1-1-2012

Stress Responses and Attachment of Listeria Monocytogenes in Food

Dongryeoul Bae

STRESS RESPONSES AND ATTACHMENT OF *LISTERIA MONOCYTOGENES* IN FOOD

By

Dongryeoul Bae

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

MAY 2012

STRESS RESPONSES AND ATTACHMENT OF *LISTERIA MONOCYTOGENES* IN FOOD

By

Dongryeoul Bae

Approved:

Chinling Wang Associate Professor of Veterinary Medicine (Director of Dissertation)	Larry A. Hanson Professor of Veterinary Medicine (Committee Member)
Janet R. Donaldson Assistant Professor of Biological Sciences (Committee Member)	Ramakrishna Nannapaneni Assistant Professor of Food Science, Nutrition and Health Promotion (Committee Member)
Keun Seok Seo Assistant Professor of Veterinary Medicine (Committee Member)	Larry A. Hanson Professor and Graduate Coordinator of Veterinary Medicine
Kent H. Hoblet Dean of the College of Veterinary Medicine	

Name: Dongryeoul Bae

Date of Degree: May 11, 2012

Institution: Mississippi State University

Major Field: Veterinary Medical Science

Major Professor: Chinling Wang

Title of Study: STRESS RESPONSES AND ATTACHMENT OF *LISTERIA MONOCYTOGENES* IN FOOD

Pages in Study: 107

Candidate for Degree of Doctor of Philosophy

The rates of the US hospitalization and mortality caused by *Listeria monocytogenes* was estimated to be the highest of 31 food-borne pathogens including *Salmonella*, *Escherichia coli*, *Campylobacter*, and *Clostridium*. This pathogen has an ability to survive under extreme conditions widely found in the natural environment and food. Among 13 serovars *L. monocytogenes* serovar 4b, 1/2a and 1/2b are mainly associated with human listeriosis outbreaks. The deadliest outbreaks of human listeriosis and massive product recalls in multi-states were associated with ready-to-eat (RTE) food products such as mexican-style cheese, turkey deli meat, cabbage, and cantaloupes contaminated with the bacterium. Thus, contamination of food products with *L. monocytogenes* is a major concern for the food industry, regulatory agents and consumers.

This study used oligonucleotide probe-based DNA array, quantitative real time RT-PCR, gene manipulation, biochemical assays, and electron microscopy techniques to better understand the molecular mechanisms of *L. monocytogenes* under stress conditions on various food matrices. The transcriptome profiles of *L. monocytogenes* via microarray

analysis and quantitative PCR identified genes that are involved in adaptation, attachment, or survival and growth of the pathogen under a stress condition on a food matrix. The mechanistic and functional studies further characterized the biological properties of *L. monocytogenes* in various RTE food products. We showed that specific genes involved in energy metabolism, biosynthesis of proteins, and cellular processes to affect listerial growth or adaptation to a RTE meat matrix were changed with no associated changes in virulence factor expression. We also reported that the effects of salt stress on the expression of genes involved in PTS and its related metabolic enzymes in *L. monocytogenes*. In addition, a novel gene involved in attachment to RTE vegetables and fruits was discovered.

The concern about the prevalence of *L. monocytogenes* in RTE food has been escalated by recent food-borne outbreaks, suggesting that the prevention of human listeriosis become the top priority for the food industry. Data from these studies help us to better understand the survival, growth and contamination of the bacterium under different conditions. The information will help the development of prevention strategies in RTE meat products, vegetables and fruits.

DEDICATION

I dedicate this dissertation to parents for making me see the world, supporting, and encouraging me to achieve my goal, to my wife (Mija Ha) for her patience and understanding, and especially to my daughter (Madison) and son (Nathan) for giving me love and pleasure.

ACKNOWLEDGEMENTS

I would like to acknowledge my major professor, Dr. Chinling Wang for her direction and support during my Ph.D work, Dr. Keun Seok Seo for his advice and academic direction, and Drs. Larry A. Hanson, Ramakrishna Nannapaneni, and Janet R. Donaldson, my committee members, for their valuable review and suggestions.

I would also like to thank Ting Zhang and Connie Liu as lab colleagues and Dr. Lesya Pinchuk for their assistance and generosity.

Finally, I would like to share my thankful acknowledgements to Dr. Frank Austin for my manuscript editing and suggestions and Amanda Lawrence for her contribution in FESEM imaging and manuscript editing, as well as our graduate students, staff, and professors for their friendship and assistance.

You made me stand here. I sincerely appreciate your help.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Listeria monocytogenes is an opportunistic and zoonotic bacterial pathogen that can cause serious clinical illness (listeriosis) in susceptible individuals such as newborn, pregnant women, and immunocompromised persons including patients with transplants, cancer, AIDS, diabetes, or alcoholism. Survey and epidemiology studies have demonstrated that most cases of human listeriosis have been linked to the consumption of ready-to-eat (RTE) products contaminated with *L. monocytogenes*. Therefore, the prevention of human listeriosis caused by the ingestion of RTE foods is a great challenge for the food industry. However, the pathogen, found in the natural environments and food, has an ability to survive under harsh conditions such as high acidity, low temperature, high osmolarity, and high hydrostatic pressure.

Studies for *L. monocytogenes* contamination or decontamination in RTE foods have been widely conducted; however, the effectiveness of the prevention strategies is rather limited. One of the primary reasons is that we lack of understanding of the components that contribute to bacterial survival, growth and attachment to food. Accordingly, the purpose of my dissertation research was to indentify genes and characterize their functions associated with attachment, virulence, or growth of *L. monocytogenes*. Data from this study may provide a better understanding of the molecular mechanisms of *L. monocytogenes* attachment, colonization, adaptation, or

survival/growth in a specific environmental stress condition or in various food matrices including turkey deli meat, leafy vegetables and fruit.

The specific objectives of my dissertation study include:

1) To examine the survival or growth of *L. monocytogenes* on RTE deli meat, leafy vegetables, or under salt stress condition.
2) To determine target genes involved in survival, adaptation, colonization, or growth of *L. monocytogenes* under environmental stress conditions.
3) To characterize the functions of the proteins encoded by the target genes involved in the survival, adaptation, colonization, or growth of *L. monocytogenes*.

The conventional and recent developed molecular biological techniques including oligonucleotide probe-based DNA array, quantitative real-time RT-PCR, gene manipulation, biochemical assays, electron microscopy techniques, *in silico* analysis, and statistical data analyses were used to address the research questions and hypotheses.

CHAPTER II

LITERATURE REVIEW

2.1 A high risk assessment of *Listeria monocytogenes* in ready-to-eat (RTE) food products

Most cases of human listeriosis have been associated with the ingestion of RTE products contaminated with *L. monocytogenes*. Deli meats, dairy products, and frankfurters in various RTE foods have been mostly linked to human listeriosis (Table 2.1). Among 13 serovars *L. monocytogenes* serovars 4b, 1/2a, and 1/2b are mainly linked to the listeriosis outbreak (*25*). According to serotype, tendency to cause death is shown to be different. The most common cause of death by serotype 4b is central nervous system (CNS) infection, whereas 1/2a and 1/2b cause bacteremia and maternal-neonatal diseases in many listeriosis cases, respectively (*26, 63*). The cost estimates for diseases caused by *L. monocytogenes* and others including *Escherichia. coli* O157:H7, non-O157 Shiga-toxin producing *E. coli, Campylobactor*, and *Samonella* approach 2.3 and 4.6 billion dollars per year in the United States, respectively (*8*). Major outbreaks of listeriosis including a recent deadliest outbreak in the United States are shown in Table 2.1. Most of the major outbreaks were also caused by the ingestion of dairy products, poultry deli meat and frankfurters, and vegetables and fruit contaminated with serotype 4b strains. Increased consumption of RTE foods will increase the prevalence of *L. monocytogenes* in RTE foods. Therefore, the contamination or cross-contamination of RTE foods with *L. monocytogenes* during harvesting, processing, distribution, and storage has been a major concern for the food industry. Accordingly, efforts (from farms

or processing facilities to retail stores) to reduce or protect the contamination of the pathogen have been triggered. In addition, extensive studies on the protection or decontamination of *L. monocytogenes* in RTE foods have been conducted to elucidate the antimicrobial effects by chemical or physical methods using a variety of sanitizers, preservatives, antimicrobial natural compounds, heat, pH, hydrostatic pressure, water activity, etc.

2.2 RTE food products associated with major human listeriosis

2.2.1 Dairy products

A recent study (*16*) showed a high prevalence of *L. monocytogenes* in healthy ruminant animals including sheep (14.2%), beef cattle (30.6%), and dairy cattle (46.3%). In addition, strain diversity of the pathogen was also indentified. *L. monocytogenes* serotype 4b (84.2%), 1/2a (13.2%), 1/2b (1.8%), and 4c (0.9%) were identified from 114 isolates from the domestic animals. The results have suggested that dairy cattle are an important reservoir for *L. monocytogenes* 4b strain, which is highly prevalent in human listeriosis (*16*). In general, milk or its derived products are generally recognized as safe food from listeriosis through proper pasteurization processes, such as ultra high temperature (UHT), high temperature short time (HTST), or low temperature long time (LTLT) and an aseptic packaging process in dairy plants (*34*). However, post-pasteurization contamination in processed dairy products can occur (*37*). In addition, unpasteurized or abnormally treated milk (i.e. inadequate pasteurization process, contaminated processing equipment, etc.) and raw milk cheese product have been linked to human listeriosis (*30*, *37*, http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5740a1.htm). In 1984, *Listeria-*

contaminated Mexican-style soft cheese, an unripened type and consumed fresh, was linked to one of the deadliest outbreaks of the human listeriosis. Consequently, *L. monocytogenes* was highlighted as a food-borne pathogen for the first time. Therefore, fluid milk or its products, which are produced without pasteurizing, can be a potential vehicle for the transmission of *L. monocytogenes* into humans.

2.2.2 Frankfurters and sliced poultry deli meat

The US Food and Drug Administration (FDA) and the Food Safety Inspection Service (FSIS) posed that poultry deli meats and frankfurters with dairy products have the greatest risk for listeriosis in comparison to other food products (*8, 22*). Several major outbreaks of human listeriosis associated with the ingestion of frankfurters (1998-1999) and RTE turkey deli meat (1998 and 2002) have occurred in the US. The majority of isolates from patients and contaminated products belong to serotype 4b. The National Alliance for Food Safety and Security (NAFSS), which consists of twenty-five research universities, extensively studied the prevalence and level of *L. monocytogenes* in samples of prepackaged deli meat, sliced deli meat, and the retail-sliced deli meat from four states including California, Maryland, Georgia, and Tennessee. The NAFSS designed a risk assessment model for *L. monocytogenes* in RTE meat and poultry deli meat by evaluating four stages (retail occurrence, growth, consumption, and dose-response) (*22*). Data indicated that the prevalence of *L. monocytogenes* was higher in retail-sliced deli meat (1.39%) than prepackaged deli meats (0.17%), and the incidence of the pathogen was relatively higher in poultry (0.65%), beef (1.28%) and pork (0.87%) deli meats than other deli meat types. The growth rate of *L. monocytogenes* in products prepackaged with antimicrobial growth inhibitor (organic acid, fatty acids, sodium nitrite, or smoke) was

predicted to decrease, resulting in increased shelf life. Furthermore, results have shown that increasing the shelf life of deli meat products resulted in a decrease in the number of deaths or illnesses per year (*22*).

2.2.3 Vegetables and fruits

Only one major outbreak of listeriosis is related to the ingestion of vegetables such as raw celery, tomatoes, and lettuce (*30*). In fact, fruits and vegetables have not been directly associated with a major outbreak of listeriosis in the US until the deadliest outbreak caused by contaminated cantaloupe consumption in 2011. At least thirty deaths from multiple states were reported. Many pathogenic microorganisms, including *L. monocytogenes*, are frequently isolated from raw vegetables (cabbage, broccoli, bean sprouts, cucumber, lettuce, pepper, potatoes, *etc*) and fruits (cantaloupes watermelon, strawberries, *etc*) (*6, 27*). Perhaps, the reasons for a high incidence of *L. monocytogenes* in raw vegetables and fruits may be due to proximity to soil or using irrigated water or fertilizer contaminated with pathogens. Lacking an effective strategy to control *L. monocytogenes* contamination in fresh vegetables and fruits would mean a high risk for humans to consume contaminated raw vegetables and fruits.

2.3 Molecular mechanisms of *Listeria monocytogenes* under environmental stress conditions

L. monocytogenes can be exposed to harsh conditions including food processing and environmental stresses by heat, acid, alkali, osmosis, oxidation, starvation, or antimicrobial agents. Nevertheless, the pathogen has shown to overcome and adapt to the stressful conditions throughout the expression of regulatory proteins, distinct properties in an advanced metabolic system (i.e. phosphoenopyruvate-dependent

phosphotransferase system, PTS and carbon catabolite repression, CCR), quorum sensing, or polycistronic gene expression pattern (*9*, *13*, *40, 51, 52*). Thus, bacteria efficiently and quickly sense a change in an environmental stress condition and synthesize gene products essential for their survival or growth under the conditions. In soil, irrigated water, decaying vegetation, or silage, the pathogen does not highly express regulatory proteins or virulence factors in comparison to human and animals (*21*). Regulatory proteins in *L. monocytogenes* encoded by *sigB* (a transcriptional regulator) or *prfA* (a central response regulator) control virulence genes in response to environmental stresses (*15, 18, 19, 20, 32, 33, 67, 68*).

2.3.1 Thermal stress

L. monocytogenes has an ability to survive in a wide range of temperatures from zero to 48°C or pasteurization temperature (65-82.8°C) in RTE food products (*34, 39, 56*). The optimal temperature for the growth of the pathogen is 30 to 37°C. The expression of virulence genes such as *prfA*, *hly*, and *actA* is severely repressed at temperatures below 30°C, while genes such as *flaA, actA, cheA,* or *cheY* involved in motility or chemotaxis are repressed at 37°C (*14, 36*). Heat shock-induced genes encode chaperones and proteases are used to protect the misfolding or degradation of proteins or enzymes in their survival maintenance or cell growth (*66*). Although much research has been conducted on heat shock or cold shock stresses, the molecular adaptive responses of *L. monocytogenes* at different temperatures are not fully understood. Physiological changes in the extended lag phase, the decreased growth rate, and the reduced cell numbers of the pathogen were observed during incubation period at refrigerator temperature (*11*). A study has demonstrated that β-ketoacyl-acyl carrier protein synthase

III (FabH) play an important role to increase the content of anteiso-branched chain fatty acids (BCFAs) at low temperature (*57*). A characteristic of the plasma membrane of gram positive bacteria including *L. monocytogenes* that it is consists of high contents of iso- and anteiso-BCFA, which have low melting points for maintaining membrane fluidity at low temperatures (*2*). Anteiso-BCFAs are produced from 2-methylbutyryl-CoA, an intermediate the metabolism of isoleucine. Singh and colleagues found that FabH had high catalytic efficiency with 2-methylbutyryl-CoA via steady-state kinetic analysis of *L. monocytogenes* at 10 and 30°C. The finding suggests that FabH increases the content of anteio-BCFAs by high substrate specificity of FabH with 2-methylbutyryl-CoA (57). Recently, a study showed that *cspA* and *pgpH* genes are associated with cold stress (*3*). Arguedas-Villa et al. (*3*) evaluated the growth of 20 *L. monocytogenes* strains from different origin (human listeriosis case, food product, or environment) at 4°C or 30°C for 2 h, and divided them into two groups (short vs. long lag time). They found that the transcription level of *sigB*, *cspA* and *pgpH* was increased in short lag phase group, suggesting that the proteins may be involved in cold resistant.

Fewer studies on the cold shock responses in *L. monocytogenes* have been conducted compared to heat shock responses involved in manufacturing RTE deli meat products because the pathogen is usually exposed to heat stress during food processing. The responses of *L. monocytogenes* to heat stress trigger the release of bacterial proteins such as heat shock proteins (Hsp70 and Hsp90), single stranded binding proteins (Ssb, DnaK, DnaJ, Dna G, and DnaB), and some other chaperones and proteases (GroEL, GroES, CplC, and CplP) (*35, 46, 53*). In particular, the function of Hsps includes the repair of injured cells and refolding of aberrant proteins by heat shock. Transcriptional

activator, SigB, also plays an important role in regulating heat shock proteins and other regulon via recognizing promoters of heat shock genes (*66*).

2.3.2 pH stress

L. monocytogenes can survive at a low-pH environment in the gastrointestinal tract or acidic foods. Acidification in RTE deli meat products is commonly used by adding acetate, lactates, diacetates, or organic acids as food preservatives. Survival at very low-pH environmental conditions is due to the acid tolerance response (ATR). ATR includes induction of proteins involved in glutamate decarboxylase (GAD) system and increase of proton efflux (*60*). The GAD operon consists of three genes, *gadA*, *gadB*, and *gadC*. *gadA* and *gadB* encode biochemically undistinguishable glutamic acid decarboxylase, and *gadC* encodes a putative glutamate or glutamate-γ-aminobutyrate (GABA) antipoter. Glutamate intracellularly transported via antipoter in *L. monocytogenes* is converted to GABA by GAD. The GAD decarboxylation reaction consumes H^+, which increase intracellular pH. The survival of GAD gene deletion-mutant (Δ*gadAB*) was shown to be reduced in synthetic human and *ex-vivo* porcine gastric fluid (*12*). Therefore, the GAD system is considered to be important for ATR. The synthesis of the chaperon GroEL and ATP synthase proteins was shown to be increased during the growth of *L. monocytogenes* at low temperature and acidic conditions (*45*). ATP synthase can generate proton-motive force to maintain the intercellular pH via translocation of H^+ (*10*). Therefore, ATP synthase and heat shock proteins also play an important role in the ATR of *L. monocytogenes*.

