins were up-regulated and 10 were down-regulated as compared to proteins expressed at 37°C. One unidentified protein with a (spot ID #44, MW of 29.2) was up-regulated by as much as 12-fold. There was only one protein (spot ID #28, MW of 50) that was up-regulated across all the three temperature treatments. No proteins were commonly expressed in cells exposed to heat shock at 60°C for 0 minute as compared to prolonged heat shock (60°C for 9 min). There was only one protein tagatose 1,6-diphosphate aldolase (lmo 0539) that was up-regulated during both thermo-tolerance and heat shock. Eight different proteins (ranging in MW between 19 kDa and 62.5 kDa) were commonly expressed at both the thermo-tolerance inducing, and the prolonged heat stress conditions. However, none of these proteins were identifiable.

7.4 Discussion

Heat shocking of *L. monocytogenes* at 60°C for 0 minute results in the down- regulation of 67% (12 out of 18) of the proteins as compared to 37°C (Table 7.1). Heat shocking at 60°C for 9 minutes results in the down-regulation of 43% (9 out of 21) proteins. When the cells were pre-exposed to 48°C for 30 minutes prior to heat shocking at 60°C for 9 minutes, only 50% (10 out of 20) of the proteins were shown to be down-regulated. The identifiable proteins that were down-regulated at 60°C for 0 minute include PdhB (Pyruvate dehydrogenase -E1 β sub-unit) (lmo 1053), GroEL, the chaperone heat shock protein (lmo 2068), and ManA (Mannose-6-β-lactamase family protein) (lmo 1577). The PdhABCD complex is involved in cellular metabolism and energy stress, specifically the transformation of pyruvate to acetyl-coenzyme A, a key component providing an energy source and a metabolite precursor (Folio et al., 2004; Chaturongakul and Boor, 2006). Salotra et al. (1995) and Duché et al. (2002a) they found that DnaK, PdhA, CysK, Gap were over-expressed after cold and salt stress. The down-regulation of GroEL at 60°C for 0 minute is also surprising given that this is a heat shock protein associated with the class 1 stress response gene

regulator, HrcA (Nair et al., 2000; Rosen and Ron, 2002; Hu et al., 2007a). Van der Veen et al. (2007) using microarray analysis reported the up-regulation of the *groEL* gene at 48°C over 40 minutes. Hu et al. (2007a) reported >5-fold down-regulation of *groEL* gene in a *hrcA* deletion mutant of a serotype 1/2a strain suggesting that the *groESL* operon is downregulated by HrcA. The up-regulated proteins include DnaN (involved in DNA replication) and Pgk (involved in metabolism). The induction of DnaN as a function of stress during the onset of stationary phase in *E. coli* has been previously reported (Villarroya et al., 1998). DnaK is a heat shock protein and its function to stabilize cellular proteins was previously reported (Hebraud and Guzzo, 2000). Only one of the unidentified proteins (spot ID # 28, MW ~ 50 KDa) was differentially expressed at 60°C for 0 min and 60°C for 9 min (as compared to 37°C) (Table 7.1).

It is evident that maintaining *Listeria monocytogenes* at 60°C for 9 minutes induces a unique set of 21 proteins as compared to 37°C. Importantly, these cells express the lipoprotein, TcsA, (which is similar to the CD4+ T cell-stimulating antigen) and Gap (type 1 glyceraldehydes 3-phosphage dehydrogenase). Previous studies show TcsA which mediates T cell activation is expressed in *L. monocytogenes* (Cabanes et al., 2002; Sanderson et al., 1995). The CD4+ T cell stimulating antigen was up-regulated 3.6-fold under our experimental conditions suggesting that this could serve as a marker for pathogens under temperature stressed conditions. Hu et al., (2007a) suggest that HrcA controls glyceraldehyde 3-phosphate dehyrdogenase (Gap) since they observed a 1.5-fold down-regulation of this gene in a hrcA deletion mutant. In this study the Gap protein was differentially expressed over 3-fold only when the cells were maintained at 60°C for 9 minutes. Schaumburg et al. (2004) have suggested that this surface protein may serve as a receptor for human plasminogen on the bacterial cell surface. One other protein that was expressed 3.3-fold was Gap. Tasara and Stephan (2007) report that even though this protein is stably expressed across multiple *L. monocytogenes* strains it is prone to wide variations under stress adaptations implying the suitability as a marker for stressed cells. Geng et al. (2006) have shown that the 43 kDa protein reacted positively with polyclonal antibodies for the detection of stressed *L. monocytogenes* cells. AtpA was up-regulated 2.3-fold. Rouquette et al. (1998) have reported that ATPAse is a general stress protein that aids in the disruption of the vacuolar membrane and the intracellular survival of *Listeria* sp. AtpA has been previously reported to be related to acid stress in *Streptococcus mutans* (Len et al., 2004).