L. monocytogenes has an ability to resist high pH conditions encountered through their systemic infection in humans. The pathogen is often exposed to the stress when it is

phagocyted in phagocytic vacuoles or in the gastrointestinal tract in presence of pancreatic secretions (*54*, *61*). Thus, how *L. monocytogenes* responds to alkaline stress is a crucial step for its survival in hosts. A recent study identified a hypothetical protein (lmo0841), named *L. monocytogenes* Ca^{2+}-dependent ATPase 1 (LMCA1) (*17*). The authors found that LMCA1 has a high homology to eukaryotic and prokaryotic sacoplasmic reticulum Ca^{2+}-ATPases (SERCA1a) and plasma membrane Ca^{2+}-ATPases (PMCA). They measured ATPase activity of LMCA1 activity in the presence of various ions. Interestingly, LMCA1 has maximal activity at about pH 8.75, whereas SERCA1a is inactive. In addition, data presented that the enzymatic activities for Ca^{2+} affinity of LMCA1 mutants were severely decreased, suggesting that H^+ is transported inversely to the direction of Ca^{2+} transport. This study suggests that LMCA1 may play an important role in maintaining intracellular pH by the transport of H^+ and removal of Ca^{2+}. Giotis et al. (*24*) found that in alkaline stress *L. monocytogenes* increased the proportions of anteiso-BCFAs as in low temperature, whereas the proportions were decreased in acidic condition, suggesting that a ratio of anteiso form to iso form of BCFAs may also play important role in pH adaptation.

2.3.3 Nutritional stress

L. monocytogenes adapts well to nutritional fluctuations in the natural environment. The bacterium has developed a starvation survival response (SSR) via physiological or morphological changes in response to glucose or multiple-nutrient starvation (*28*). A rapid decrease of the number of viable cells is one of the characteristics during long-term bacterial cell maintenance. This phenomenon may help for the maintenance of viable cells by utilizing nutrients from the dead cells (*28*). *L.*

monocytogenes may synthesize proteins only needed for the cell maintenance because starvation survival can be dependent on their limited intracellular amino acids and carbon sources. Especially, bacterial intracellular proteins (i.e. various PTS permeases) to uptake extracellular carbohydrates can be mainly used for sugar utilization by the fluctuations of preferred carbohydrates available (*62*). Unlike eukaryotes, *L. monocytogenes* can utilize various carbohydrates such as glucose, fructose, mannose, and cellobiose as a primary carbon source (*50*). Moreover, whole genome sequences of *L. monocytogenes* also present genes encoding proteins involved in the uptake of various sugars. Bacteria including *L. monocytogenes* can mimic their energy expenditure via a carbon catabolite repression (CCR) system (*47*). Glucose is the most preferred energy carbon source. Thus, the transcription level of genes involved in the synthesis or uptake of other carbon sources can be decreased until glucose is depleted (*64*). Phosphoenopyruvate-dependent phosphotransferase (PTS) system is an important and distinct means for extracellular sugar uptake using energy from phosphoenopyruvate in bacteria. A mechanistic study has shown that the growth of a *ptsH* mutant that lacks HPr, was repressed by impaired PTS (*51*). Interestingly, our recent data presented that the transcripts of PTS genes were also decreased with increased NaCl concentration (*4*).

2.3.4 Oxidative stress

Aerobic organisms contain superoxide dismutase, catalase, and peroxidase to eliminate O_2^- and H_2O_2 produced during respiratory reactions (*41*). *L. monocytogenes* can be exposed to nitric oxide (NO), superoxide (O_2^-), or hydrogen peroxide (H_2O_2) in phagocytic cells such as neutrophils or macrophages during the infection (*41*). Most of invasive bacteria are killed by those oxidant molecules in the phagosome of neutrophils

and macrophages. However, some bacterial cells tolerate it. In *E. coli*, Dps (an iron-binding protein) was synthesized abundantly to diminish DNA damage through binding Fe (II) and inhibiting the formation of hydroxyl radicals (*70*). Uhlich and colleagues (*65*) investigated the functions of Crp/Fnr family of *L. monocytogenes* strain F2365 using transposon mutagenesis. They suggested that Crp/Fnr family in *L. monocytogenes* strain F2365 is involved in oxidative stress.

2.3.5 Osmotic stress

In the food industry, salt is widely used as a general preservative or antibacterial agent because of its inhibitory effects on bacterial growth. Salt can damage the bacterial cells via the disruption of the bacterial maintenance of osmotic pressure between the cytoplasmic and the extracellular environments. However, *L. monocytogenes* can mediate the change of transporter proteins such as glycine betaine and carnitine encoded by *betL*, *gbu* operon, and *opuC* operon, resulting accumulation of osmoprotectants in the cell (*1*). However, the exact mechanism of osmoprotectants to osmotic stress is still unknown. In addition, RelA, HtrA, KdpE, LisRK, ProBA, BtlA, and Ctc involved in osmotic stress have been identified to be expressed in response to high osmolarity (*5, 7, 22, 43, 58, 59, 69*). In particular, the two-component regulatory system Kdp is involved in osmotic stress. KdpE of the Kdp two-component regulatory system is associated with the uptake of K^+ (*31*). The *orfX* of Kdp operon is also involved in osmotic stress adaptation. The K^+ uptake by the Kdp system was shown to have a protective effect on the pathogen to osmotic stress (*7*). Interestingly, a recent study showed that the cold shock proteins (Csps) are involved in salt stress response (*55*). They found that the growth of *csps* deletion mutants grown in BHI with 3% NaCl (w/v) for 2 h at 4°C or 37°C significantly

inhibited, and the transcription level of *csps* were significantly decreased in the mutant strain compared to wild type *L. monocytogenes.* Data suggest that Csps can promote an adaptive ability of *L. monocytogenes* to osmotic stress.

2.3.6 Antimicrobial stress

The increase in prevalence of antimicrobial resistance in pathogens has become a major concern. Antibiotics can mostly damage pathogens via inhibition of cellular function, resulting in cytocidal or cytostatic effects. The wide use of antibiotics for human and animal treatment or animal growth promotion can increase the development of antimicrobial resistance (*26*). In addition, sanitizers or disinfectants used in food processing can also lead to the resistance (*44*). Antibiotic resistance genes in foodborne pathogens can be transferred through transformation, conjugation, or transposon. *L. monocytogenes* may develop antibiotic resistance via plasmid transfer from other gram-positive bacteria (*48*). The treatment of pathogens with sub-lethal amounts of antibiotic can enhance the adaptation or development of antibiotic resistance in pathogens, resulting in higher levels of antibiotic or multiple antibiotics. According to a recent study, twenty-one isolates of *L. monocytogenes* in human food samples from farms and food processing plants were tested for antibiotic resistance (*49*). Twenty out of 21 isolates were resistant to multi antibiotics. The effects of the combination of bacteriocin, chemical or physical treatment (i.e. nisin and carbon dioxide, carbon dioxide and cold shock, or nisin and cold shock) on *L. monocytogenes* have been investigated (*37, 42, 56*). The study showed that CO_2^- increased the permeability of cell membrane via changing the lipid composition of cell membrane, resulting in the pore formation by nisin.

Table 2.1 Major outbreaks of listeriosis in the United States

Year	Serotype	Sources/Locations	Cases	Deaths	Recall
1979	4b	Raw celery, tomatoes, lettuce / MA	20	5	
1983	4b	Pasteurized milk/ MA	49	14	
1985	4b	Mexican-fresh soft cheese / CA	142	94	
1986	4b, 1/2a, 1/2b, 3b	Ice cream and salami / PA	36	16	
1998-1999	4b	Frankfurters, RTE meat, and poultry products / 11 states	≈100	6 deaths, 2 miscarriages	150 million lbs.
1998	4b	Frankfurters and luncheon / MI (Sara Lee)	≈500	Miscarriages, stillbirth, and >61 deaths	
1999		Turkey deli meat / 10 states	29	3 miscarriages and 4 deaths	
2000-2001	4b	Mexican cheese / NC	12	5	
2000	1/2a	Delicatessen sliced turkey / USA	29	7	
2002	4b	Turkey deli meat / 7 states	44	3 miscarriages and stillbirth; 7 deaths	27.4 million lbs.
2007		Milk / MA	11	1 stillbirth, 1 premature, and 3 deaths	
2011	1/2a, 1/2b	Cantaloupe/28 states	146	30 deaths and 1 miscarriage	

Sources from CDC, "*Listeria*" 2nd ed. by Chris, B. and K. Alec, and Ho et al., 1986

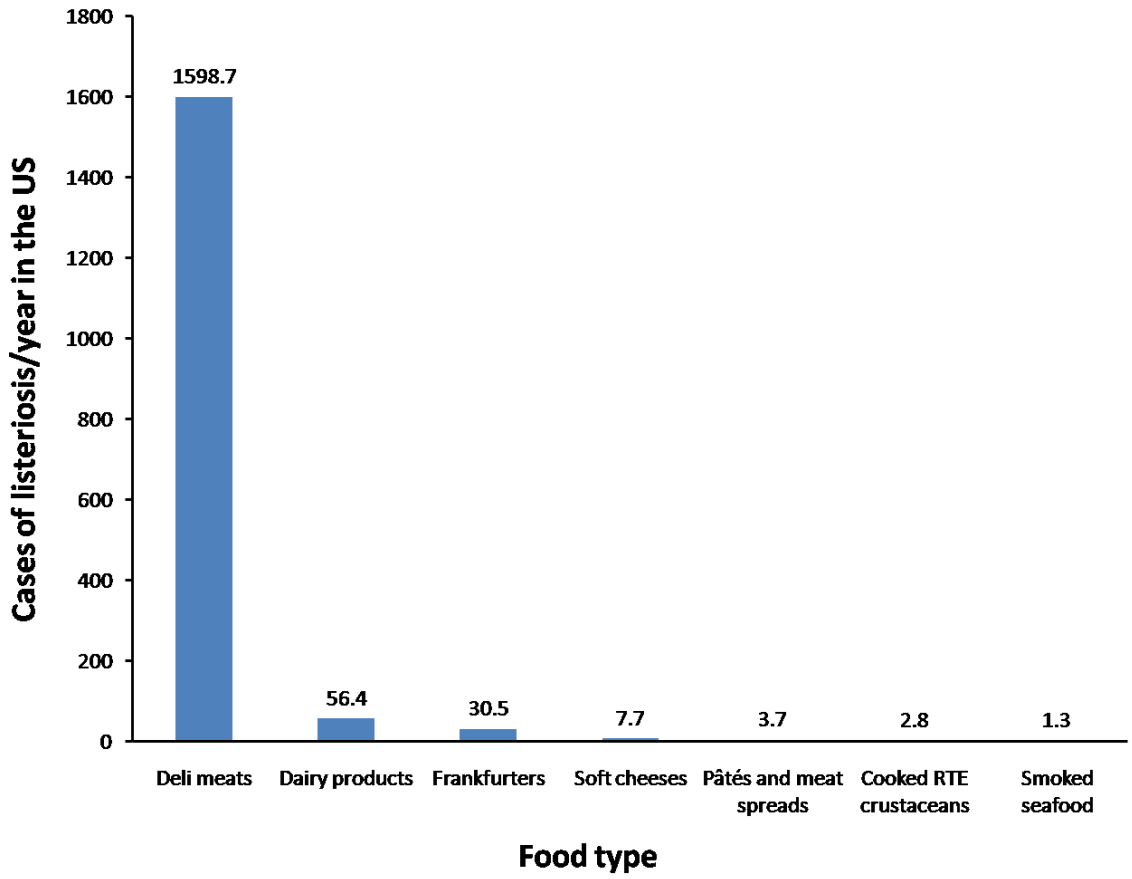

Figure 2.1 The risk of listeriosis for selected RTE foods.

Deli meats, dairy products, and frankfurters have been shown to be a high risk of listeriosis. Data were from FDA, CFSAN 2003, Quantitative Assessment of Relative Risk to Public Health from Foodborne *Listeria monocytogenes,* Among Selected Categories of Ready-to-Eat Foods.

2.4 References

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

2. Annous, B.A., L.A. Bexker, D.O. Bayles, D.P. Labeda, and B.J. Wilkinson, 1997. Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. *Appl Environ Microbiol*. 63:3387–3894.

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

4. Bae, D., C. Liu, T. Zhang, M. Jones, S. N. Peterson, and C. Wang. 2012. Global Gene Expression of *Listeria monocytogenes* to Salt Stress. *J Food Prot*, in press.

5. Begley, M., C. Hill, and C. G. Gahan. 2003. Identification and disruption of *btlA*, a locus involved in bile tolerance and general stress resistance in *Listeria monocytogenes. FEMS Microbiol Lett*. 218:31-38.

6. Beuchat, L. R. 1996. Pathogenic Microorganisms Associated with Fresh Produce. *J Food Prot.* 59:204-216.

7. Brondsted, L., B. H. Kallipolitis, H. Ingmer, and S. Knochel. 2003. *kdpE* and a putative RsbQ homologue contribute to growth of *Listeria monocytogenes* at high osmolarity and low temperature. *FEMS Microbiol Lett*. 219:233-239.

8. Buzby, J. C. 2001. Children and microbial foodborne illness. *Food Review.* 24:32-37.

9. Camilli, A., L. G. Tilney, and D. A. Portnoy. 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol Microbiol.* 8:143-157.

10. Chris, B. and K. Alec. 2005. Factors affecting the growth and survival of *monocytogenes*, p. 62-69. In *Listeria*, 2nd ed. Blackwell Publishing Ltd, Oxford, UK.

11. Cotter, P. D. and C. Hill. Surviving the acid test: Responses of gram-positive bacteria to low pH. 2003. *Microbiol Mol Biol Rev.* 67:429-453.

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

13. Dons, L., J. E. Olsen, and O. F. Rasmussen. 1994. Characterization of two putative *Listeria monocytogenes* genes encoding polypeptides homologous to the sensor protein CheA and the response regulator CheY of chemotaxis. *DNA Seq.* 4:301–311.

14. Dons, L., O. F. Rasmussen, and J. E. Olsen. 1992. Cloning and characterization of a gene encoding flagellin of *Listeria monocytogenes*. *Mol Microbiol.* 6:2919–2929.

15. Dramsi, S., C. Kocks, C. Forestier, and P. Cossart. 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature, and the pleiotropic activator *prfA*. *Mol Microbiol.* 9:931-941.

16. Esteban, J. I., B. Oporto, G. Aduriz, R. A. Juste, and A. Hurtado. 2009. Faecal shedding and strain diversity of *Listeria monocytogenes* in healthy ruminants and swine in Northern Spain. *BMC Vet Res.* 5:2-30.

17. Faxén, K., J. L. Andersen, P. Gourdon ,N. Fedosova, J. P. Morth, P. Nissen, and J. V. Møller. 2011. Characterization of a *Listeria monocytogenes* Ca(2+) pump: a SERCA-type ATPase with only one Ca(2+)-binding site. *J Biol Chem.* 286:1609-17.

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

19. Fraser, K. R., D. Sue, M. Wiedmann, K. J. Boor, and C. P. O'Byrne. 2003. Role of σ^B in regulating the compatible solute uptake systems of *Listeria monocytogenes*: osmotic induction of *opuC* is σ^Bdependent. *Appl Environ Microbiol*. 69:2015-2022.

20. Freitag, N. E., L. Rong, and D. A. Portnoy. 1993. Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. *Infect Immun*. 61:2537-2544.

21. Freitag, N. E., G. C. Port, and M. D. Miner. 2009. *Listeria monocytogenes* – from saprophyte to intracellular pathogen. *Nat Rev Microbiol.* 7:623–628.

22. FSIS. 2010. FSIS Comparative Risk Assessment for *Listeria monocytogenes* in Ready-to-eat Meat and Poultry Deli Meats Report. http://www.fsis.usda.gov/PDF/Comparative_RA_Lm_Exec_Summ_May2010.pdf

23. Gardan, R., O. Duche, S. Leroy-Setrin, and J. Labadie. 2003. Role of *ctc* from *Listeria monocytogenes* in osmotolerance. *Appl Environ Microbiol*. 69:154-161.

24. Giotis, E. S., D. A. McDowell, I. S. Blair, and B. J. Wilkinson. 2007. Role of Branched-Chain Fatty Acids in pH Stress Tolerance in *Listeria monocytogenes. Appl Environ Microbiol*. 73:997–1001.

25. Goulet,V., C. Hedberg, A. Le Monnier, and H. de Valk. 2008. Increasing incidence of listeriosis in France and other European countries. *Emerg Infect Dis*. 14:734–740.

26. Granier, S. A., C. Moubareck, C. Colaneri, A. Lemire, S. Roussel, T. Dao, P. courvalin, and A. Brisabois. 2011. Antimicrobial Resistance of *Listeria monocytogenes* Isolates from Food and the Environment in France over a 10-Year Period. *Appl Environ Microbiol.* 77:2788–2790.

27. Heisick, J. E., D. E. Wagner, M. L. Nierman, and J. T. Peeler. 1989. *Listeria* spp. found on fresh market produce. *Appl Environ Microbiol.* 55:1925-1927.

28. Herbert, K. C. and S. J. Foster. 2001. Starvation survival in *Listeria monocytogenes*: characterization of the response and the role of known and novel components. *Microbiology.*147:2275–2284.

29. Ho, J. L., K. N.Shands, G.Friedland, P.Eckind, and D. W.Fraser. 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. *Arch Intern Med.* 146:520-4.

30. Jakobsen, R. A., R. Heggebø, E. B. Sunde, and M. Skjervheimb. 2011. *Staphylococcus aureus* and *Listeria monocytogenes* in Norwegian raw milk cheese production. *Food Microbiol.* 28:492-496.

31. Kallipolitis, B.H. and H. Ingmer. 2001. *Listeria monocytogenes* response regulators important for stress tolerance and pathogenesis. *FEMS Microbiol Lett.* 204:111–115.

32. Kazmierczak, M. J., M. Wiedmann, and K. J. Boor. 2006. Contributions of *Listeria monocytogenes* sigmaB and PrfA to expression of virulence and stress response genes during extra- and intracellular growth. *Microbiology.* 152:1827-38.

33. Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann. 2003. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. *J Bacteriol*. 185:5722-34.

34. Knabel, S. J., H. W. Walker, P. A. Hartman, and A. F. Mendonca. 1990. Effects of Growth Temperature and Strictly Anaerobic Recovery on the Survival of *Listeria monocytogenes* during Pasteurization. *Appl Environ Microbiol.* 56:370-376.

35. Krüger, E., D. Zühlke, E. Witt, H. Ludwig, and M. Hecker. 2001. Clp-mediated proteolysis in gram-positive bacteria is autoregulated by the stability of a repressor. *EMBO J.* 20:852-863.

36. Leimeister-Wächter, M., E. Domann, and T. Chakraborty. 1992. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. *J Bacteriol.* 174:947–952.

37. Li, J., G. L. Kolling, K. R. Matthews, and M. L. Chikindas. 2003. Cold and carbon dioxide used as multi-hurdle preservation do not induce appearance of viable but non-culturable *Listeria monocytogenes*. *J Appl Microbiol.*94:48-53.