One of the proteins was up-regulated 6.8-fold under prolonged heat shock conditions; however, it was not identifiable. As mentioned previously only 1 protein (which was unidentified) was up-regulated across all three temperature treatments. Only three proteins (ID # 27, ID # 33, ID # 47) were differentially expressed at thermo-tolerance inducing conditions but not at the heat shock

conditions. Unfortunately, none of these proteins were identifiable. These proteins could serve as valuable markers for screening those *L. monocytogenes* cells that have been exposed to thermotolerant conditions (such as 48°C for 30 minutes). Phan-Thanh and Gormon (1995, 1997) examined stress proteins expression in *L. monocytogenes* under different conditions such as heat, cold shock, acid, SDS, and ethanol. They report the induction of about 32 different heat shock protein with some of them up-regulated by as much as 50-fold under heat shock. However, there were no common stress proteins that were expressed as a result of the different stressors even though some of them were induced under two or three stress conditions. Sokolovic et al. (1990) examined listeriolysin production in *L. monocytogenes* serogroup1/2a under 48°C for 30 minute heat shock. They report that at least 5 different heat shock proteins were differentially coinduced with listeriolysin production in the strains that they studied. In comparison with the microarray study which was performed using the same experimental treatments (Chapter VI), only 1 protein (lmo 0539) was common between the heat shock and prolonged heat shock conditions. However, the gene associated with that particular protein was not found to be significantly differentially expressed using the microarray analysis. When comparing the prolonged heat exposure and thermo-tolerance inducing conditions, eight proteins (all of them are unidentifiable) were differentially expressed.

7.4.1 Conclusions

This study was aimed at understanding those soluble proteins that are differentially expressed at thermo-tolerance inducing, heat shock, and prolonged heat shock conditions. The results indicate that *L. monocytogenes* is capable of uniquely modulating its proteome to survive sub-lethal temperatures. Thus, during food processing sub-lethal temperature exposure could induce the over expression of unique heat stress-related proteins such as TcsA, Gap, and AtpA. These candidate marker proteins may have value in lateral flow ELISA assays for identifying *L. monocytogenes* cells that have been heat stressed. The use of predictive modeling to link microarray results with proteomic results can lead to a systems biology approach of understanding the ecology of *L. monocytogenes*.

Table 7. 1. Proteins that were found to be differentially expressed in *L. monocytogenes* under thermo-tolerance inducing, heat shock and prolonged heat shock conditions.

Spot #	Protein Characteristics		Protein Identity	Lmo #	Protein Designation	Differential Protein Expression at different temperature treatments		
	Isoelectric Point (pI) Observed/Theoretical	Molecular Weight (kDa) Observed/Theoretical				60°C-0 min heat shock	60°C - 9 min	48°C 30 min and 60°C for 9 min
	Replication, recombination and repair							
4	5.01/4.7	46.0/42.4	DNA polymerase III subunit beta	lmo0002	DnaN	2.9		
	Translation							
21	5.62/5.5	30.5/27.9	Methionine aminopeptidase	lmo1709	Map		-3.6	
	Posttranslational modification, protein turnover, chaperones							
6	4.99/4.7	70.0/57.4	Chaperonin GroEL	lmo2068	GroEL	-4.2		
	Energy production and conversion							
1	5.05/4.8	36.0/35.3	Pyruvate dehydrogenase (E1 beta subunit)	lmo1053	PdhB	-2.0		
20	5.4/5.3	54.7/55.1	H+-transporting ATP synthase chain alpha	lmo2531	AtpA		2.3	
	Amino acid transport and metabolism							
5	4.94/4.7	48.0/45.0	Aminopeptidase	lmo1711		3.1		

10	5.13/5.0	28.0/24.8	Tetrahydrodipicolinate succinylase	lmo1011		2.3	
12	5.18/5.0	44.0/39.0	Aminopeptidase P	lmo1354			-5.6
19	5.5/5.4	32.0/32.2	Cysteine synthase A	lmo0223	CysK	-4.8	
	Carbohydrate transport and metabolism						
3	5.25/5,1	42.0/36.3	Glyceraldehyde-3-phosphate dehydrogenase, type I	lmo2459	Gap	3.3	
9	5.11/4.8	37.0/35.4	Mannose-6-phosphate isomerase, class I	lmo2110	ManA	-3.2	
11	5.18/4.9	42.0/37.7	Tagatose 1,6-diphosphate aldolase	lmo0539		2.8	2.6
13	5.2/4.9	46.0/42.1	Phosphoglycerate kinase	lmo2458	Pgk	2.2	
14	5.26/5.1	86.0/71.8	Transketolase	lmo1305	Tkt		-5
16	5.37/5.1	64.0/56.1	Phosphoglyceromutase	lmo2456	Pgm	-2.9	
17	5.44/5.3	25.0/23.8	Ribulose-phosphate 3-epimerase	lmo1818		-2.1	
	Coenzyme transport and metabolism						
18	5.41/5.3	34.0/31.7	Pyridoxine biosynthesis protein	lmo2101		-2.3	
	Lipid transport and metabolism						
22	5.66/5.7	31.5/26.7	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	lmo1086	IspD	-2.2	-2.3
	Nucleotide transport and metabolism						
23	5.83/5.5	24.6/22.9	Uracil phosphoribosyltransferase	lmo2538	Upp	2.8	
	General function prediction only						
2	5.0/5.0	37.0/38.4/	CD4+ T cell-stimulating antigen, lipoprotein	lmo1388	TcsA	3.6	
8	5.07/4.8	31.0/24.6	Metallo-beta-lactamase family protein	lmo1577		-2.4	