38. Little, C. L., J. R. Rhoades, S. K. Sagoo, J. Harris, M. Greenwood, V. Mithani, K. Grant, and J. McLauchlin. 2008. Microbiological quality of retail cheeses made from raw, thermized or pasteurized milk in UK. *Food Microbiol.* 25:304–312.

39. Lou, Y. and A. E. Yousef. 1999. Characteristics of *Listeria monocytogenes* important to food producers, p. 131-224. In E. T. Ryser and E. H. Marth (ed.), *Listeria*, Listeriosis, and Food Safety, 2nd ed. Marcel Dekker Inc., New York, NY.

40. Marilyn, K. 2005. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene.* 361:13–37.

41. Nathan, C., and M. U. Shiloh. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci USA.* 97:8841-8848.

42. Nilsson L., Y. Chen, M. L. Chikindas, H. H. Huss, L. Gram, and T. J. Montville. Carbon dioxide and nisin act synergistically on *Listeria monocytogenes*. *Appl Environ Microbiol.*66:769–774.

43. Okada, Y., S. Makino, T. Tobe, N. Okada, and S. Yamazaki. 2002. Cloning of *rel* from *Listeria monocytogenes* as an osmotolerance involvement gene. *Appl Environ Microbiol*. 68:1541-1547.

44. Pan,Y., F. Breidt, and S. Kathariou. 2006. Resistance of *Listeria monocytogenes* Biofilms to Sanitizing Agents in a Simulated Food Processing Environment. *Appl Environ Microbiol.* 72:7711–7717.

45. Phan-Thanh, L. and F. Mahouin. 1999. A proteome approach to study the acid response in *Listeria monocytogenes*. *Electrophoresis.* 20:2214-2224.

46. Phan-Thanh, L. and T. Gormon. 1995. Analysis of heat and cold shock proteins in *Listeria* by two-dimensional electrophoresis. *Electrophoresis.* 16:444-450.

47. Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart. 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect Immun.* 60:1263–1267.

48. Poyart-Salmeron, C., C. Carlier, P. Trieu-Cuot, A. L. Courtieu, and P. Courvalin. 1990. Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes*. *Lancet.* 335:1422–1426.

49. Prazak, M. A., E. A. Murano, I. Mercado, and G. R. Acuff. 2002. Antimicrobial resistance of *Listeria monocytogenes* isolated from various cabbage farms and packing sheds in Texas. *J Food Prot.* 66: 1796-1799.

50. Premaratne, R. J., W. Lin, and E. A. Johnson. 1991. Development of an Improved Chemically Defined Minimal Medium for *Listeria monocytogenes*. *Appl Environ Microbiol.* 57:3046-3048.

51. Reizer, J., C. Hoischen, F. Titgemeyer, C. Rivolta, R. Rabus, J. Stulke, D. Karamata, M. H. Saier, Jr., and W. Hillen. 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. *Mol Microbiol*. 27:1157-1169.

52. Riedel, C. U., I. R. Monk, P. G. Casey, M. S. Waidmann, C. G. Gahan, and C. Hill. 2009. AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in *Listeria monocytogenes*. *Mol Microbiol.* 71:1177–1189.

53. Rosen, R. and E. Z.Ron. 2002. Proteome analysis in the study of the bacterial heat shock response. *Mass Spectrometry Rev.* 21:244-265.

54. Segal, A. W., M. Geisow, R. Garcia, A. Harper, and R. Miller. 1981. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature.* 290:406–409.

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

56. Shannon, E. M., S. R. Milillo, M. G. Johnson, and S. C. Ricke. 2011. Inhibition of *Listeria monocytogenes* by Exposure to a Combination of Nisin and Cold-Pressed Terpeneless Valencia Oil. *J Food Sci.* 76: M600–M604.

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

58. Sleator, R. D., and C. Hill. 2005. A novel role for the LisRK two-component regulatory system in listerial osmotolerance. *Clin Microbiol Infect*. 11:599-601.

59. Sleator, R. D., C. G. Gahan, and C. Hill. 2001. Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. *Appl Environ Microbiol*. 67:2571-2577.

60. Stack, H. M., C. Hill, and C. G. M. Gahan. 2008. Stress Responses, p. 61-96. In Liu, D. (ed.), Handbook of *Listeria monocytogenes*. Taylor & grancis Group, Boca Raton, FL.

61. Stancik, L. M., D. M. Stancik, B. Schmidt, D. M. Barnhart, Y. N. Yoncheva, and J. L. Slonczewski, 2002. pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J Bacteriol*. 184:4246–4258.

62. Stoll, R., and W. Goebel. 2010. Identification of the major PEP-phosphotransferase system (PTS) for glucose, mannose, and cellobiose of *Listeria monocytogenes* and their significance for extra- and intracellular growth. *Microbiology*. 156:1069–1083.

63. Swaminathan, B., and P. Gerner-Smidt. 2007. The epidemiology of human listeriosis. *Microbes Infect.* 9:1236-1243.

64. Titgemeyer, F. and W. Hillen. 2002. Global control of sugar metabolism: a gram-positive solution. *Antonie van Leeuwenhoek.* 82**:**59-71.

65. Uhlich, G. A., Wonderling, L. D. & Luchansky, J. B.2006. Analyses of the putative Crp/Fnr family of transcriptional regulators of a serotype 4b strain of *Listeria monocytogenes*. *Food Microbiol.* 23:300–306.

66. van der Veen, S., T. Hain, J. A. Wouters, H. Hossain, W. M. de Vos, T. Abee, T. Chakraborty, and M. H. Wells-Bennik. 2007. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. *Microbiology.* 153:3593-3607.

67. Wemekamp-Kamphuis, H. H., J. A. Wouters, P. P. L. A. de Leeuw, T. Hain, T. Chakraborty, and T. Abee. 2004. Identification of sigma factor σ^B-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogenes* EGD-e. *Appl Environ Microbiol*. 70:3457-3466.

68. Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor. 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. *J Bacteriol*. 180:3650-3656.

69. Wonderling, L. D., B. J. Wilkinson, and D. O. Bayles. 2004. The *htrA* (*degP*) gene of *Listeria monocytogenes* 10403S is essential for optimal growth under stress conditions. *Appl Environ Microbiol*. 70:1935-1943.

70. Zhao, G., P. Ceci, A. Ilari, L. Giangiacomo, T. M. Laue, E. Chiancone, and N. D. Chasteen. 2002. Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J Biol Chem*. 277:27689-27696.

CHAPTER III

TRANSCRIPTOME ANALYSIS OF *LISTERIA MONOCYTOGENES* GROWN ON A READY-TO-EAT MEAT MATRIX[1]

3.1 Abstract

The contamination of ready-to-eat (RTE) meat products with *Listeria monocytogenes* is a major concern for the food industry. For a better understanding of the adaptation and survival ability of *L. monocytogenes* grown on turkey deli meat, the transcriptome of *L. monocytogenes* strain F2365 was determined using a microarray. Microarray data were validated using a quantitative real time RT-PCR assay. Based on the microarray data, 39 and 45 genes from *L. monocytogenes* were transcriptionally up-regulated and down-regulated, respectively. The genes regulated at the transcriptional level were mainly involved in energy metabolism, fatty acid and phospholipid metabolism, biosynthesis of proteins, transport and binding proteins, DNA metabolism, cellular processes, and regulatory functions. There was no significant change in the expression of genes encoding for known virulence factors such as *sigB, prfA, inlA, inlB, plcA, plcB,* and *hly*. This study suggests that *L. monocytogenes* grown on RTE deli meat changes its transcription involved in its metabolic pathways to obtain an energy source or to adapt to environmental change without altering the transcription levels of virulence

[1] Reprint with permission (Appendix) from Bae, D., M. R. Crowley, and C. Wang. 2011. Transcriptome analysis of *Listeria monocytogenes* grown on a ready-to-eat meat matrix. *J Food Prot.* 74:1104-11.

factors. The global transcriptome profiles provide a better understanding of the growth or adaptation of *L. monocytogenes* in RTE meat products.

3.2 Introduction

The Gram-positive facultative intracellular bacterium *Listeria monocytogenes* is a food-borne pathogen that causes human listeriosis. The pathogen, found in the natural environment and food, has an ability to survive and proliferate in food products under extreme conditions such as high acidity, low temperature, high osmolarity, and high hydrostatic pressure *(5, 6, 26)*. The concern about the prevalence of *L. monocytogenes* in ready-to-eat (RTE) food has been escalated by food-borne outbreaks through the consumption of RTE meat products, in particular turkey deli slices. Risk assessment by the US Food and Drug Administration reported that turkey deli meat is one of the potential sources for *L. monocytogenes* infection. The Food Safety and Inspection Service (FSIS) recommends that turkey deli meat be consumed within five days after opening the package. Among the 13 serotypes of *L. monocytogenes*, serotype 4b is commonly associated with human outbreaks of listeriosis in susceptible individuals *(10, 29)*.

The RTE meat products contain a higher level of protein and about 1 to 2% (w/w) of sodium chloride with microbial growth inhibitors such as potassium lactate, sugar, sodium phosphates, potassium chloride, sodium acetate, sodium ascorbate, and sodium nitrite compared to Brain Heart Infusion (BHI) agar, the standard laboratory growth agar. Salt in particular has been considered as an antibacterial agent as an essential additive to enhance flavor, texture, and shelf life of meat products *(20)*. In addition, antimicrobial growth inhibitors including lactates and diacetates used in retail-sliced or prepackaged RTE deli meat play an important role in controlling *L. monocytogenes* growth during

refrigerated storage *(15, 18)*. A previous study demonstrated the limited inhibitory effects of salt and organic acids on the growth of *L. monocytogenes* during the processing of RTE turkey meat products *(14)*. Additionally, the effects of acidic, osmotic, or nutritional stresses on the survival or adaptation of *L. monocytogenes* were elucidated when it was grown in BHI or conditional medium *(22, 23)*. Burnett and colleagues showed that RTE meat products were a good source for the growth of *L. monocytogenes (2)*. However, little is known about how *L. monocytogenes* adapts and multiplies in a RTE meat matrix and how the adaptive changes affect its ability to cause disease in humans. The high concentration of *L. monocytogenes* in RTE foods has been shown to cause human listeriosis *(9)*. Therefore, the objective of this study was to determine the differential transcriptome profiles of *L. monocytogenes* grown on delicatessen turkey meat or BHI using a microarray to identify genes that contribute to listeriosis.

3.3 Materials and methods

3.3.1 Bacterial cultivation and growth

Commercial packages of RTE smoked turkey breast deli meat slices containing 0.9% sodium were purchased and stored at 4°C until use. *L. monocytogenes* strain F2365 purchased from the American Type Culture Collection was maintained in a BHI medium (Difco Laboratories, Detroit, MI) broth with 20% glycerol at -80°C until use. Fifty microliters of F2365 from a frozen stock was inoculated and grown in 5 ml of BHI broth at 37°C overnight as previously described *(17)*. Twenty microliters of this bacterial culture (approximately 1.0×10^6 CFU/ml) was inoculated into 5 ml of a fresh BHI medium and incubated at 37°C for 7 h in a MaxQ™ 4000 incubating and orbital shaker (Barnstead/Lab-Line, Dubuque, IA) at 180 rpm untill the optical density (O.D.) reached

$A_{600} \approx 0.8$. Turkey deli meat slices were placed into a sterile stomacher bag. The surface area of a turkey deli meat slice was about five times larger than that of BHI agar plate. Thus, two-hundred microliters or 1ml of the inoculum was spread on 10 cm diameter BHI agar plates or on both sides of a turkey meat slice (about 5.9×10^5 CFU/cm^2) in a stomacher bag, respectively. Bacteria were then incubated at 15°C for 5 days in a BOD10 refrigerated incubator (Thermo Fisher Scientific-Revco, Asheville, NC) to mimic the temperature abuse condition. For the bacterial growth curve, 5.5×10^3/cm^2 cells were placed on a turkey deli meat or a BHI agar plate at 15°C up to 5 days. Bacteria were washed and collected from BHI agar plates and turkey deli meat slices using 10 ml and 40 ml of PBS (pH 7.4) [Invitrogen, Gland Island, NY], respectively. Bacterial cells were then enumerated by plate counts. Data were obtained from two independent experiments using quadruplicate plates per each experiment at different time points (8, 16, 24, 48, 72, 96, and 120 h) [$n = 8$]. Data for the bacterial growth were analyzed by the procedures of analysis of variance (ANOVA) using SAS program (version 9.1.3; SAS Institute, Cary, NC).

3.3.2 Cell collection and RNA isolation

L. monocytogenes grown on BHI agar or turkey deli meat was collected after 5 days of incubation at 15°C. Bags containing turkey deli meat and PBS were shaken using a reciprocal shaker (Model 3506, Lab-Line Instruments, Melrose Park, IL) at maximum speed for 5 min. The bacterial suspension was collected using a syringe and mixed with RNA Protect® (Qiagen, Valencia, CA) at a ratio of 1:2 (bacterial suspension : RNA protect). The mixture was incubated for 10 min at RT and centrifuged at 7,000 × g at 4°C for 10 min. Total RNA was extracted from the bacterial pellet and purified using Trizol™

(Invitrogen, Carlsbad, CA) and the RNeasy® Mini Kit (Qiagen), respectively. The quality and concentration of the RNA was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, DE) and the Nanodrop®ND1000 UV-Vis spectrophotometer (Nonodrop Technologies, Wilmington, DE), respectively. RNA Integrity Numbers of RNA samples from BHI agar and turkey deli meat were 8.65 ± 0.46 (SEM) and 8.32 ± 0.23 (SEM), respectively. The range of the OD values of RNA samples at 260/280 nm and A260/230 nm were between 1.8 and 2.0.

3.3.3 Microarray analysis

L. monocytogenes strain F2365 microarray slide (version 3) was obtained from the Pathogen Functional Genomic Research Centre (PFGRC). The microarray slide represents 2847 open reading frames (GenBank Accession no. AE017262). Total RNA was extracted, purified, and reverse transcribed into complementary DNA (cDNA) according to a slightly modified microarray protocols provided by the Institute for Genomic Research (TIGR) (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). Briefly, about 2.5 µg of the total RNA for cDNA synthesis was reverse transcribed using 2 µl of Smart® reverse transcriptase (Clontech, Palo Alto, CA) and 1 µl of random hexamers in the presence of 0.1 M dithiothreitol (DTT), 25 mM dNTP with a 3:2 ratio of aminoallyl-dUTP, and dTTP (Ambion, Austin, TX) with incubation at 42°C for 16 h in water bath. After completion of cDNA synthesis, the cDNA was purified, and unincorpotated aa-dUTP was removed using a MinElute® PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified cDNA was resuspended in 0.1 M sodium carbonate buffer (pH 9.3), labeled with cyanine 3 (cy3) or cyanine 5 (cy5) dyes (Amersham Pharmacia Biotech, Piscataway, NJ), and then purified using a

MinElute® PCR purification kit (Qiagen). The labeling reaction on the purified cDNA was analyzed by measuring absorbance readings at 260 nm, 650 nm, and 550 nm in the Nanodrop. About 6.0 µg of each cDNA labeled with cy3 and cy5 were hybridized, dried, and resuspended in a 50 µl hybridization buffer (40% formamide, 5× sodium chloride/sodium citrate buffer, 0.1% SDS, and 0.6 µg/µl salmon sperm DNA). The resuspended samples were loaded on a *L. monocytogenes* strain F2365 microarray slide v3, and the slides were incubated at 42°C for 16 h. Finally, the slides were washed with a low (2× SSC and 0.1% SDS), medium (0.1× SSC and 0.1% SDS), and high (0.1× SSC) stringency buffers containing 0.1mM DTT (Invitrogen), respectively.

3.3.4 Microarray data analysis

The two-channel microarray slides labeled with cy3 and cy5 were scanned at 10 µm resolution with around 700 photomultiplier tube on the cy3 (532 nm) and cy5 (635 nm) channels using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA). TM4 software developed by TIGR, including Spotfinder, Ginkgo, and Magnolia programs, were used to analyze microarray data. Tiff image files were converted to data files. The signal intensity of spots on the slides was adjusted and quantified by background subtraction using the Spotfinder program. Data LOWESS normalization was performed using the Ginkgo program. Simple Omnibus Format in Text (SOFT), converted from the processed data file by Magnolia 1.2, was used to deposit data to Gene Expression Omnibus (GEO). Values from the normalized data were transformed to a $\log_2$ scale. Three independent experiments using quadruplicate samples per each experiment (n = 12) were conducted. Each independent experiment included a set of flip dye assay. Significant differential expression was derived as a mean signal of

each gene of ≥ 1.5-fold on the $\log_2$ scale and a $P < 0.05$. The range of 10% to 14.29% trimmed mean was used to eliminate extreme observations. The $\log_2$ ratio for each gene was analyzed by Student's *t*-test using SAS program. An annotation file provided from PFGRC was used to classify genes (http://cmr.jcvi.org/cgi-bin/CMR/shared/MakeFrontPages.cgi?page=searches&crumbs=searches). The microarray data are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20274.

3.3.5 Quantitative real time reverse transcription polymerase chain reaction

Primers listed in Table 3.1 were designed using Gene Runner software (http://www.generunner.net/), and the primers were synthesized by MWG Biotech Inc (Huntsville, AL). The transcription levels of selected up-regulated and down-regulated genes were validated by qRT-PCR. cDNA was synthesized by a two-step reverse transcription kit (Applied Biosystems, Foster City, CA) from the same samples used for the microarray experiments. The cDNA concentration was measured using the Nanodrop spectrophotometer. Briefly, 70 ng of the cDNA in 25 µl of final volume with a Power SYBR® Green PCR Master Mix (Applied Biosystems) was amplified by PCR. The amplification was performed using a Mx3005P ™ Real-Time PCR System (Stratagene Inc., La Jolla, CA). The mixture was initially incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 45 sec, and 72°C for 30 sec for PCR. *rpoD* (F:5'-TGGATTCGTCAAGCGATAACC-3', R:5'-GCACCGGAATACGGATIGTT-3') and *gap* (F:5'-ACCAGTGTAAGCGTGAA-3', R:5'-TCACAGCGCAAGACAAA-3') were used as internal controls to normalize the expression rate of each gene *(24, 28)*. mRNA expression levels of the target genes were calculated by using delta-delta Ct method. Four

independent experiments using triplicate RNA samples per each experiment were conducted (n = 12). Data for the mRNA expression levels of the target genes were analyzed by Student's t-test using SAS program.