15	5.33/5.0	25.0/20.6	Acetyltransferase	lmo0664		-2.6		2.2
	Function unknown							
7	5.1/4.8	25.0/27.3	Phospho-beta-glucosidase	lmo0191	BglA		-6.7	
	Not identified							
24	5.06/nd	60.9/ nd	not identified				6.8	5.0
25	5.18/ nd	22.0/ nd	not identified				-4.8	
26	5.2/ Nd	23.3/ Nd	not identified			-2.0		
27	5.18/ Nd	50.0/ Nd	not identified					5.0
28	5.2/ Nd	50.0/ Nd	not identified			3.7	2.4	7,1
29	5.23/ Nd	51.5/ Nd	not identified				2.6	4.4
30	5.21/ Nd	62.5/ Nd	not identified				-2.1	-2.1
31	5.23/ Nd	71.0/ Nd	not identified				2.1	
32	5.36/ Nd	19.0/ Nd	not identified				-2.6	-4.3
33	5.3/ Nd	34.0/ Nd	not identified					2.2
34	5.41/ Nd	32.5/ Nd	not identified			-3.1		
35	5.41/ Nd	28.0/ Nd/	not identified				4.6	3.6
36	5.41/ Nd	31.0/ Nd	not identified				2.3	
37	5.5/ Nd	35.5/ Nd	not identified					-3.2
38	5.43/ Nd	56.2/ Nd	not identified			2.3		
39	5.48/ Nd	61.7/ Nd/	not identified					-2.5
40	5.78/ Nd	24.1/ Nd	not identified				-2.0	-2.2
41	5.65/ Nd	54.7/ Nd	not identified					-2
42	5.6/ Nd	29.0/ Nd/	not identified			-3.7		

43	5.66/ Nd	42.7/ Nd	not identified			-2.7
44	5.78/ Nd	29.2/ Nd	not identified		4.3	12.1
45	Nd/5.77	Nd/34.5	not identified		-3.1	
46	5.76/ Nd	41.0/ Nd	not identified	-2.3		
47	5.9/ Nd	51.5/ Nd	not identified			2.8

Nd= not determined since the protein was unidentifiable; ID[a] refers to spots shown in Fig. 7.1,7.2,7.3 and 7.4.; [b]Isoelectric point (pI) and molecular weight (MW) obtained in the experiment (practical); [c] The theoretical pI and MW were obtained from the Agronne Gelbank database of *L. monocytogenes* EGDe (serotype ½a); (http://gelbank.anl.gov/cgi-bin/proteomes/peptide_search_MWPI.pl); [d]Fold difference in the protein expression of the different treatments compared to control

NEW SCIENTIFIC RESULTS

1. Combination of Modified Atmosphere Packaging (MAP) with 2 kGy of (γ) gamma irradiation reduces the natural bioburden of fresh alfalfa and radish sprouts to low levels, and improves microbiological safety and shelf-life. The D_{10}-value of *L. monocytogenes* 4ab strain on alfalfa sprouts was found to be between 0.46 kGy (when packaged in a gas mixture containing 3-5% oxygen, 10-15% carbon dioxide and balanced with nitrogen) and 0.58 kGy when packaged in an oxygen-carbon dioxide-nitrogen (2%-4%-94%) gas mixture.
2. Enhanced heat resistance was demonstrated in two *L. monocytogenes* strains (4ab and ATCC 43256) when they were exposed to sub-lethal heat conditions. The heat resistance of the strains was higher when exposed to 48°C for 30 minutes followed by 60°C treatment conditions as compared to a direct 60°C heat treatment. The D-value increased from 3.03 min to 6.72 min in case of *L. monocytogenes* 4ab, and from 3.74 minutes to 4.55 minutes in *L. monocytogenes* ATCC 43256.
3. This is the first report demonstrating that *L. monocytogenes* differentially expresses genes when exposed to sub-lethal heat conditions as compared to a direct heat stress. When the transcriptome of *L. monocytogenes* under optimal temperature (37°C) was used as base-line, heat shock (60°C for 0 minute) caused the up-regulation of 55 genes. Twenty (20) genes were up-regulated at prolonged heat shock (60°C for 9 minutes) conditions, and 17 genes were up-regulated under thermo-tolerance inducing (48°C for 30 minutes prior to exposure to 60°C for 9 minutes) conditions.
4. This study has demonstrated for the first time that sub-lethal temperature (48°C for 30 min) induces the over expression of unique heat stress-related proteins in *L. monocytogenes* ATCC 43256. Eighteen different proteins were differentially expressed at 60°C for 0 minute, 21 proteins were differentially expressed when the cells were exposed to 60°C for 9 minutes, and 20 proteins were differentially expressed when cells were initially exposed to 48°C for 30 minutes prior to 60°C for 9 minutes.
5. My dissertation has demonstrated that *L. monocytogenes* ATCC 43256 is capable of entering into a viable but non-culturable (VBNC) condition when exposed to sub-lethal temperature conditions. When the cells were exposed to 48°C for 30 minutes prior to exposure for 9 minutes at 60°C, only 1% was viable (per culture methods) as compared to greater than 99% viability (based on a microscopic viability assay).

ÚJ TUDOMÁNYOS EREDMÉNYEK

1. A módosított atmoszférás csomagolás (MAP) és a 2 kGy gamma (γ) sugárzás kombinált alkalmazása csökkenti a friss lucerna és retek csíra természetes mikrobiotáját, növeli a mikrobiológiai biztonságot és hosszabb eltarthatóságot eredményez. A *Listeria monocytogenes* 4ab D-értéke lucernacsírán 0.46 kGy 3-5% O_2, 10-15% CO_2, N_2–vel kiegyenlített légtérben, míg 2% O_2, 4% CO_2, 94% N_2 gázösszetétel mellett 0.58 kGy.
2. Megállapítottam, hogy a szubletális hőhatás megnövekedett hőrezisztenciát okoz a vizsgált *L. monocytogenes* törzsek (4ab és ATCC 43256) esetében. A 48°C, 30 perces enyhe hőstressz megnövekedett rezisztenciát eredményezett a 60°C-os hőkezeléssel szemben *L. monocytogenes* 4ab és *L. monocytogenes* ATCC 43256 esetében. A *L. monocytogenes* 4ab D_{60}-értéke 3,03 percről 6,72 percre, a *L. monocytogenes* ATCC 43256 D_{60}-értéke 3,74 percről 4,55 percre nőtt.
3. Vizsgálataim alapján bizonyítást nyert, hogy a szubletális hőstressz a direkt hőhatáshoz képest *Listeria monocytogenes*ben eltérő génexpressziót okoz. Az optimális hőmérsékleti körülményekhez (37°C) viszonyítva hősokk hatására (60°C, 0 perc) a *L. monocytogenes* transzkriptomban 55 gén aktiválása volt megfigyelhető. A 60°C-os 9 perces hőkezelés 20 gén, a 48°C 30 perces enyhe hőstressz alkalmazása a 60°C, 9 perces hőkezelést megelőzően 17 gén aktiválását eredményezte.
4. Elsőként igazoltam, hogy a szubletális hőmérséklet (48°C, 30 perc) egyedi, hőstresszel kapcsolatba hozható fehérjék túlexpresszióját indukálta *L. monocytogenes* ATCC 43256 törzsben. Tizennyolc fehérje eltérően expresszálódott 60°C, 0 perc hatására; a 60°C-os 9 perces hőkezelés 21 fehérje, a 48°C 30 perces enyhe hőstressz alkalmazása a 60°C, 9 perces hőkezelést megelőzően 20 fehérje eltérő expresszióját eredményezte.
5. Megállapítottam, hogy a *L. monocytogenes* ATCC 43256 szubletális hőmérsékleti feltételek mellett képes élő, de nem tenyészthető (VBNC) formává alakulni. A 60°C, 9 perces hőkezelést megelőző 48°C 30 perces enyhe hőstressz hatására tenyésztéses módszerrel a sejtek csupán 1 %-a, míg mikroszkópos módszerrel több, mint 99%-a bizonyult életképesnek.

OVERALL CONCLUSIONS AND SUGGESTIONS

Overall, my studies show that the combination of low-dose γ (gamma) irradiation with Modified Atmosphere Packaging (MAP) and refrigerated storage on alfalfa and radish sprouts can improve the microbiological safety and shelf-life of these high value commodities. Combination of MAP with 2 kGy γ irradiation was able to reduce the natural microbiota to low levels and no further population increase was detected for up to 10 days storage at 5°C. The study also demonstrated that the impedimetric method can be used to detect and enumerate *L. monocytogenes* within 24 hours if they are present in numbers higher than 10^3CFU/g. For the presence-absence test, however, an enrichment step and confirmation is needed. Further studies are necessary to optimize MAP conditions packaged produce to prevent the re-growth of surviving pathogens such as *L. monocytogenes* during storage.