3.4 Results and discussion

Various levels of *L. monocytogenes* in RTE food products collected from patients' refrigerators, retail stores, or plants have been shown through epidemiologic studies on human listeriosis *(9)*. A study conducted by Farber et al. concluded that contaminated RTE foods, which caused listeriosis in healthy adults, contained various levels of *L. monocytogenes* ranging from 1.3×10^6 to 2.1×10^9 CFU/g *(9)*. In this study, we inoculated 1.1×10^7 CFU/g (5.9×10^5 CFU/cm^2) of *L. monocytogenes* F2365 on a sliced turkey deli meat followed by incubation at 15°C for 5 days. Extended cool temperature storage may be associated with outbreaks of listeriosis because these temperatures allow the growth of *L. monocytogenes* in contaminated food *(19)*. Therefore, the study was conducted to determine the global transcriptome profiles of *L. monocytogenes* grown on a RTE meat matrix under the conditions of high initial concentration of *L. monocytogenes*, cool temperatures and a maximum storage time suggested by US FDA regarding human listeriosis associated with consumption of RTE deli meat products. A previous study first reported global transcriptome profiling of *L. monocytogenes* strain F2365 grown in a liquid food using custom-made oligonucleotide microarray chips. A previous study first reported global transcriptome profiling of *L. monocytogenes* strain F2365 grown in a liquid food using custom-made oligonucleotide microarray chips *(13)*. The current study using the same strain examined gene transcriptional levels of bacteria grown on a solid food matrix, compared to an agar culture medium.

The growth of *L. monocytogenes* on a turkey deli meat slice compared to BHI agar was significantly decreased after 8 h incubation in Figure 3.1 ($P < 0.05$ by ANOVA). Thirty-nine and forty-five genes from strain F2365 grown on a turkey deli meat were up-regulated and down-regulated, respectively, when compared to the bacterium grown on BHI agar (Tables 3.2 and 3.3). Microarray data were validated by qRT-PCR. A positive linear correlation ($R = 0.89$) between the qRT-PCR and the microarray data (Table 3.4) was determined. The significantly up- or down-regulated genes were classified into groups based on their cellular functions using annotation of *L. monocytogenes* strain F2365 provided by PFGRC. The up- and down-regulated genes were largely categorized into groups based on the functions of their end products including energy metabolism, fatty acid and phospholipid metabolism, biosynthesis of proteins, transport and binding proteins, DNA metabolism, cellular processes, and regulatory functions (Tables 3.2 and 3.3). Results indicate that *L. monocytogenes* grown on RTE deli meat changes its transcription involved in its metabolic pathways, suggesting that the changed gene transcriptional activity of the pathogen may be involved in the survival or adaptation to environmental change.

Regarding energy metabolism, the transcript levels of LMOf2365_0366, LMOf2365_1074, LMOf2365_1395, and LMOf2365_1641 genes were increased, whereas the transcription levels of LMOf2365_2429, LMOf2365_2430, and LMOf2365_2431 genes were down-regulated. D-amino acid aminotransferase (LMOf2365_1641) catalyzes the conversion of pyruvate and D-glutamate to D-alanine and 2-oxoglutarate or vice versa. Interestingly, the two amino acids, D-gultamate and D-alanine, are essential components of peptidoglycan in the cell wall of Gram-positive bacteria *(1)*. Thompson et al. showed that the growth of *L. monocytogenes* with a double

mutant of genes (alanine racemase, *dal* and D-amino acid aminotransferase, *dat*), which are involved in the reaction of L-alanine oxidation to pyruvate via D-alanine, was notably decreased in the culture media supplemented with D-alanine *(25)*. Therefore, the increased transcript of LMOf2365_1641 gene may be implicated in an increase in synthesizing the bacterial cell wall for the proliferation of *L. monocytogenes* on a turkey matrix. In contrast, all three genes (LMOf2365_2429, LMOf2365_2430, and LMOf2365_2431) that participate in a glycolytic pathway to catalyze the conversion of glyceraldehyde-3-phosphate from dihydroxyacetone phosphate to pyruvate used as a substrate in the Krebs cycle were down-regulated. The decreased transcription level of glycolytic enzymes may be caused by decreased energy needs from the bacteria grown under the environment that contains a high concentration of ATP, phosphenolpyuvate (PEP), or carbohydrates.

Our data also showed that the expression of LMOf2365_1023, encoding phosphocarrier protein HPr, was transcriptionally up-regulated. HPr is involved in the phosphorylation of a transport and binding protein of *L. monocytogenes* to uptake carbohydrates, which are chemically modified and entered into cells via the phosphorylation cascade reaction of the components of the PEP-dependent phosphotransferase system (PTS). The increased HPr induces sugar uptake *(7)*. Therefore, the increased transcript of LMOf2365_1023 gene in *L. monocytogenes* grown on a turkey matrix may induce the phophosporylation activity of PTS components, resulting in the induction of more sugar uptake. Interestingly, the transcript of LMOf2365_1317 (glutamine synthetase type I) gene was increased by 3.42-fold, but that of LMOf2365_2134 (glutamine aminotransferase) gene was decreased by 4.40-fold. It has been shown that glutamine is an essential amino acid for protein synthesis and an

energy source in cell growth or division *(27)*. *De novo* nucleotide biosynthesis is important for the growth of bacteria *(21)*. Additionally, genes encoding proteins associated with the synthesis of purines and pyrimidine as well as transport and binding of ribonucleotides (LMOf2365_1864, LMOf2365_1865, LMOf2365_1867, LMOf2365_868, and LMOf2365_2746) were transcriptionally up-regulated. Perhaps, an increased glutamine concentration through the changes in transcriptional activities of genes may affect bacterial growth.

The transcription of LMOf2365_0054 and LMOf2365_2045 (*divIVA*) were up-regulated. Those up-regulated genes are involved in encoding single-strand binding and cell division proteins, respectively. A study demonstrated that the single-stranded DNA-binding proteins (SSBs) bind DNA or interact with enzymes for the maintenance of genome integrity by participating in DNA replication, recombination, or repair *(16)*. The growth of *S. pneumoniae* with mutant of *divIVA*, encoding a cell division protein was severely inhibited with morphological changes and abnormal cell division *(8)*. The transcription of LMOf2365_0544, LMOf2365_1004, LMOf2365_1479, LMOf2365_1826, LMOf2365_2224, LMOf2365_2263, and LMOf2365_2461 were down-regulated. The down-regulated genes encoding a universal stress protein family and cellular detoxification protein were shown to be increased by harsh environmental conditions *(3)*. The data suggest that *L. monocytogenes* may not be damaged severely in the turkey deli meat incubation condition.

The present study reveals the physiological changes of *L. monocytogenes* grown on a RTE meat matrix at the transcriptional level. Our previous study showed that *L. monocytogenes* grown on a RTE turkey meat matrix was more invasive to mouse macrophage cell line J774A.1 when compared to the bacteria grown on BHI *(12)*. *sigB*

and *prfA* transcriptional regulator genes can be activated under an extracellular (e.g. osmotic, oxidative, acidic, or nutritional stress) or intracellular conditions. The stress-response or virulence genes such as *cspD, lmo1601, bsh, bilE* operon*, inlA, inlB, plcA, plcB, actA,* and *hly* have shown to be mediated by *sigB* or/and *prfA* activity *(4, 11)*. However, the transcript levels of genes (*inlA, inlB, plcA, plcB, actA, hly, prfA,* and *sigB*) associated with stress-responses and virulence in *L. monocytogenes* grown on a turkey deli meat when compared to the bacterium grown on a BHI agar plate in the study were not significantly changed. This may be due to the RTE meat deli product used in the study. The commercial product contains less than 2% salt and organic acid compounds by weight. The amount of the antilisterial agents may contribute to resistance of *L. monocytogenes* to a RTE turkey deli meat.

In conclusion, outbreaks of listeriosis due to ingestion of contaminated RTE meat products have become a major concern. In the United States, the food industry has made a tremendous effort to achieve zero tolerance for *L. monocytogenes* in RTE meat products. The treatment on RTE products with salt, organic acids, or high pressure is commonly used to inhibit the bacterial cell growth or prevent a cross-contamination *(14)*. We report that specific genes involved in energy metabolism, biosynthesis of proteins, and cellular processes to affect listerial growth or adaptation to a RTE meat matrix were changed with no associated changes in virulence factor expression. The microarray database provides a baseline for further study of how *L. monocytogenes* behaves under the conditions with a high level of *L. monocytogenes* contamination, extended cool temperatures, and a recommended maximum storage time linked to a delicatessen turkey meat. This study may also contribute to the development of novel strategies in RTE deli meat preservation and storage.

3.5 Acknowledgments

This work was supported by grants from the USDA/ARS #58-6402-7-230, the Pathogen Functional Genomics Resource Center at the J. Craig Venter Institute, and the Mississippi Agriculture and Forestry Experiment Station. We thank Dr. Frank W. Austin for his valuable review and suggestions.

Table 3.1 The design of primers used for the quantitative real time RT-PCR to validate the changed genes of *L. monocytogenes* strain F2365 grown on RTE turkey deli meat as compared to growth of the same strain on BHI agar plates

Gene	Locus	Forward Primer	Reverse Primer	References
rpoD	LMOf2365_1473	TGGATTCGTCAAGCGATAACC	GCACCGGAATACGGATIGTT	(*28*)
gap	LMOf2365_2432	ACCAGTGTAAGCGTGAA	TCACAGCGCAAGACAAA	(*24*)
	LMOf2365_1443	TGTCGCTGGTATTGAGGATG	ACAAACGGCGCACTACTGG	this study
	LMOf2365_1826	TAGTAGAAATGCGGTTGGTG	CGAGCCGCATTACTATTCAA	this study
	LMOf2365_1876	TTGGAAATGTGCTTGCGGTG	CACTCACTGCTCCAAATGTA	this study
	LMOf2365_2133	TTTGCTGCTGGTGGTGTTGC	TTCTGGGTTAAGGCGAGACA	this study
	LMOf2365_2134	GAACTTGTGCTGGGCTTGTC	CACAGCTACCTCATTACTCG	this study
	LMOf2365_0627	GGTATGACAGCAGGAATTGG	GAGAATCCCTAATAACGCCG	this study
	LMOf2365_0766	AACTGCGATGACTGCTGCTG	AGCAAGTACACCTGGAACGA	this study
glnA	LMOf2365_1317	CGTCGCGATATTGTGCTTG	TCCCTGCTAAGAAATGATAAG	this study
divIVA	LMOf2365_2045	CTGCCGAAGAAGTGAAAGC	GACGCTAATTCTGTTGCATC	this study
rpsJ	LMOf2365_2606	AGGTGCTTCTGTATCTGGTC	GCAAGTCTAAACGCATCAAGC	this study

Table 3.2 The up-regulated genes of *L. monocytogenes* F2365 grown on RTE Turkey deli meat as compared to growth of the same strain on BHI agar plates using cDNA microarray analysis

Category and gene[1]	Products[2]	Fold change[3]	*P*-value
Energy metabolism: Glycolysis/gluconeogenesis			
LMOf2365_0366	triosephosphate isomerase	1.86	<0.01
Energy metabolism: Amino acids and amines			
LMOf2365_1641	D-amino acid aminotransferase	1.81	<0.01
Energy metabolism: Pentose phosphate pathway			
LMOf2365_1395	6-phosphogluconate dehydrogenase, decarboxylating	2.03	<0.01
Energy metabolism: Pyruvate dehydrogenase			
LMOf2365_1074	pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase beta subunit	2.33	<0.01
Fatty acid and phospholipid metabolism: Biosynthesis			
LMOf2365_0368	dihydroxyacetone kinase	1.98	<0.01
Amino acid biosynthesis			
LMOf2365_1317	glutamine synthetase, type I	3.42	<0.01
LMOf2365_1621	chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase	1.91	<0.01
Protein synthesis: Translation factors			
LMOf2365_2632	translation elongation factor Tu	1.76	<0.01
LMOf2365_2633	translation elongation factor G	1.86	<0.01
Protein synthesis: Ribosomal proteins: synthesis and modification			
LMOf2365_0055	ribosomal protein S18	2.11	<0.01
LMOf2365_0261	ribosomal protein L1	1.96	<0.01
LMOf2365_1499	ribosomal protein S20	2.40	<0.01
LMOf2365_1561	ribosomal protein L21	2.16	<0.01
LMOf2365_1679	ribosomal protein S2	1.67	0.03
LMOf2365_1814	ribosomal protein L19	1.92	<0.01
LMOf2365_1824	ribosomal protein S16	2.92	<0.01
LMOf2365_2602	ribosomal protein L2	2.13	<0.01
LMOf2365_2603	ribosomal protein L23	1.93	<0.01
LMOf2365_2605	ribosomal protein L3	1.91	<0.01
LMOf2365_2606	ribosomal protein S10	2.76	<0.01
LMOf2365_2634	ribosomal protein S7	3.03	<0.01
LMOf2365_2635	ribosomal protein S12	1.88	<0.01

Table 3.2 (continued)

Category and gene[1]	Products[2]	Fold change[3]	*P*-value
Transport and binding proteins: Carbohydrates, organic alcohols, and acids			
LMOf2365_1023	phosphocarrier protein HPr	1.63	0.01
LMOf2365_1264	putative transporter	2.51	<0.01
Transport and binding proteins: Nucleosides, purines and pyrimidines			
LMOf2365_1867	uracil permease	2.10	0.03
DNA metabolism: DNA replication, recombination, and repair			
LMOf2365_0054	single-strand binding protein	1.74	0.01
Cellular processes: Cell division			
LMOf2365_2045	cell division protein DivIVA	2.13	<0.01
Purines and pyrimidine ribonucleotide biosynthesis			
LMOf2365_1864	carbamoyl-phosphate synthase, small subunit	2.00	<0.01
LMOf2365_1865	dihydroorotase, multifunctional complex type	1.69	<0.01
LMOf2365_2746	inosine-5-monophosphate dehydrogenase	2.23	<0.01
Regulatory functions: RNA interactions			
LMOf2365_1868	pyrimidine operon regulatory protein PyrR	1.78	0.01
Hypothetical proteins: Conserved			
LMOf2365_0369	conserved hypothetical protein	2.06	0.03
LMOf2365_0371	conserved hypothetical protein	2.42	<0.01
LMOf2365_0766	conserved hypothetical protein	2.26	<0.01
LMOf2365_1823	conserved hypothetical protein	2.37	<0.01
Unknown function: General			
LMOf2365_0626	cyclic nucleotide-binding protein	1.71	0.02
LMOf2365_0627	BioY family protein	2.78	<0.01
LMOf2365_2161	CAAX amino terminal protease family protein	1.99	<0.01
Unknown function: Enzymes of unknown specificity			
LMOf2365_1991	pyridine nucleotide-disulfide oxidoreductase family protein	1.84	0.01

[1, 2] The category for genes and products were based on annotations provided by TIGR.
[3] The fold differences indicate changes in the transcription of *L. monocytogenes* F2365 grown on turkey deli meat as compared to growth of the same strain on BHI agar plates. The fold change was calculated by using delta-delta Ct method.

Table 3.3 The down-regulated genes of *L. monocytogenes* F2365 grown on RTE turkey deli meat as compared to growth of the same strain on BHI agar plates using cDNA microarray analysis

Category and gene[1]	Products[2]	Fold change[3]	*P*-value
Transport and binding proteins: Cations and iron carrying compounds			
LMOf2365_1443	transporter, NRAMP family	-3.71	<0.01
LMOf2365_1875	ABC transporter, manganese-binding protein		
LMOf2365_1876	manganese ABC transporter, permease protein	-3.60	<0.01
LMOf2365_1877	manganese ABC transporter, ATP-binding protein	-2.85	<0.01
Transport and binding proteins: Amino acids, peptides and amines			
LMOf2365_0303	D-methionine ABC transporter, permease protein	-2.75	0.01
LMOf2365_0304	D-methionine ABC transporter, ATP-binding protein	-2.13	0.04
Transport and binding proteins: Unknown substrate			
LMOf2365_1993	ABC transporter, permease protein	-2.05	0.01
Energy metabolism: Glycolysis/gluconeogenesis			
LMOf2365_2429	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	-1.91	<0.01
LMOf2365_2430	triosephosphate isomerase	-2.07	<0.01
LMOf2365_2431	phosphoglycerate kinase	-1.93	<0.01
Biosynthesis of cofactors, prosthetic groups, and carriers: Pyridoxine			
LMOf2365_2133	pyridoxine biosynthesis protein	-4.32	<0.01
Biosynthesis of cofactors, prosthetic groups, and carriers: Pantothenate and coenzyme A			
LMOf2365_1929	aspartate 1-decarboxylase	-1.97	0.03
LMOf2365_1931	3-methyl-2-oxobutanoate hydroxymethyltransferase	-1.85	<0.01
Amino acid biosynthesis: Histidine family			
LMOf2365_2134	glutamine amidotransferase, SNO family	-4.40	<0.01
DNA metabolism: DNA replication, recombination, and repair			
LMOf2365_1479	recombination protein O	-2.36	<0.01
LMOf2365_2461	excinuclease ABC, A subunit	-1.79	0.05

Table 3.3 (continued)

Category and gene[1]	Products[2]	Fold change[3]	*P*-value
Transcription: Transcription factors			
LMOf2365_2434	putative RNA polymerase sigma-54 factor	-1.87	<0.01
Regulatory function: DNA interactions			
LMOf2365_0266	transcriptional regulator, DegA family	-1.95	0.03
Regulatory functions: Other			
LMOf2365_2433	putative transcriptional regulator	-2.19	<0.01
Cellular processes: Detoxification			
LMOf2365_1004	glutathione peroxidase	-1.64	0.05
LMOf2365_2224	arsC family protein	-1.82	<0.01
LMOf2365_2263	putative arsenate reductase	-3.26	<0.01
Cellular processes:Adaptations to atypical conditions			
LMOf2365_0544	universal stress protein family	-1.86	0.02
Cell envelope: Other			
LMOf2365_1826	cell wall surface anchor family protein, authentic frameshift	-2.61	<0.01
Central intermediary metabolism: Amino sugars			
LMOf2365_0976	N-acetylglucosamine-6-phosphate deacetylase	-1.74	0.02
LMOf2365_0977	glucosamine-6-phosphate isomerase	-1.73	0.01
Central intermediary metabolism: Sulfur metabolism			
LMOf2365_1532	carbon-sulfur lyase	-1.73	<0.01
Hypothetical proteins: Conserved			
LMOf2365_0200	conserved hypothetical protein	-1.72	0.02
LMOf2365_0235	conserved hypothetical protein	-1.89	<0.01
LMOf2365_0392	conserved hypothetical protein	-2.08	<0.01
LMOf2365_0393	conserved hypothetical protein	-2.21	<0.01
LMOf2365_0605	conserved hypothetical protein	-2.00	0.02
LMOf2365_0684	conserved hypothetical protein	-1.83	<0.01
LMOf2365_0817	conserved hypothetical protein	-2.55	<0.01
LMOf2365_0933	conserved hypothetical protein	-1.92	0.01
LMOf2365_0984	conserved hypothetical protein	-2.17	0.02
LMOf2365_1223	conserved hypothetical protein	-2.15	<0.01
LMOf2365_1442	conserved hypothetical protein	-3.49	<0.01
LMOf2365_2146	conserved hypothetical protein	-1.89	<0.01
LMOf2365_2166	conserved hypothetical protein	-2.18	0.01

Table 3.3 (continued)

Category and gene[1]	Products[2]	Fold change[3]	*P*-value
Unknown function: General			
LMOf2365_1598	CBS domain protein	-2.20	<0.01
LMOf2365_2223	MecA family protein	-2.12	<0.01
LMOf2365_2302	yhzC protein	-2.03	0.03
Unknown function: Enzymes of unknown specificity			
LMOf2365_0842	oxidoreductase, aldo/keto reductase family	-1.94	0.01
LMOf2365_1275	hydrolase, alpha/beta fold family	-1.78	0.03

[1, 2] The category for genes and products were based on annotations provided by TIGR.
[3] The fold differences indicate changes in the transcription of *L. monocytogenes* F2365 grown on turkey deli meat as compared to growth of the same strain on BHI agar plates. The fold change was calculated by using delta-delta Ct method.

Table 3.4 Validation of microarray data by quantitative real-time polymerase chain reaction

	Fold change [1]	
Locus	**cDNA microarray [2]**	**qRT-PCR [3]**
LMOf2365_0627	2.78	3.90
LMOf2365_0766	2.26	1.31
LMOf2365_1317	3.42	8.54
LMOf2365_1443	-3.71	-1.47
LMOf2365_1826	-2.61	-5.96
LMOf2365_1876	-3.60	-2.10
LMOf2365_2045	2.13	4.31
LMOf2365_2133	-4.32	-3.53
LMOf2365_2134	-4.40	-4.64
LMOf2365_2606	2.76	3.50

[1] The fold differences indicate changes in the transcription of *L. monocytogenes* F2365 grown on turkey deli meat as compared to growth of the same strain on BHI agar plates. The fold change was calculated by using delta-delta Ct method.
[2, 3] Data were obtained from three independent experiments using quadruplicates RNA samples per experiment (n = 12).

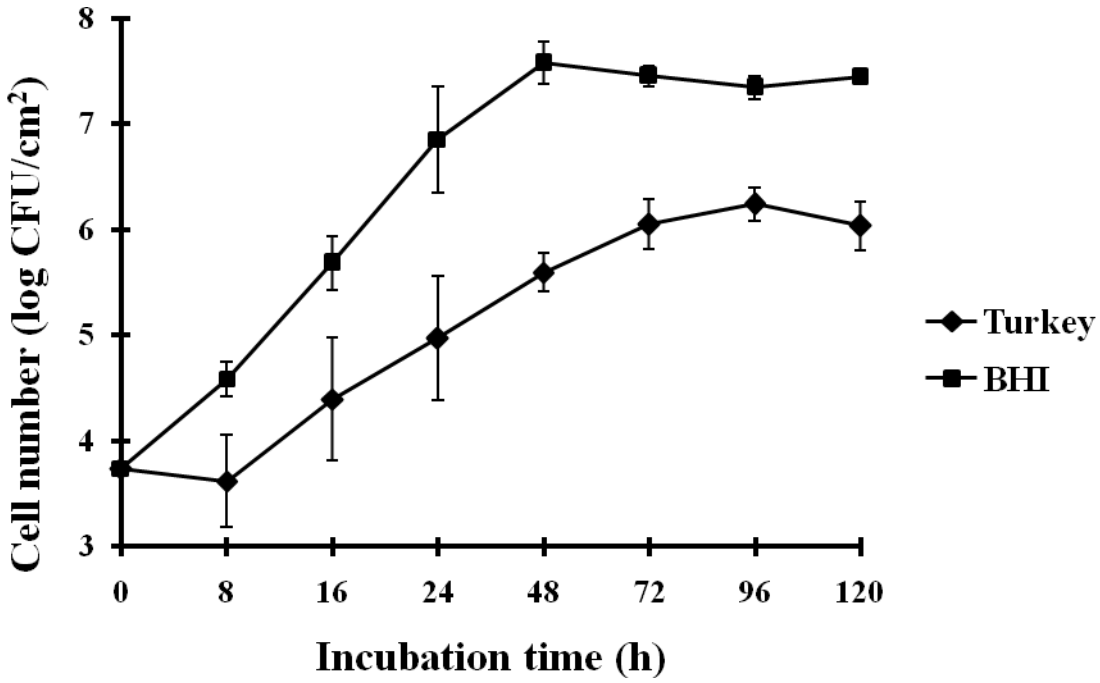

Figure 3.1 The growth of *L. monocytogenes* strain F2365 on a turkey deli meat or BHI agar at 15°C.

Data were obtained from two independent experiments using quadruplicate samples per each experiment via plate counts at different time points (n = 8).

3.6 References

1. Boneca, I. G. 2005. The role of peptidoglycan in pathogenesis. *Curr Opin Microbiol*. 8:46-53.

2. Burnett, S. L., E.L.Mertz, B.Bennie, T.Ford, A.Starobin. 2005. Growth or Survival of *Listeria monocytogenes* in Ready-to-Eat Meat Products and Combination Deli Salads During Refrigerated Storage. *Journal of Food Science*. 70:301-304.

3. Chatterjee, S. S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, S. Machata, E. Domann, T. Chakraborty, and T. Hain. 2006. Intracellular gene expression profile of *Listeria monocytogenes. Infect Immun*. 74:1323-38.

4. Chaturongakul, S., S. Raengpradub, M. Wiedmann, and K. J. Boor. 2008. Modulation of stress and virulence in *Listeria monocytogenes. Trends Microbiol*. 16:388-96.

5. Chen, H. 2007. Temperature-assisted pressure inactivation of *Listeria monocytogenes* in turkey breast meat. *Int J Food Microbiol*. 117:55-60.

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

7. Deutscher, J., C. Francke, and P. W. Postma. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev*. 70:939-1031.

8. Fadda, D., C. Pischedda, F. Caldara, M. B. Whalen, D. Anderluzzi, E. Domenici, and O. Massidda. 2003. Characterization of *divIVA* and other genes located in the chromosomal region downstream of the *dcw* cluster in *Streptococcus pneumoniae. J Bacteriol*. 185:6209-14.

9. Farber, J. M. 2000. Present situation in Canada regarding *Listeria monocytogenes* and ready-to-eat seafood products. *Int J Food Microbiol*. 62:247-51.

10. Gillespie, I. A., J. McLauchlin, K. A. Grant, C. L. Little, V. Mithani, C. Penman, C. Lane, and M. Regan. 2006. Changing pattern of human listeriosis, England and Wales, 2001-2004. *Emerg Infect Dis*. 12:1361-6.

11. Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann. 2003. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. *J Bacteriol*. 185:5722-34.

12. Lin, C. S., C. Wang, H. J. Tsai, and C. H. Chou. 2010. Growth of *Listeria monocytogenes* on a RTE-meat matrix enhances cell invasiveness to mouse J774A.1 macrophages. *Int J Food Microbiol*. 144:199-201.

13. Liu, Y., and A. Ream. 2008. Gene expression profiling of *Listeria monocytogenes* strain F2365 during growth in ultrahigh-temperature-processed skim milk. *Appl Environ Microbiol*. 74:6859-66.

14. Lloyd, T., C. Z. Alvarado, M. M. Brashears, L. D. Thompson, S. R. McKee, and M. Berrang. 2009. Control of *Listeria monocytogenes* in turkey deli loaves using organic acids as formulation ingredients. *Poult Sci*. 88:2235-9.

15. Mbandi, E., and L. A. Shelef. 2002. Automated measurements of antilisterial activities of lactate and diacetate in ready-to-eat meat. *J Microbiol Methods*. 49:307-14.

16. Mijakovic, I., D. Petranovic, B. Macek, T. Cepo, M. Mann, J. Davies, P. R. Jensen, and D. Vujaklija. 2006. Bacterial single-stranded DNA-binding proteins are phosphorylated on tyrosine. *Nucleic Acids Res*. 34:1588-96.

17. Mujahid, S., T. Pechan, and C. Wang. 2008. Protein expression by *Listeria monocytogenes* grown on a RTE-meat matrix. *Int J Food Microbiol*. 128:203-11.

18. Pradhan, A. K., R. Ivanek, Y. T. Grohn, I. Geornaras, J. N. Sofos, and M. Wiedmann. 2009. Quantitative risk assessment for *Listeria monocytogenes* in selected categories of deli meats: impact of lactate and diacetate on listeriosis cases and deaths. *J Food Prot*. 72:978-89.

19. Rosset, P., M. Cornu, V. Noel, E. Morelli, and G. Poumeyrol. 2004. Time-temperature profiles of chilled ready-to-eat foods in school catering and probabilistic analysis of *Listeria monocytogenes* growth. *Int J Food Microbiol*. 96:49-59.

20. Ruusunen, M. a. P., E. 2005. Reducing sodium intake from meat products. *Meat Science*. 70:531-541.

21. Samant, S., H. Lee, M. Ghassemi, J. Chen, J. L. Cook, A. S. Mankin, and A. A. Neyfakh. 2008. Nucleotide biosynthesis is critical for growth of bacteria in human blood. *PLoS Pathog*. 4:e37.

22. Shabala, L., S. H. Lee, P. Cannesson, and T. Ross. 2008. Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels on inactivation kinetics. *J Food Prot*. 71:1169-77.

23. Stoll, R., S. Mertins, B. Joseph, S. Muller-Altrock, and W. Goebel. 2008. Modulation of PrfA activity in *Listeria monocytogenes* upon growth in different culture media. *Microbiology*. 154:3856-76.

24. Tasara, T., and R. Stephan. 2007. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *FEMS Microbiol Lett*. 269:265-72.

25. Thompson, R. J., H. G. Bouwer, D. A. Portnoy, and F. R. Frankel. 1998. Pathogenicity and immunogenicity of a *Listeria monocytogenes* strain that requires D-alanine for growth. *Infect Immun*. 66:3552-61.

26. Toepfl, S., Mathys, A., Heinz, V., Knorr, D. 2006. Potential of High Hydrostatic Pressure and Pulsed Electric Fields for Energy Efficient and Environmentally Friendly Food Processing. *Food Reviews International*. 22:405-423.

27. Vander Heiden, M. G., L. C. Cantley, and C. B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 324:1029-33.

28. Werbrouck, H., K. Grijspeerdt, N. Botteldoorn, E. Van Pamel, N. Rijpens, J. Van Damme, M. Uyttendaele, L. Herman, and E. Van Coillie. 2006. Differential *inlA* and *inlB* expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Appl Environ Microbiol*. 72:3862-71.

29. Yildirim, S., W. Lin, A. D. Hitchins, L. A. Jaykus, E. Altermann, T. R. Klaenhammer, and S. Kathariou. 2004. Epidemic clone I-specific genetic markers in strains of *Listeria monocytogenes* serotype 4b from foods. *Appl Environ Microbiol*. 70:4158-64.

CHAPTER IV

GLOBAL GENE EXPRESSION OF*LISTERIA MONOCYTOENES* TO SALT STRESS[2]

4.1 Abstract

Outbreaks of listeriosis caused by the ingestion of *Listeria*-contaminated ready-to-eat (RTE) foods have been reported worldwide. Many RTE foods, such as deli meat products, contain high amounts of salt, which can disrupt the maintenance of osmotic balance within bacterial cells. To understand how *L. monocytogenes* adapts to salt stress, we examined the growth and global gene expression profiles of *L. monocytogenes* strain F2365 under salt stress using oligonucleotide probe-based DNA array and quantitative real-time PCR (qRT-PCR) analyses. The growth of *L. monocytogenes* in Brain Heart Infusion (BHI) medium with various concentrations of NaCl (2.5, 5, and 10%) was significantly inhibited ($P < 0.01$) when compared to growth in BHI with no NaCl supplementation. Microarray data indicated that growth in BHI medium with 1.2% NaCl up-regulated four genes and down-regulated 24 genes in *L. monocytogenes*, which were confirmed by qRT-PCR. The transcript levels of genes involved in the uptake of glycine betaine/L-proline were increased, whereas genes associated with a putative phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), metabolic enzymes, and virulence factor were down-regulated. Specifically, the transcription levels of PTS transport genes were shown to be dependent on NaCl concentration. To further

[2] Reprint with permission (Appendix) from Bae, D., C. Liu, T. Zhang, M. Jones, S. N. Peterson, and C. Wang. 2012. Global Gene Expression of *Listeria monocytogenes* to Salt Stress. *J Food Prot*, in press.

examine if the down-regulation of PTS genes is related to decreased cell growth, the transcript levels of genes encoding components of Enzyme II, involved in the uptake of various sugars used as the primary carbon source in bacteria, were also measured using qRT-PCR. Our results suggest that the decreased transcript levels of PTS genes may be caused by salt stress or reduced cell growth through salt stress. Here, we report global transcriptional profiles of *L. monocytogenes* in response to salt stress, contributing to an improved understanding of osmotolerance in this bacterium.

4.2 Introduction

The gram-positive bacterium *L. monocytogenes* is a widely-distributed pathogen that causes listeriosis. A recent paper has reported that the hospitalization and mortality rates caused by the food-borne pathogen in the United States reached 94% and 15.9%, respectively *(25)*. *L. monocytogenes* severely affects neonates, pregnant women, elderly men, and immunocompromised patients, often resulting in stillbirths, abortions, meningitis, septicemia, and encephalitis secondary to the initial listeriosis infection *(13, 32)*. This pathogen can withstand extreme conditions, including high acidity, low temperature, high osmolarity, low energy status, oxidative stress, high hydrostatic pressure, and antibacterial agents *(5, 6, 26, 35)*.

In the food industry, salt is often used as a general preservative and an antibacterial agent because of its inhibitory effects on bacterial growth in ready-to-eat (RTE) meat, seafood, and fermented foods (*8*). In addition, salt has been considered an essential additive to enhance flavor, texture, and shelf-life of meat products *(24)*. Salt can cause damage to bacterial cells by disrupting their osmotic balance between the cytoplasmic and extracellular environments (*7*). Specific changes in cellular morphology

and gene expression resulting from the adaption of *L. monocytogenes* to osmotic stress have been documented *(29)*. *L. monocytogenes* can mediate the change in expression of transporter genes such as *betL*, the *gbu* operon, and the *opuC* operon, which encode proteins involved in the transport of glycine, betaine, and carnitine, in order to adapt to osmotic stress *(1)*. In addition, RelA, HtrA, KdpE, LisRK, ProBA, BtlA, and Ctc, proteins potentially involved in osmotic stress resistance, have been identified in *L. monocytogenes* under conditions of high osmolarity *(3, 4, 9, 20, 28, 30, 37)*.

The mechanism by which *L. monocytogenes* adapts to osmotic stress is not fully understood. Because RTE deli meat products typically contain a 1.2% salt concentration, we used this concentration in our microarray experiments to examine the global gene regulation of *L. monocytogenes* under salt stress condition. In addition, to better understand the molecular mechanism by which *L. monocytogenes* adapts to salt stress, we analyzed the growth levels of *L. monocytogenes* strain F2365 at various NaCl concentrations. The objective of this study was to compare differential gene expression profiles of *L. monocytogenes* grown under normal and high salt stress conditions in order to identify genes that allow this bacterium to persist in 1.2% NaCl concentration typically found in RTE foods.

4.3 Materials and methods

4.3.1 Bacterial strain and cultivation

L. monocytogenes serotype 4b strain F2365 frozen in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI) with 20% glycerol at -80°C was used. Fifty microliters of the F2365 frozen stocks were inoculated in 5 ml of BHI broth. The culture was then incubated at 37°C overnight (18 h) as previously described *(19)*. Twenty-five

microliters of the activated bacterial culture (approx. 7.3×10^6 CFU/ml) were inoculated in 5 ml of fresh BHI broth with or without the addition of 1.2% NaCl and incubated at 37°C for 6 h in the MaxQ 4000 orbital shaker (Barnstead/Lab-Line, Dubuque, IA) at 180 rpm until the O.D. reached $A_{600} \approx 0.7$ for microarray analysis. To evaluate cell growth, the A_{600} value was measured after 2, 4, 8, 16, and 24 h of incubation. In addition, CFUs were counted at 2, 4, 8, 16, and 24 h after incubation by plate counts to generate a bacterial growth curve. Data from each time point (two independent experiments performed in quadruplicate) were subjected to an analysis of variance (ANOVA) using SAS 9.1.3 (SAS Institute, Cary, NC). For qRT-PCR to measure the transcription levels of PTS genes (LMOf2365_1038, LMOf2365_0442, LMOf2365_0115, and LMOf2365_0938), twenty-five microliters of the bacterial culture (about 5.9×10^6 CFU/ml) were inoculated in 5 ml of BHI medium with 2.5, 5.0, and 10.0% NaCl or without NaCl and incubated at 37°C with shaking at 180 rpm. Finally, bacterial cells were collected at 6 h after incubation.

4.3.2 RNA isolation and measurement

RNA Protect (Qiagen, Valencia, CA) was added to bacterial cultures at a ratio of 2:1 (v/v). The mixture was incubated for 10 min at RT and centrifuged at 7,000 × g for 10 min at 4°C. Total RNA was extracted from the bacterial pellet as follows: the cell pellet resuspended with Trizol (Invitrogen, Carlsbad, CA) and 1 ml of the resulting solution was transferred to a Lysing Matrix B tube (MP Biomedicals, Solon, Ohio), disrupted by shaking for 5 min using the Disruptor Genie (Scientific Industries, Inc., Bohemia, NY), and incubated at RT for 10 min. Total RNA was then extracted and purified using the RNeasy Mini Kit (Qiagen). The purity and concentration of RNA was determined using

the Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, DE) and a Nanodrop ND1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA Integrity Numbers of all RNA samples were greater than 8.0, and the range of the OD ratios at A_{260}/A_{280} nm and $A_{260}/_{230}$ nm were between 1.8 and 2.1.