My studies show that *L. monocytogenes* exhibits unique physiological, genomic, and proteomic responses when exposed to sub-lethal temperatures. Specifically, my results demonstrate that *Listeria monocytogenes* 4 ab (a meat isolate) exhibits enhanced heat resistance (D-values) at 60°C after exposure to sub-lethal temperature conditions. If the cells were pre-exposed to different temperatures, heat injury in the cells was observed. This observation was based on the difference in plate counts obtained using Tryptic Soy Agar and Tryptic Soy Agar amended with NaCl. Based on the observation that increasing exposure to heat stresses can result in bacterial injury, it is recommended that culture media for enumeration of *L. monocytogenes* in heat processed foods be carefully chosen to avoid obtaining misleadingly low counts.

I performed laboratory studies to understand the genomic, and proteomic responses using a virulent strain of *L. monocytogenes* (ATCC 43256). These studies were performed during my research stay at Texas A&M University in the United States. Increased thermo-tolerance was observed when the cells of this virulent strain was exposed to 48°C for 30 minutes followed by 60°C heat treatment in broth media. The D-value increased from 5.01 minutes to 5.65 minutes. This increase in the D-value was in agreement with my previous studies using the (avirulent) *L. monocytogenes* 4ab strain and with a number of other published studies. Not only did the D-value increase after the sub-lethal heat exposure, the surviving cells converted into what could be termed as “viable but non-culturable” (VBNC). I verified the existence of such cells using a combination of fluorescent microscopy, digital image analysis, and fluorescence spectroscopy. These experimental approaches verified the existence of the majority (>99%) of the cells being in the VBNC state. The VBNC state can be problematic for the food industry when culture-based methods are used to detect and enumerate these organisms in heat-processed foods.

My results suggest that the current culture-plate based detection methods could be significantly underestimating the actual numbers of viable *Listeria* spp. in heat processed foods. More research is thus needed to better understand the VBNC state of *L. monocytogenes* in foods and develop appropriate detection tools to detect such cells.

Whole genome microarray studies were performed to identify the differentially expressed genes produced during heat stress. The transcriptome of *L. monocytogenes* ATCC 43256 under optimal temperature (37°C) conditions was compared to the transcriptome occuring at heat shock (60°C for 0 minute) conditions, prolonged heat shock (60°C for 9 minutes), and thermo-tolerance inducing (48°C for 30 minutes prior to exposure to 60°C for 9 minutes) conditions. When the cells were exposed to 60°C for 0 minute heat shock conditions, 91 out of 6347 genes were differentially expressed. When the cells were maintained at 60°C for 9 minutes (prolonged heat shock), 80 out of 6347 were differentially expressed. When the cells were pre-exposed to 48°C for 30 minutes prior to prolonged heat exposure (ie.. thermo-tolerance inducing conditions), 71 genes out of 6347 genes were differentially expressed. The highest numbers of differentially expressed genes were those that were associated with translation, transcription, amino acid transport, carbohydrate transport and metabolism, general function, replication, recombination and repair, inorganic ion transport, and metabolism respectively. Ten (10) genes were commonly expressed across the 3 treatments. The results highlights the fact that this pathogen responds to different temperature conditions very differently. Additional research is needed to better understand the functional genomics of the organism so that the differential expression of the genes during food processing can be attributed to a specific change in function, virulence or phenotype.

Two dimensional gel electrophoresis coupled with MALDI-TOF were performed to understand the proteomic response of *L. monocytogenes* when exposed to sub-lethal heat shock. Sub-lethal heat exposure was found to induce heat shock proteins in *L. monocytogenes* ATCC 43256. The proteome of this organism under four different experimental conditions was investigated. The experimental treatments included 37°C (control), heat shock conditions (60°C for 0 minute), prolonged heat shock conditions (60°C for 9 minutes) and thermo-tolerance inducing conditions (48°C for 30 minutes followed by exposure to 60°C for 9 minutes) conditions. A total of 47 different proteins were differentially expressed across the 4 experimental treatments. Eighteen (18) proteins were differentially expressed when cells were exposed to 60°C for 0 minute as compared to the control. Out of these 18 proteins, 6 proteins were unidentifiable. When cells were exposed to 60°C for 9 minutes, 21 proteins were differentially expressed. One of these proteins was up-regulated 6.8 times. Under the thermo-tolerance inducing conditions, 20 proteins were differentially expressed. One of them showed a 12-fold up-regulation. There was no commonly expressed protein