4.3.3 Microarray assay

Aminoallyl (aa)-labeled *L. monocytogenes* serotype 4b strain F2365 microarray slides (Version 3) were provided by the Pathogen Functional Genomics Resource Center (PFGRC). The slides consist of spots containing 70-mer oligonucleotides probe that represents 2847 open reading frames (ORFs) in the *L. monocytogenes* (GenBank Accession no. AE017262). cDNAs were synthesized from the purified *L. monocytogenes* RNA using a slightly modified microarray protocol provided by the Institute for Genomic Research (TIGR) [http://pfgrc.jcvi.org/index.php/microarray/protocols.html]. Briefly,2.5 µg of RNA was reverse transcribed using 2 µl of Smart reverse transcriptase (Clontech, Palo Alto, CA), 1 µl of random hexamers in the presence of 0.1 M dithiothreitol (DTT, Invitrogen), and a 25 mM dNTPs containing a 3:2 ratio of aa-dUTP and dTTP (Ambion, Austin, TX). The mixture was incubated overnight at 42°C. The resulting cDNA was purified, and unincorporated aa-dUTP was removed using a MinElute PCR purification kit (Qiagen) following the manufacturer's instructions. The cDNA purification, labeling, and reading were carried out as described by Bae et al. *(2)*.

4.3.4 Microarray data analysis

The hybridized slides were scanned at 10 µm resolution within the range of the photomultiplier tube between 650 and 750 on two channels using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA). The TM4 including Spotfinder,

Ginkgo and Magnolia softwares developed by TIGR was used. Image files from the slides were analyzed using the Spotfinder. The signal intensity of spots on the slides was adjusted and quantified by background subtraction, and data normalization was performed using Ginkgo. Magnolia 1.2, which exports the data in SOFT format, was used for depositing data into the Gene Expression Omnibus (GEO). The data from the normalized ratio of query to reference signal for each spot were transformed to a $\log_2$ scale. Data were obtained from three biological replicates with four technical replicates (n = 12). Each independent experiment included a set of flip dye assays to observe dye bias during cDNAs labeling. The means of each gene on the 12 slides were transformed to $\log_2$ scale, and the $\log_2$ ratio for each gene was analyzed by the Student's t-test. Differences of ≥ 2-fold changes in gene expression levels were considered significant (P < 0.05). An annotation file provided by TIGR was used (ftp://ftp.jcvi.org/pub/data/PFGRC/MAIN/microarray/annotation/L_monocytogenes/version3/L_monocytogenes_3_4b_F2365.ann). The microarray data are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19570.

4.3.5 Quantitative real-time reverse transcription PCR

To confirm the microarray data, LMOf2365_0914, LMOf2365_2479, LMOf2365_2694, and several significantly up- and down-regulated genes were selected for qRT-PCR analysis. For qRT-PCR to measure the transcription levels of PTS genes, LMOf2365_1038, LMOf2365_0442, LMOf2365_0115, and LMOf2365_0938 were also used to investigate a correlation between the growth and the transcription levels of PTS genes of *L. monocytogenes*. Primers were designed using Gene Runner software (http://www.generunner.net/) and purchased from MWG Biotech Inc. (Huntsville, AL).

Primer sequences for target genes are shown in Table 4.1. The cDNA was synthesized from 1 μg of the same RNA samples. Reverse transcription PCR was performed using Taq Man Reverse Transcription Reagents with random hexamers (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The cDNA concentration was quantified by measuring OD at A_{260} nm with ssDNA-33 option using a Nanodrop spectrophotometer (Nanodrop Technologies). qRT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) by the manufacturer's instructions. Approximately 100 ng of cDNA, 1X SYBR Green master mix, and 250 nM of forward and reverse primers were used in 25 μl final volume for qRT-PCR. Amplification was performed as follows: initial incubation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 55-60°C for 30 sec, and 72°C for 30 sec for PCR. mRNA expression levels were determined using the average Ct values of *gap* and *tuf* as internal controls to normalize gene expression *(33, 36)*. Data were analyzed by ANOVA followed by a Tukey test. Three and two independent experiments using quadruplicate RNA samples were conducted for validating microarray data and measuring mRNA expression levels of PTS genes, respectively. The correlation coefficient (*R*) between data from the microarray and qRT-PCR experiments was analyzed using SAS 9.1.3.

4.4 Results

The differential global gene expression levels of *L. monocytogenes* in response to salt stress were examined in the cells grown in BHI medium or in BHI supplemented with 1.2% NaCl. Based on microarray data, four genes were up-regulated and 24 genes were down-regulated in *L. monocytogenes* grown in medium supplemented with 1.2% NaCl. These genes were grouped into predicted functional categories according to the

annotation of *L. monocytogenes* strain F2365 provided by TIGR. To validate the microarray analysis, qRT-PCR was performed for several genes significantly up- or down-regulated, as well as those that remained unchanged (Table 4.1). The correlation coefficient between qRT-PCR and microarray analysis was found to be $R = 0.92$ (Figure 4.1), suggesting that the microarray analysis was valid.

Results show that the transcript levels of LMOf2365_1035, LMOf2365_1036, and LMOf2365_1037, loci associated with the uptake of glycine betaine, were up-regulated at 2.82-, 2.64-, and 2.31-fold, respectively in the presence of 1.2% NaCl (Table 4.2). Twenty-four down-regulated genes in *L. monocytogenes* grown under the salt stress condition are shown in Table 3.3. At least seven of these down-regulated genes (LMOf2365_0420, LMOf2365_0440, LMOf2365_0442, LMOf2365_0444, LMOf2365_0937, LMOf2365_2645, and LMOf2365_2647) are associated with a phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), LMOf2365_0421, LMOf2365_0445, and LMOf2365_0939, loci involved in the biosynthesis and degradation of polysaccharides for energy metabolism, were also down-regulated. The proteins encoded by LMOf2365_0421, LMOf2365_0445, and LMOf2365_0939 were classified in glycosyl hydrolase family 38 or as a β-glucosidase that catalyzes the cleavage of glucosidic bonds in β-D-glucosides. Transcript levels of LMOf2365_2644, associated with fermentation, were also down-regulated. Interestingly, the transcript level of LMOf2365_0282 (*inlD*) encoding internalin D associated with pathogenesis was decreased. The transcripts for LMOf2365_1147, LMOf2365_2096, LMOf2365_2097, and LMOf2365_2618 encoding conserved hypothetical proteins were significantly down-regulated. LMOf2365_0422 containing a PTS regulatory domain (PRD/PTS system IIA 2 domain protein), LMOf2365_2648 (PTS system IIA 2 domain

protein), LMOf2365_2620 (phosphotriesterase family protein), and LMOf2365_2788 (similar to dehydrogenase) were also down-regulated. However, the functions of these proteins or the putative enzymes are still unknown.

The growth of *L. monocytogenes* in media with various concentrations of NaCl (2.5, 5, and 10%) was significantly inhibited ($P < 0.01$, ANOVA) [Figure 3.2]. Along with reduced cell growth, the decreased transcript levels of PTS genes, LMOf2365_1038, LMOf2365_0442, LMOf2365_0115, and LMOf2365_0938**,** encoding Enzyme II cytoplasmic subunits for the transport of major carbon sources (glucose, fructose, mannose, and cellobiose) are shown in Figure 3.3. The relative transcriptional levels of the PTS genes (except LMOf2365_0115) were shown to be salt-concentration dependent.

4.5 Discussion

Salt can be used to inhibit bacterial growth or enhance the flavor, texture, and shelf-life of food products. However, *L. monocytogenes* has the ability to adapt to osmotic stress by modulating transcription levels of transporter genes such as *betL*, *gbu*, and the *opuC* operons (*1, 11, 14*). Other studies also revealed that osmotic stress induces elevated uptake of glycine betaine in *L. monocytogenes* by the activation of the *Gbu* porter (*14, 18*). According to our microarray data, the transcripts of LMOf2365_1035 (glycine betaine/L-proline ABC transporter, ATP-binding protein), LMOf2365_1036 (glycine betaine ABC transporters, permease), and LMOf2365_1037 (glycine betaine/L-proline ABC transporter, glycine betaine/L-proline-binding protein) associated with the uptake of glycine betaine were up-regulated under 1.2% salt stress condition (Table 3.2). The data indicate that these up-regulated transporter genes may be involved in the osmotolerance of *L. monocytogenes* grown in medium with 1.2% NaCl, a common salt

concentration found in many RTE foods. In general, a transcriptional or phenotypic response of *L. monocytogenes* exposed to 1.2% salt in BHI broth (liquid) or on RTE deli meat (solid phase) would be different. Therefore, a fixed concentration of salt used in BHI medium would be required to consider when translating this to an experiment on RTE deli meat regarding a response difference of *L. monocytogenes* by different physical phases.

We also found that many genes down-regulated during osmotic stress are associated with PTS components, metabolic enzymes, and transport and binding proteins (Table 3.3). Specifically, several of the down-regulated genes identified in this study (LMOf2365_0420, LMOf2365_0440, LMOf2365_0442, LMOf2365_0444, LMOf2365_0937, LMOf2365_2645, and LMOf2365_2647) are associated with the PTS pathway, which is involved in the transport and phosphorylation of sugars as carbon sources, such as glucose, mannose, and cellobiose *(10, 15, 21, 23)*. Therefore, PTS is an important and distinct means for extracellular sugar uptake using energy from phosphoenopyruvate. The system includes cytoplasmic components and membrane-integrated proteins, enzyme I (EI), histidine protein (HPr), and enzyme II (EII). The PTS is a complex system that can be controlled by signal transduction, metabolic status, or the phosphorylation status of various PTS components *(34)*. In both our microarray and qRT-PCR data, the transcript level of *ptsH* was not significantly decreased, but the transcription level of *ptsH* tended to be down-regulated in response to NaCl concentration. Marr et al. *(17)* demonstrated that PTS activity was decreased by the overexpression of *prfA* in *L. monocytogenes* grown in glucose-supplemented minimal medium, suggesting that *prfA*, a transcriptional regulator in virulence, interferes with PTS-dependent sugar uptake and carbon source availability. Other groups also

demonstrated that down-regulation of *prfA* was observed in *L. monocytogenes* grown in the presence of PTS sugars *(12, 31)*. These studies showed growth changes resulting from an interaction between PTS and *prfA* activities. However, our data showed no such correlation between PTS and *prfA* gene expression levels.

The growth of *L. monocytogenes* was significantly inhibited when incubated in media with various concentrations of NaCl, as indicated in Figure 4.2. Genes encoding proteins associated with the pathways for β-glucosides (LMOf2365_0030), galactitol (LMOf2365_2645, LMOf2365_2647), and a putative sugar (LMOf2365_0440) uptake as well as transporters for the uptake of fructose (LMOf2365_0444), cellobiose (LMOf2365_0937), and an unknown substrate (LMOf2365_2807) were significantly down-regulated with other decreased transcripts of PTS genes (Table 4.3). These data led us to determine if the down-regulation of PTS genes is related to decreased cell growth. Thus, we examined bacterial cell growth using BHI containing various NaCl concentrations and measured transcription levels of genes encoding components of EII involved in the uptake of glucose, fructose, mannose, and cellobiose used as the primary carbon source in bacteria by qRT-PCR. Interestingly, our results indicated that the transcription levels of PTS transport genes (LMOf2365_1038, LMOf2365_0442, and LMOf2365_0938 except LMOf2365_0115) were dependent on NaCl concentration (Figure 4.3). Along with decreased cell growth under high salt concentration, the decreased transcript levels of PTS genes may imply that salt stress may inhibit *L. monocytogenes* growth by modulating the expression of PTS components and transport genes, resulting in the decreased carbohydrate uptake and availability. In addition, our data indicate that only LMOf2365_1038 among the PTS genes seemed to be cell growth-dependent regarding a changes of the expression of PTS genes by reduced cell growth.

The transcript of the gene was steadily increased during log growth phases. The data suggest that down-regulation of PTS genes in *L. monocytogenes* under salt stress can be caused by an effect of reduced cell growth through salt stress.

L. monocytogenes can invade non-phagocytic cells using surface proteins called internalins. *inlA* and *inlB* play major roles in the entry into host cells (*16*, *27*). Our microarray data showed that the transcription of *inlD* (-2.34-fold) but not *inlA* (-1.66-fold) and *inlB* (-0.76-fold) was significantly reduced by osmotic stress; however, the function of *inlD* associated with intracellular infection of human host cells is still unclear. We also examined changes in virulence factor transcripts of *L. monocytogenes* in response to salt stress. There were no significant changes in the transcription levels of *prfA, sigB, inlA, inlB, plcA, plcB, actA,* or *hly*. To our knowledge, this is the first report detailing the effects of salt stress on the expression of genes involved in PTS and its related metabolic enzymes in *L. monocytogenes*. Data from this study may provide aid in determining the mechanism of osmotolerance in *L. monocytogenes* under conditions typically present in RTE foods. Further studies into the inhibitory effects of high salt concentrations against *L. monocytogenes* are needed to elucidate the potential relationship between salt stress and the functions of various PTS pathway components.

4.6 Acknowledgments

This work was supported by grants from USDA/ARS (#58-6402-7-230) and the Mississippi Agriculture and Forestry Experiment Station. We thank Dr. Michael Crowley for assistance with scanning images, Dr. Jerry Li for data analysis, and Dr. Stephen Pruett for his valuable review and suggestions.

Table 4.1 Quantitative real time RT-PCR primers used in salt study

Gene	Locus	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')	References
tuf	LMOf2365_2632	CTGAAGCTGGCGACAACA	CTTGACCACGTTGGATATCTTCAC	*(36)*
gap	LMOf2365_2432	ACCAGTGTAAGCGTGAA	TCACAGCGCAAGACAAA	*(33)*
sigB	LMOf2365_0914	TCATCGGTGTCACGGAAGAA	TGACGTTGGATTCTAGACAC	this study
InlD	LMOf2365_0282	GCTGGTATGATGCGAAAACT	CGTTGTTGGGTCTCATTGGA	this study
gbuB	LMOf2365_1036	TGGTATTTGGATGGCGAA	CAATTACGACCATGGAAAGT	this study
ftsX	LMOf2365_2479	TGTTTGTGTGGGCTCTTTAG	ATTTAGGACTGTAGGAAGAC	this study
	LMOf2365_1035	TTGAAAAAGATGGTCCTCG	ATCTTCGGTTACAGCAATCG	this study
	LMOf2365_0440	ACAACTCCGATGATAACAGC	TGGGGACTTCGATTAGCG	this study
	LMOf2365_0442	GAAGAAATGGCAGAAATG	GTCAGAATCAGTAATCGCCA	this study
	LMOf2365_2694	ATCAGTTAATGTTGCGGTG	CATTCTCATTGGCAGCA	this study
	LMOf2365_2644	ATAATGCAAAGTCGCCCGCT	AAAGATCAAGTTCGTGTCG	this study
	LMOf2365_1038	GGCTTAGAAACCGTATCCTT	CCTGCTTCTGCCTTAGTTAC	this study
	LMOf2365_0115	GCAGAAGTTGATGATGTAGCAAGCCGAATTCTTGTGTATACC		this study
	LMOf2365_0938	ACGCAAGAAATGACCTAGTG	GCTGTCATGAGTGTATCTTG	this study

Table 4.2 Classification of up-regulated genes of *L. monocytogenes* strain F2365 grown in BHI medium with 1.2% NaCl

Category and gene[1]	Products [2]	Fold change [3]	*P*-value
Transport and binding proteins: Amino acids, peptides and amines			
LMOf2365_1035	glycine betaine/L-proline ABC transporter, ATP-binding protein	2.82	<.001
LMOf2365_1036	highly similar to glycine betaine ABC transporters (permease)	2.64	0.001
LMOf2365_1037	glycine betaine/L-proline ABC transporter, glycine betaine/L-proline-binding protein	2.31	0.017
Protein synthesis: Ribosomal proteins: synthesis and modification			
LMOf2365_2602	ribosomal protein L2	2.32	0.003

[1, 2] Category for genes and products are based on annotations provided by TIGR.
[3] The fold change was calculated by using delta-delta Ct method. Data were obtained from three independent experiments using quadruplicate RNA samples (n = 12).

Table 4.3 Classification of down-regulated genes of *L. monocytogenes* strain F2365 grown in BHI medium with 1.2% NaCl

Category and gene[1]	Products [2]	Fold change [3]	*P*-value
Energy metabolism: Biosynthesis and degradation of polysaccharides			
LMOf2365_0421	glycosyl hydrolase, family 38	-3.21	<.001
LMOf2365_0445	glycosyl hydrolase, family 38	-3.49	<.001
LMOf2365_0939	β-glucosidase	-3.23	<.001
Energy metabolism: Fermentation			
LMOf2365_2644	alcohol dehydrogenase, zinc-dependent	-2.93	<.001
Energy metabolism: Amino acids and amines			
LMOf2365_2619	putative creatinine amidohydrolase	-2.68	0.017
Transport and binding proteins: Carbohydrates, organic alcohols, and acids			
LMOf2365_0030	similar to PTS system, β-glucosides specific enzyme IIABC component	-3.12	<.001
LMOf2365_0420	PTS system, IIABC component, degenerate	-2.96	<.001
LMOf2365_0440	similar to *Staphylococcus xylosus* glucose uptake protein	-2.74	<.001
LMOf2365_0442	PTS system, fructose-specific, IIA component	-3.18	<.001
LMOf2365_0444	PTS system, fructose-specific, IIC component	-3.17	<.001
LMOf2365_0937	PTS system, IIC component	-2.87	<.001
LMOf2365_2645	similar to PTS system galactitol-specific enzyme IIC component	-2.83	<.001
LMOf2365_2647	PTS system, IIA component	-3.00	<.001
Transport and binding proteins: Unknown substrate			
LMOf2365_2807	major facilitator family transporter	-2.55	0.027
Protein fate: Degradation of proteins, peptides, and glycopeptides			
LMOf2365_2808	putative carboxypeptidase	-3.84	<.001
Cellular processes: Pathogenesis			
LMOf2365_0282	internalin D	-2.46	0.001
Hypothetical proteins: Conserved			
LMOf2365_1147	conserved hypothetical protein	-2.41	0.004
LMOf2365_2096	conserved hypothetical protein	-2.66	<.001
LMOf2365_2097	conserved hypothetical protein	-2.31	0.001
LMOf2365_2618	conserved hypothetical protein	-2.80	0.007

Table 4.3 (continued)

Category and gene[1]	Products [2]	Fold change [3]	*P*-value
Unknown function: General			
LMOf2365_0422	PRD/PTS system IIA 2 domain protein	-2.85	<.001
LMOf2365_2648	PTS system IIA 2 domain protein	-2.95	<.001
Unknown function: Enzymes of unknown specificity			
LMOf2365_2620	phosphotriesterase family protein	-2.82	0.013
LMOf2365_2788	similar to dehydrogenase	-2.96	<.001

[1, 2] Category for genes and products are based on annotations provided by TIGR.
[3] The fold change was calculated by using delta-delta Ct method. Data were obtained from three independent experiments using quadruplicate RNA samples (n = 12).

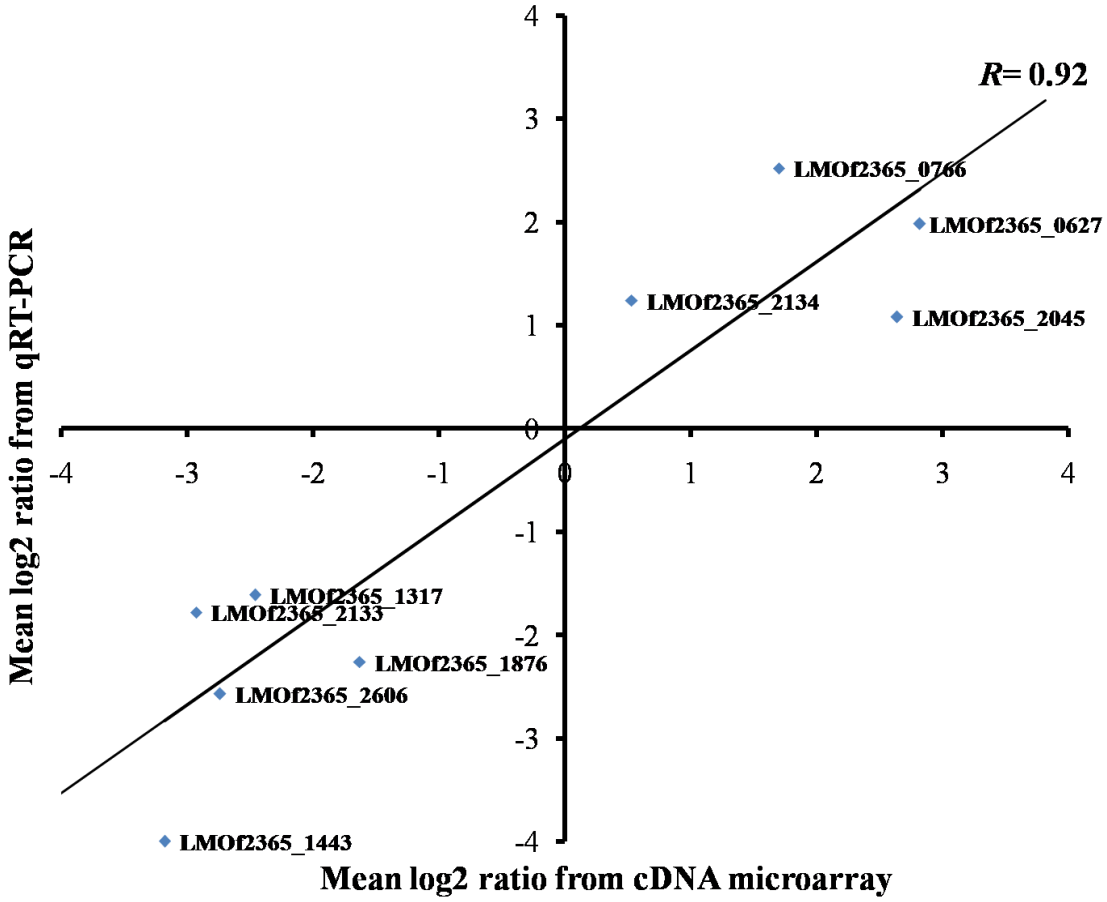

Figure 4.1 Validation of microarray analysis with quantitative real-time RT-PCR.

The x-axis indicates the log2 ratio from the microarray analysis. Y-axis indicates the log2 ratio from the qRT-PCR analysis. Data were obtained from three independent experiments using quadruplicate RNA samples per each experiment for microarray analysis (n = 12).

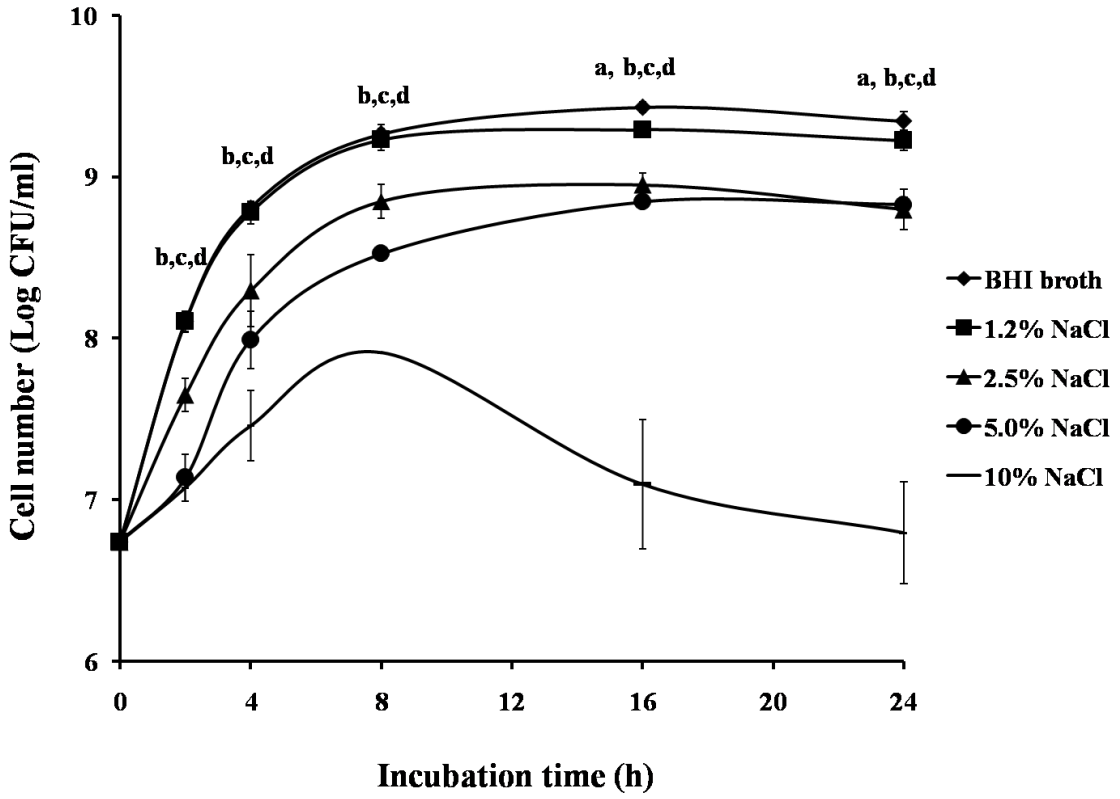

Figure 4.2 The growth of *L. monocytogenes* strain F2365 in BHI medium and the medium supplemented with 1.2, 2.5, 5, or 10% NaCl.

Data were obtained from two independent experiments via plate counts at different time points ($n = 8$). Values represent the mean ± SEM. The growth of *L. monocytogenes* was significantly decreased by salt stress after 16 h incubation compared to all the other groups with various salt concentrations ($P < 0.05$, ANOVA). Each point represents P value(s) as indicated: [a] $P < 0.05$, control vs. 1.2% NaCl; [b] $P < 0.05$, control vs. 2.5% NaCl; [c] $P < 0.05$, control vs. 5.0% NaCl; [d] $P < 0.05$, control vs. 10.0% NaCl.

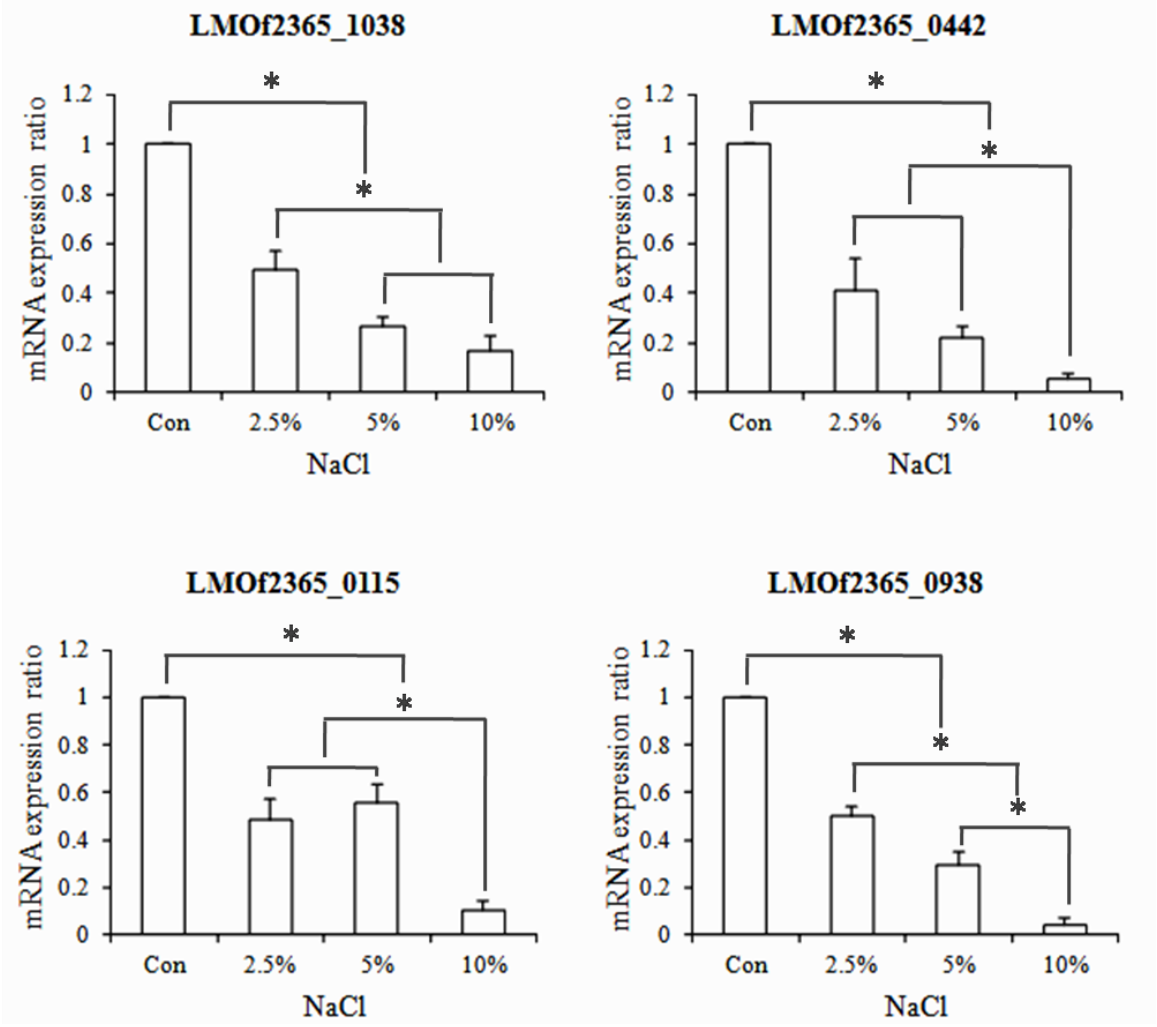

Figure 4.3 The ratio of mRNA expression level of PTS transport genes involved in sugar uptake.

Cells were grown in BHI medium without NaCl (control) and with 2.5, 5, and 10% of NaCl. Bacterial cells were collected to extract total RNA at 6 h after incubation. LMOf2365_1038, LMOf2365_0442, LMOf2365_0115, and LMOf2365_0938 are components of EII involved in the uptake of glucose, fructose, mannose, and cellobiose, respectively. Values are expressed as fold change from the control (value 1.0). $n = 8$; bar = SEM. The symbol (*) represents that means are significantly different between groups ($P < 0.05$, ANOVA).

4.7 References

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

2. Bae, D., M. Crowley, and C. Wang. 2011. Transcriptome analysis of *Listeria monocytogenes* grown on a ready-to-eat meat matrix. *J Food Prot*. 74:1104-1111.

3. Begley, M., C. Hill, and C. G. Gahan. 2003. Identification and disruption of *btlA*, a locus involved in bile tolerance and general stress resistance in *Listeria monocytogenes. FEMS Microbiol Lett*. 218:31-38.

4. Brondsted, L., B. H. Kallipolitis, H. Ingmer, and S. Knochel. 2003. *kdpE* and a putative RsbQ homologue contribute to growth of *Listeria monocytogenes* at high osmolarity and low temperature. *FEMS Microbiol Lett*. 219:233-239

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

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

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

8. Desmond, E. 2006. Reducing salt: A challenge for meat industry. *Meat Science*. 74:188-196.

9. Gardan, R., O. Duche, S. Leroy-Setrin, and J. Labadie. 2003. Role of *ctc* from *Listeria monocytogenes* in osmotolerance. *Appl Environ Microbiol*. 69:154-161.

10. Geerse, R. H., F. Izzo, and P. W. Postma. 1989. The PEP: fructose phosphotransferase system in *Salmonella typhimurium*: FPr combines enzyme IIIFru and pseudo-HPr activities. *Mol Gen Genet*. 216:517-525.

11. Gerhardt, P. N., L. T. Smith, and G. M. Smith. 1996. Sodium-driven, osmotically activated glycine betaine transport in *Listeria monocytogenes* membrane vesicles. *J Bacteriol*. 178:6105-6109.

12. Gilbreth, S. E., A. K. Benson, and R. W. Hutkins. 2004. Catabolite repression and virulence gene expression in *Listeria monocytogenes. Curr Microbiol*. 49:95-98.

13. Gillespie, I. A., J. McLauchlin, K. A. Grant, C. L. Little, V. Mithani, C. Penman, C. Lane, and M. Regan. 2006. Changing pattern of human listeriosis, England and Wales, 2001-2004. *Emerg Infect Dis*. 12:1361-136.

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

15. Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate Bound to Histidine in a Protein as an Intermediate in a Novel Phospho-Transferase System. *Proc Natl Acad Sci U S A*. 52:1067-1074.

16. Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *EMBO J*. 18:3956-3963.

17. Marr, A. K., B. Joseph, S. Mertins, R. Ecke, S. Muller-Altrock, and W. Goebel. 2006. Overexpression of PrfA leads to growth inhibition of *Listeria monocytogenes* in glucose-containing culture media by interfering with glucose uptake. *J Bacteriol*. 188:3887-3901.

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

19. Mujahid, S., T. Pechan, and C. Wang. 2008. Protein expression by *Listeria monocytogenes* grown on a RTE-meat matrix. *Int J Food Microbiol*. 128:203-211.

20. Okada, Y., S. Makino, T. Tobe, N. Okada, and S. Yamazaki. 2002. Cloning of *rel* from *Listeria monocytogenes* as an osmotolerance involvement gene. *Appl Environ Microbiol*. 68:1541-1547.

21. Parker, C., and R. W. Hutkins. 1997. *Listeria monocytogenes* Scott A transports glucose by high-affinity and low-affinity glucose transport systems. *Appl Environ Microbiol*. 63:543-546.

22. Reizer, J., A. Reizer, M. J. Merrick, G. Plunkett, 3rd, D. J. Rose, and M. H. Saier, Jr. 1996. Novel phosphotransferase-encoding genes revealed by analysis of the *Escherichia coli* genome: a chimeric gene encoding an Enzyme I homologue that possesses a putative sensory transduction domain. *Gene*. 181:103-108.

23. Romano, A. H., J. D. Trifone, and M. Brustolon. 1979. Distribution of the phosphoenolpyruvate:glucose phosphotransferase system in fermentative bacteria. *J Bacteriol*. 139:93-97.

24. Ruusunen, M., and E. Puolanne. 2005. Reducing sodium intake from meat products. *Meat Science*. 70:531-541.

25. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. *Emerg Infect Dis*. 17:7-15.

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

27. Shen, Y., M. Naujokas, M. Park, and K. Ireton. 2000. InIB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell*. 103:501-510.

28. Sleator, R. D., C. G. Gahan, and C. Hill. 2001. Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. *Appl Environ Microbiol*. 67:2571-2577.

29. Sleator, R. D., and C. Hill. 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS Microbiol Rev*. 26:49-71.

30. Sleator, R. D., and C. Hill. 2005. A novel role for the LisRK two-component regulatory system in listerial osmotolerance. *Clin Microbiol Infect*. 11:599-601.

31. Stoll, R., S. Mertins, B. Joseph, S. Muller-Altrock, and W. Goebel. 2008. Modulation of PrfA activity in *Listeria monocytogenes* upon growth in different culture media. *Microbiology*. 154:3856-3876.

32. Swaminathan, B., and P. Gerner-Smidt. 2007. The epidemiology of human listeriosis. *Microbes Infect*. 9:1236-1243.

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

34. Tetsch, L., and K. Jung. 2009. How are signals transduced across the cytoplasmic membrane? Transport proteins as transmitter of information. *Amino Acids*. 37:467-477.

35. Toepfl, S., A.Mathys, V.Heinz, and D.Knorr. 2006. Potential of High Hydrostatic Pressure and Pulsed Electric Fields for Energy Efficient and Environmentally Friendly Food Processing. *Food Rev Int*. 22:405-423.

36. Werbrouck, H., K. Grijspeerdt, N. Botteldoorn, E. Van Pamel, N. Rijpens, J. Van Damme, M. Uyttendaele, L. Herman, and E. Van Coillie. 2006. Differential *inlA* and *inlB* expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Appl Environ Microbiol*. 72:3862-3871.

37. Wonderling, L. D., B. J. Wilkinson, and D. O. Bayles. 2004. The *htrA* (*degP*) gene of *Listeria monocytogenes* 10403S is essential for optimal growth under stress conditions. *Appl Environ Microbiol*. 70:1935-1943.