in the heat shocked cells at 60°C for 0 minute compared to the cells that were exposed to prolonged heat shock conditions. However, 8 different proteins were commonly expressed at both thermo-tolerance inducing conditions and prolonged heat shock conditions. However, none of them were identifiable. The protein DnaN, a previously identified stress protein was up-regulated almost 3-fold at 60°C for 0 minute. Similarly, TcsA, a lipoprotein (CD4+ T cell stimulating antigen) and Gap (glyceraldehydes-3-phosphate-dehydrogenase) were selectively expressed under prolonged heat shock conditions suggesting their potential as a candidate marker proteins targets for identifying temperature-stressed *L. monocytogenes* cells. The results indicate that the protein expression profiles of the organism are different when the cells are exposed to different temperature conditions.

Overall, the experimental results support my original hypothesis that *Listeria monocytogenes* elicits unique physiological, genomic and proteomic responses as part of its stress adaptive response to sub-lethal temperature stress.

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http://gelbank.anl.gov/cgi-bin/proteomes/peptide_search_MWPI.pl

APPENDIX A

RNA ISOLATION FROM *LISTERIA* SPECIES FOR MICROARRAY ANALYSIS

Cell Collection and RNeasy kit total RNA isolation from *Listeria* Species for Microarray

*****Modeled after Mark Kazmierczak's original RNA Prep with RNeasy Midi Kit Instructions under Microarray Protocols

**All spectrophotometer readings will be made with the Beckman spectrophotometer.

The minimum volume for disposable cuvettes is ~600 μL, but use 800 μL to account for bubbles.

Cell Collection

1. After cells have reached desired growth phase or after exposure to stress, harvest cells by adding 2 volumes of RNAprotect to 1 volume of culture. Vortex and incubate at RT for 5 minutes.
2. Pellet each treatment in Beckman centrifuge at 5000 rpm for 5 minutes.
3. Remove supernatant by dumping, then with vacuum pump. Store pellets at -80˚C.

RNA Isolation

4. Prepare:
   - 50 mg/mL lysozyme in TE Buffer. Keep on ice.
   - 70% EtOH with DEPC-H_2O, aliquotted into 15 mL conical tubes. 4 mL per 1X
   - Buffer RLT + β-mercaptoethanol (βME). Per 1X, 4 mL RLT + 40 μL βME
5. Thaw pellets at RT (Room Temperature) . Resuspend each pellet w/ 200 μL of lysozyme-TE. Pipet to mix.
6. Vortex tubes for 10s intervals, every 2 minutes for 10 minutes total at RT to keep cells in suspension. Put tubes on ice**.**
7. In chemical hood, add 3.9 mL of RLT-βME to each tube. Vortex ~10 sec, then ice tube.
8. Sonication treatment on ice. Program 4 → three 30-second sonication intervals (output 18-24 watts).

9. After sonication, spin 5000 rpm, 5 min in Beckman centrifuge to pellet any cell debris.
10. Dump supernatants into tubes containing 4 mL of 70% EtOH. Vortex to mix.
11. In hood, transfer cell lysate to RNeasy Midi column (4 mL/column capacity).
    Spin for 3 min in Sorvall. (3200 rpm, 25°C). Discard flow-through.

    Repeat with remaining volume, spinning 5 minutes this time. Discard flow-through.

Go to QIAGEN RNAprotect® Bacteria Reagent Handbook page 34, and follow the procedure from point 2. to 6. and then come back to point 18. and you can do the further steps based on this protocol.

12. Pipet 2.0 mL of Buffer RW1 into the RNeasy column. Let stand for 5 minutes at RT.
    Spin for 5 min in Sorvall (3200 rpm, 25°C) to wash. Discard flow-through.
13. Pipet 2.5 mL of RPE into each column. Spin 3 minutes at 3200 rpm. Discard flow-through.
14. Pipet 2.5 mL of RPE into each column. Spin 5 minutes at 3200 rpm. Discard flow-through.
15. Place columns into a NEW collection tubes.
16. Pipet 200 µL RNase-free H_2O onto membrane. Let stand 1 min. Spin 3200 rpm, 3 min.
17. Repeat step 21. Transfer RNA into chilled 1.5 mL microcentrifuge tubes.
18. Quantify each RNA eluate separately w/ Nanodrop. Keep RNA on ICE. (5 µL)
19. Precipitate RNA from the final aqueous state (RNA in water) using 1/10 volume 3M Sodium Acetate and 2.5 volumes of ice-cold EtOH (100%). Store at -80°C.

DNase treatment and phenol:chloroform extractions

20. Pellet RNA for 30 minutes at 12,000 rpm in centrifuge at 4˚C. Decant supernatant.
21. Wash pellet w/ 800 µL of 70% cold EtOH. Vortex briefly. Centrifuge 12,000 rpm, 20 min.
22. Decant supernatant. Quick spin, then further remove EtOH by pipetting (long pipet tips).
    spin it again, then further remove EtOH by pipetting
    keep the tubes open to dry the pellet
    add 100 µL RNase-free H_2O, resuspend the pellet → take samples to NanoDrop
    keep the tubes on ice
23. (Resuspend 1st pellet in 175 µL RNase-free H_2O. Use that volume to resuspend any other tubes of the same RNA.)
24. Add: 10 µL RNasin (add immediately after resuspending in H_2O)
    25 µL 10X DNase buffer
    40 µL RQ1 DNase

After adding each reagent, pipet briefly then flick. TAP to mix. DO NOT VORTEX!

25. Incubate at 1 hour at 37˚C.
26. Transfer to screw-cap tube.
27. Mix w/ 0.5 volume phenol and 0.5 volume chloroform. Vortex 30 seconds.
    *If using phase-lock gel tubes for subsequent steps, only need to centrifuge <u>for 5 min</u>. Remember to centrifuge phase-lock gel tubes briefly prior to use.
28. Centrifuge for 20 minutes at 12,000 rpm.
29. Remove aqueous layer to new screw-cap tube.
30. Repeat steps 32-34 on aqueous layer.
31. Mix aqueous layer with equal volume of chloroform. Vortex 30 seconds.
32. Centrifuge 20 minutes at 12,000 rpm.
33. Precipitate aqueous layer with 1/10 volume 3M Sodium Acetate and 2.5 volumes of ice-cold EtOH (100%). Store at -80˚C.

Prior to microarray experiment, RNA integrity must be checked via gel electrophoresis and purity assessed by A260 and A280 readings with the Nanodrop.

APPENDIX B

PROTEIN ANALYSIS USING TWO DIMENSIONAL GEL ELECTROPHORESIS

Extraction of soluble protein fraction from *Listeria monocytogenes* cells

- Take 1.5 mL of logarithmic phase grown culture of *L. monocytogenes* cells in micro centrifuge tube.
- Centrifuge the tube at maximum rpm for 3 min.
- Discard the supernatant carefully
- Again add 1.5 mL of logarithmic phase grown culture of *Listeria monocytogenes* cells in the same centrifuge tubes
- Centrifuge the tube at maximum rpm for 3 min.
- Discard the supernatant carefully.
- To ensure that supernatant is completely removed, re centrifuge the pellet for 1 min and remove leftover supernatant
- Total volume of culture centrifuges for one tube will be 3.0 mL

Cell lysis using B-per cell lysis buffer.

- Add 300 microliter of B-per cell lysis buffer in the micro-centrifuge tube containing cell pellet.
- Vortex the content vigorously for 5 min.
- Centrifuge the tube at maximum rpm for 3 min
- Collect the supernatant and place into new micro-centrifuge tube. This content represents soluble protein fraction.
- Discard the pellet

Protein clean-up using ReadyPrep 2-D cleanup kit

The purpose of using commercially available ReadyPrep 2-D cleanup kit was to enhance the suitability of prepared protein samples for 2DGE by reducing the ionic contamination. Moreover, this procedure also results in concentration of diluted samples thereby allowing higher protein loads during gel electrophoresis. The detailed procedure used in the sample clean-up is as follow.

- Three hundred microliter of prepared soluble protein extracts were mixed with 300 µL precipitating agent 1 in eppendorf tube followed by incubation on ice for 15 min.

- At the end of incubation period, 300 µL precipitating agent 2 was added to the mixture of protein and precipitating agent 1 followed by proper mixing using vortexer and centrifugation of the eppendorf tubes at maximum speed for 5 min. The supernatants were removed immediately and tubes were placed again for 1 min centrifugation. At the end of second centrifugation any remaining supernatants were removed.
- At this time point 40 µL of wash reagent 1 on top of the pellet and tubes were centrifuge at maximum speed (>12,000 x g) for 5 min followed by discarding supernatant. After removing supernatant, 25 µL of ultrapure water was added on the top of the pellet and tubes were vortexed for 30 sec (prepared protein pellet does not dissolve in water).
- Protein pellets were further treated by adding 1 mL of prechilled (20°C) wash reagent 2, 5 µL of wash 2 additive, and tubes were vortexed for 1 min. The tubes were finally incubated at -20°C for 30 min with intermediate vortexing at every 10 min. After the incubation period, tubes were centrifuged at maximum speed for 5 min and supernatant was discarded. Tubes were placed again for 1 min centrifugation to discard any remaining supernatants and formed protein pellets were air dried for 5 min.

Resuspending protein pellet in sample/rehydration buffer.

- The protein pellets achieved after step 3 were used in this step.
- Take 100 microliter of sample/ rehydration buffer and re-suspend protein pellet into it.
- Vortex very very vigorously for complete solubalization.
- Centrifuge the content at maximum speed for 1 min.
- Take out the supernatant portion in fresh tube and discard the tube containing cell debris.
- The supernatant portion is ready to use for Bradford bioassay

Bradford Bioassay.

Bradford bioassay for the protein quantification was performed in a 96-well microtiter plate. The Bradford assay is a colorimetric assay in which acidic solution of Bradford dye reagent (Coomassie) shifts from 465 nm to 595 nm when binding with protein occurs. Increased absorbance at 595 nm is proportional to the amount of bound dye and thereby to the amount of protein present in the sample. The detailed procedure of the Bradford bioassay for the protein quantification is described below.

In order to determine the protein concentration of unknown samples, standard protein curve was prepared using known protein concentration. Protein standards to prepare a standard curve were prepared from known concentration of bovine serum albumin (BSA). Protein standards were prepared by dissolving different concentrations of BSA (0 to 2000 µg/mL) in deionized water. Five

microliter of this prepared protein standards were mixed with 200 µL of the Bradford dye reagent and absorbance reading (580 nm) were reported. Obtained absorbance reading were plotted against concentration of the protein standards using excel spread sheet and equation of line was derived. To measure the concentration of unknown protein samples, known volume of protein sample was mixed with 200 µL of the Bradford dye reagent and absorbances reading were obtained. Later the protein concentrations from the unknown protein samples were determined by solving the line equation using A580 nm of the unknown protein samples.

Rehydration of the strips.

- Take 35 µg of protein load (based on Bradford assay concentration determination) and male the total volume of 125 µL using rehydration buffer (note, above mentioned concentration of 35 µg protein is for 7 cm IPG strips. When bigger gels are run for spot excision purpose the protein load should be ~800 µg).
- Carefully overlay prepared 125 µL content in rehydration tray and put the IPG strips carefully on this content. Overlay 1 mL of mineral oil to prevent drying out of strips.

Focusing on IPG strips in first dimension.

- Remove the rehydrated strips from rehydration tray and remove mineral oil by vertically tapping on soft paper.
- Place small pieces of filter paper on focusing tray electrodes to avoid burning of IPG strips during 1st dimension voltage application.
- Place strips on focusing tray, add 1 mL of mineral oil on strip and perform 1st dimensional separation for desired voltage (S1: 500 V-h in 15 min, S2: 4000 V-h in 2 h, S3: 24,000 V-h for 2.5 h, and holding at 500 V-h).
- Take out the IPG strips and try to remove mineral oil by vertically tapping on tissue paper).

Equilibration of IPG strips.

- Add 150 mg of DTT in the 1st equilibration buffer tube (10 mL content) and dissolve it completely
- Place the IPG strips in tube and shake it for 15 min on shaker.
- Add 300 mg of iodoacetamide in the 2nd equilibration buffer tube (10 mL content) and dissolve it completely
- Take the IPG strips from 1st tube and place it on 2nd tube. Shake it for another 15 minutes.

SDS-PAGE for 2nd dimensional separation.

- Use 10% SDS-PAGE gel to cast in 1 mm spacer glass assembly (should not have comb). Add methanol on the top of gel layer (immediately after pouring). Addition of methanol helps to get straight top layer of gel.
- Insert the IPG strip carefully (should touch perfectly to the top layer of SDS-PAGE gel) and run for the second dimension on SDS- PAGE
- Condition for the SDS-PAGE: at 125 volts for 1 h.

Staining.

- After running the SDS-PAGE, take out the gels in clean tray and wash it with water for 3 times (5 minutes each).
- Replace the water with fixing solution and fix the gel for 15 min.
- Add staining reagent (Sypro Ruby) and keep on shaker for overnight.
- Remove the staining reagent with washing solution and keep on shaker for 15 min.
- Gels are ready for imaging.

Recipes

Sample/rehydration buffer.

9.5M urea	5.7 g
2% CHAPS	200 mg
18mM DTT	0.05 g
0.5% ampholytes	50 microliter

1 tablet of protease inhibitor

Few grains of bromophenol blue

Make volume up to 10 mL and dissolve completely

Equilibration buffer.

50mM Tris- HCL, pH 8.8	6.7 mL of 1.5M
6M urea	72 g
2% SDS	4 g

Few grains of bromophenol blue

Make volume up to 200 mL and dissolve completely

Running buffer.

25mM Tris-base	3 g
192mM glycines	14.4 g
0.1% SDS	1 g

Make volume up to 1 L and dissolve completely

Fixing solution.

50% methanol	500 mL
7% glacial acetic acid	70 mL

Make volume up to 1 L using DI water.

Washing solution.

10% methanol	100 mL
7% glacial acetic acid	70 mL

Make volume up to 1 L using DI water.

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