CHAPTER V

A NOVEL GENE OF *LISTERIA MONOCYTOENES*, *lcp*, ASSOCIATED WITH ATTACHMENT TO VGEGETABLES AND FRUITS

5.1 Abstract

A study to determine factors involved in attachment of *L. monocytogenes* serotype 4b strain F2365 (WT) to leafy vegetables and fruits was conducted. To evaluate candidate proteins involved in adhesion to lettuce leaf, 32 genes encoding surface proteins and lipases of the strain were screened using quantitative qRT-PCR. Among 32 genes, transcription levels of LMOf2365_0413, LMOf2365_0498, LMOf2365_0859, LMOf2365_2052, and LMOf2365_2812 were significantly up-regulated on lettuce leaf. *In silico* analysis showed that LMOf2365_0859 has a putative cellulose binding domain. Thus, we hypothesized that this gene may be involved in attachment to leafy vegetables and named the gene *Listeria* Cellulose-binding Protein (LCP). *lcp* mutant (Δ*lcp*) and *lcp* complement (Δ*lcp*+pMAD_*lcp*) strains were generated using homologous recombination events. The attachment of WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp* on lettuce leaves was determined. Results showed that the attachment of WT to lettuce was significantly higher than Δ*lcp*. The attachment of the complement strain was not significantly different from the WT. Similar results were observed in other fresh food such as baby spinach and cantaloupe. Fluorescence microscopy and field emission scanning microscopy analysis further support these findings. Binding ability to cellulose was determined by using cellulose acetate-coated plates. Results showed that a binding ability of Δ*lcp* was

significantly lower than the WT. Therefore, these results strongly suggest that LCP plays an important role in its attachment ability to vegetables and fruits. Results of this study help us to understand the attachment mechanism by *L. monocytogenes* to vegetables and fruits. The information can contribute to the development of strategies in vegetables decontamination, preservation, or storage.

5.2 Introduction

L. monocytogenes is a life-threatening food borne pathogen that attributes to the highest rates in hospitalization (94.0%) and mortality (15.9%) among 31 pathogens including major food-borne pathogens, such as *Salmonella*, *E. coli*, *Campylobacter*, and *Clostridium* (*32*). *L. monocytogenes* is an opportunistic and zoonotic bacterium found in the natural environments and foods and it has an ability to survive under extreme conditions such as high acidity, low temperature, high osmolarity, and high hydrostatic pressure (*2, 4, 14, 15*). Epidemiologic studies on human listeriosis have shown that the various concentrations of *L. monocytogenes* were detected from RTE food products collected from patient's refrigerators, retail stores, or plants (*19*). Most cases of human listeriosis have been linked to the consumption of RTE products contaminated with *L. monocytogenes*. *L. monocytogenes* serotypes 4b, 1/2a, and 1/2b of the 13 serotypes are mainly associated with human listeriosis outbreaks in susceptible individuals (*25, 37*). Numerous studies have been conducted on the adhesion, invasion, and/or virulence regulation of *L. monocytogenes* in animal hosts and their derived food products as vehicles of human listeriosis (*6, 9, 10, 13, 17*). However, studies on basic molecular mechanisms of *L. monocytogenes* attaching or adhering to RTE or vegetables are rather limited. Studies have shown the attachment of *L. monocytogenes* and *Salmonella enterica*

to intact or cut cabbage and lettuce leaves (*18, 28*). Therefore, the purpose of this study was to indentify genes associated with the attachment of *L. monocytogenes* on the leaf surface of raw vegetables and fruits.

The pathogen has a multilayered cell wall linked to peptidoglycan, to which LPXTG surface proteins are anchored. Based on genomic and proteomic data analysis, more than 40 genes encoding surface or surface-anchored proteins, which interact with host tissues or cells are found in *L. monocytogenes* (*8, 16, 26*). In particular, the roles of virulence and surface proteins (i.e. SigB, PrfA, ActA, InlA, InlB, InlC, InlH, or LPXTG family) of *L. monocytogenes* on pathogenesis have been well characterized in different hosts and cell types (*12, 22, 24, 30, 31, 35*). These studies have demonstrated that *L. monocytogenes* utilizes a specific host-parasite interaction that is mediated by a specific interaction between listerial surface proteins (ligands) and host cell receptors.

Currently, the consumption of fresh or minimally-processed vegetables has increased annually due to a change in diet habits (*29*). According to the Economic Research Service of United States Department of Agriculture (ERS/USDA) report in 2004, the US per capita consumption of fresh vegetables has increased approximately 51% from 52.0 kg in 1976 to 78.6 kg in 2003 (http://ucce.ucdavis.edu/files/datastore/234-66.pdf). Survey and epidemiology studies reported that *L. monocytogenes* has been found in raw or minimally processed vegetables, such as cabbage, broccoli, bean sprouts, cucumber, lettuce, peppers, and potatoes in many countries (*7*). Moreover, outbreaks of human listeriosis associated with ingestion of shredded cabbage, diced celery, lettuce, and tomatoes contaminated with the pathogen has been reported *(21, 33*). It has been shown that *L. monocytogenes* can grow in vegetables stored at refrigerated temperatures (*15*). Other studies also demonstrated that vegetables (cabbage, lettuce, sprouts, etc.) can

be served as vehicles for human listeriosis due to ingestion of *L. monocytogenes*-contaminated fresh vegetables or salads (*1, 7, 21, 34*). Recently, a deadly outbreak of human listeriosis led to 30 deaths and 1 miscarrage in a multi-state outbreak was caused by contaminated cantaloupes (http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html).

Despite of the increase in consumption of vegetables and outbreaks of human listeriosis associated with the ingestion of vegetables and fruits, less is known about survival, growth, and virulence factors of *L. monocytogenes* on vegetables in comparison to meat products. Transcription of listerial surface proteins was found to be highly increased when *L. monocytogenes* was cultured on vegetables. Of interest, one of up-regulated genes (LMOf2365_0859) contains a putative cellulose binding domain (CBD). We named this gene as *Listeria* Cellulose binding Protein (LCP) and investigated the role of LCP in attachment to vegetables and fruits.

5.3 Materials and methods

5.3.1 Bacterial strain and cultivation

L. monocytogenes strain serotype 4b F2365 was grown and maintained in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) with 20% glycerol at -80°C until use. Thirty microliters of F2365 frozen stock was dispensed into 14 ml Polystyrene Round-Bottom Tube (BD Falcon, Franklin Lakes, NJ) containing 3 ml of BHI broth and were incubated at 37°C for overnight. Thirty microliters of the activated bacterial culture (approximately 1.0×10^6 CFU/ml) was added to 3 ml of a fresh BHI medium and incubated at 37°C for 16 h in a MaxQ™ 4000 incubating and orbital shaker (Barnstead/Lab-Line, Dubuque, IA) at 180 rpm.

5.3.2 Vegetables and fruit preparation

Fresh iceberg lettuce heads, bagged baby spinach, and cantaloupes were purchased from a local retail grocery and stored at 4°C until used within 2 days after purchase. The adaxial side of about five inner iceberg lettuce and baby spinach leaves were used. The edge of leaves far from the stem was cut to an appropriate size (5 × 5 cm or 1 × 1 cm). Cantaloupe skin was cut (1 × 1 cm) into thin pieces. Cut vegetable leaves and cantaloupe skin were washed with sterile phosphate buffered saline (PBS, pH 7.4) [Invitrogen, Gland Island, NY] three times before inoculation. Washed vegetable leaves and cantaloupe skins used in this study were negative for bacterial culture. No contamination in the homogenized samples was found on a growth agar plate.

5.3.3 Bacterial growth, collection, and RNA isolation

Two-hundred microliters (1.55×10^7 CFU) of the overnight cultured inoculums was suspended in 5 ml of PBS (as a control) or spread on lettuce leaf (about 5 × 5 cm) using Whirl-Pak Bags (Nasco, Fort Atkinson, WI). The inoculated leaves or PBS containing bacteria were then incubated at 4°C up to 4 days. To determine the growth curve, bacterial numbers were enumerated at 0, 8, 16, 24, 48, 72, and 96 h after incubation via a standard plate count. To determine differential expressed genes encoding for surface proteins and lipases, bacterial cells were collected at 8 and 16 h. After incubation the lettuce leaves were gently washed with 25 ml of PBS. Supernatant containing unattached bacterial cells was discarded. Finally, lettuce leaves with attached listerial cells were vigorously washed with 25 ml of PBS using a Mini Vortex Mixer™ (VWR, Radnor, PA). The bacterial suspension from the washed lettuce leaves or PBS control was mixed with RNA Protect® (Qiagen, Valencia, CA) at a ratio of 1:2 (bacterial suspension : RNA Protect). The mixtures were incubated for 10 min at RT and

centrifuged at 7,000 × g at 4°C for 10 min. Cells were resuspended using 1ml of Trizol™ (Invitrogen, Carlsbad, CA), transferred to Lysing Matrix B tube (MP Biomedicals, Germany), disrupted by shaking for 5 min using the Disruptor Genie® (Scientific Industries, Inc., Bohemia, NY), and incubated at RT for 10 min. Total RNA was extracted from the bacterial cell pellet according to Bae et al (*5*). The concentration of the RNA was measured using the Nanodrop®ND1000 UV-Vis spectrophotometer (Nonodrop Technologies, Wilmington, DE) and agarose gel electrophoresis.

5.3.4 Quantitative real-time reverse transcription PCR for screening genes

cDNAs were synthesized from 1μg of total RNA samples from bacteria grown on lettuce leaves or control cells using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Then, about 100 ng of the cDNA was mixed with 1X Power SYBR® Green PCR Master Mix (Applied Biosystems) and 400 nM of forward and reverse primers in a final volume of 25 μl and was amplified by PCR. The amplification was performed using a Mx3005P ™ Real-Time PCR System (Stratagene Inc., La Jolla, CA) with the mixture initially incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 15 sec. *gap* was used as an internal control to normalize the expression rate of each gene. The relative mRNA expression levels of the target genes to *gap* were calculated by using delta-delta Ct method. The primer sequences of genes encoding the surface proteins and lipase of *L. monocytogenes* are shown in Table 5.1. Primers were designed using DNASTAR Lasergene 8 (DNASTAR, Inc., Madison, WI).

5.3.5 *In silico* analysis

The protein domains of LMOf2365_0859 (accession no. Q721X5) were predicted using the National Center for Biotechnology Information (NCBI) 3D molecular structure database with the protein GenInfo Identifier (GI) no. 46907073. The prediction of the protein domains is available at http://www.ncbi.nlm.nih.gov/Structure/cblast/cblast.cgi? ClustalW2 software provided from the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI) and ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) were used to align LCP amino acid sequences and a CBD of Endoglucanase D from *Clostridium cellulovorans*.

5.3.6 Construction of LMOf2365_0859 (*lcp*; listerial cellulose binding protein) mutant

A *lcp* deletion mutant (Δ*lcp*) and a complemented strain were generated by allelic replacement as described previously (*3*). In brief, a selectable marker gene (*cat*; chloramphenicol acetyltransferase) was cloned from pMK4 using *cat* forward and reverse primers (catF and catR; Table 5.2) and inserted to pMAD (a temperature-sensitive integration vector) for the construction of pMAD_*cat*. Insert (*cat*) and vector (pMAD) were digested with *Sal*I and *Eco*RI and ligated with T4 DNA ligase (NEB, Beverly, MA). The PCR products (about 1.0 kb) from adjacent 5′ and 3′ flanking regions of LMOf2365_0859 were amplified with the chromosomal DNA of F2365 using *Taq* polymerase (Applied Biosystems). The primer pairs, 0859UF and 0859UR and 0859DF and 0859DR were used for 5′ and 3′ flanking regions. The primers were designed using DNASTAR Lasergene 8 program (DNASTAR, Inc.) [Table 5.2]. The generated up- and down-DNA fragments were digested with restriction endonuclease pairs *Bam*HI and *Sal*I and *Eco*RI and *Bgl*II, respectively. pMAD_*cat* was also digested with the same restriction

enzymes. The digested DNA fragments were ligated with pMAD_*cat* digested using T4 DNA ligase. pMAD_*lcp* harboring homologous DNA fragments of LMOf2365_*cat* were transformed into DH5α. The recombinant plasmid was extracted from DH5α culture and then transformed into *L. monocytogenes* F2365 competent cells. The recombinant plasmid was first incorporated into the chromosome of F2365 by homologous recombination event. Colonies retaining the chromosome incorporated with the recombinant plasmid were grown at 42°C and selected on BHI agar plate containing chloramphenicol (10 μg/ml). The F2365 harboring pMAD_*lcp* in it chromosome was subcultured at 30°C to screen for the *lcp* deletion mutants. The subcultures were plated on BHI agar plates containing erythromycin (5 μg/ml) or chloramphenicol (10 μg/ml). *lcp* mutant strain was selected by a phenotype showing chloramphenicol resistance and erythromycin susceptibility. The deletion mutant strain, Δ*lcp*, was confirmed by PCR product (180 bp) amplification from genomic DNA using 0859F and 0859R primers designed from a deleted part of *lcp* nucleotide sequences (Table 5.2). Genomic DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen). The complementation of LMOf2365 deletion mutant (Δ*lcp*+pMAD_*lcp*) was generated after first incorporation of the recombinant plasmid into the chromosome of F2365.

5.3.7 Bacterial growth and field emission scanning electron microscopy (FESEM) analyses

Bacterial cells were cultured as described previously. Bacterial cell growth of F2365 WT and Δ*lcp,* and Δ*lcp*+pMAD_*lcp* was determined. Thirty microliters of WT, Δ*lcp,* and Δ*lcp*+pMAD_*lcp* inoculums containing approximately 4.33 ± 0.18 log10 bacterial cells were dispensed into 3 ml of BHI medium and incubated at 37°C for 24 h in a MaxQ™ 4000 incubating and orbital shaker (Barnstead/Lab-Line) at 180 rpm. Bacterial

cell numbers were determined at 2, 4, 8, 16, and 24 h after incubation using total plate counts. A field emission scanning electron microscope was used to visualize bacterial attachment on the leaves. Twenty microliters (approximately 1.0×10^6 CFU/ml) of *L. monocytogenes* WT, Δ*lcp,* and Δ*lcp*+pMAD_*lcp* were inoculated onto 1 × 1 cm pieces of lettuce leaves. After 2 hours incubation, the leaves were fixed in 2.5% glutaradehyde in 0.1 M sodium cacodylate buffer pH 7.2 at 4°C. The fixed leaves were then rinsed, post fixed in 2% osmium tetraoxide in 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series, and critical point dried using a Polaron Critical Point Dryer (Quorum Technologies, Newhaven UK). Dried specimens were mounted on aluminum stubs with carbon adhesive, and coated with platinum using an ES150T ES sputter coater (Electron Microscopy Sciences, Hatfield, PA). The attachment of WT, Δ*lcp,* and Δ*lcp*+pMAD_*lcp* strains was visualized using a JEOL JSM-6500F scanning electron microscope (JEOL USA, Peabody, MA) at 5kv.

5.3.8 Adhesion to plant and fluorescence microscopy analysis

F2365 WT (6.72, 6.80, and 5.54 log10/ml) and Δ*lcp* (6.62, 6.73, and 5.65 log10/ml), and Δ*lcp*+pMAD_*lcp* (6.66, 6.76, and 5.86 log10/ml) bacterial cells were used for the attachment assay with lettuce leaf, spinach, and cantaloupe, respectively. Twenty microliters of inoculum of each of the strains were evenly placed on 1 × 1 cm squares of the leafy vegetables and cantaloupe skins and were incubated for 2 h at RT. The leaves and cantaloupe skin were washed twice with 25 ml of PBS using a Mini Vortex Mixer™ (VWR). Supernatant containing unattached bacterial cells was discarded. To determine the number of attached bacteria, the inoculated lettuce, spinach leaves, and cantaloupe skins were homogenized using a mortar and pestle after washing. For the

detection of *L. monocytogenes* attached on the lettuce leaf, *L. monocytogenes* WT, Δ*lcp,* and Δ*lcp*+pMAD_*lcp* were labeled with 5-(and -6)-carboxy-fluorescein diacetate succinimidyl ester (CFSE) [Molecular Probes, Eugene, OR]. One ml of bacterial cultures was centrifuged at 7,000 × g for 5 min at RT. Cell pellet was resuspended with PBS and washed three times to remove BHI medium. Bacterial cells were then resuspended in 1 ml of sterile PBS with CFSE dye to a final concentration of 5 nM and incubated at 37°C in darkness for 30 min. The CFSE-labeled bacterial cells were then washed three times with 1 ml of PBS. Twenty microliters of the CFSE-labeled cell (1×10^9 CFU/ml) suspensions were placed on lettuce leaves (1 × 1 cm) and incubated at RT in darkness for 2 h. After incubation, the lettuce leaves were mounted with Vectashield® H-1400 Mounting Medium (Vector Labs, Burlingame, CA) on a glass slides, and covered. CFSE-labeled bacterial cells were observed under a fluorescent microscope (Nickon, Tokyo, Japan) with FITC filter at 20× magnification.

5.3.9 Adhesion to human cells

Hep-G2 and Caco-2 cells were seeded to 24 well plates and incubated in a 37°C 5% CO_2 incubator. Mid-log phase *L. monocytogenes* was collected by centrifugation, washed twice with PBS, and resuspended with RPMI 1640 medium. Cells were infected with bacteria at multiplicity of infection of ~50:1 for 60 min. For adhesion assay, cells were washed five times with PBS and lysed with 0.2% Triton X-100. Cell lysate was diluted and plated on BHI agar to count the viable bacteria.

5.3.10 Cellulose binding assay

To identify a component of lettuce leave that interacts with LCP, the attachment of *L. monocytogenes* to cellulose acetate was measured using crystal violet staining.

Ninty-six-well plates (Nalge NuncInternational, Rochester, NY) were coated with 1% (w/v) cellulose acetate (Sigma- Aldrich, St. Louise, MO) dissolved in glacial acetic acid according to the method of Wierzba et al (*37*). Cellulose acetate-coated wells were washed with PBS twice using an orbital shaker (Lab-Line Instruments, Inc., Melrose Park, Illinois) prior to the binding assay. One hundred microliters of the inoculums of F2365 WT, Δ*lcp,* and Δ*lcp*+pMAD_*lcp* (about 1.0×10^7 CFU/ml) were then placed in a 96 well coated plate and incubated for 16 h at RT. Bacterial cells unbound to cellulose acetate were carefully washed with PBS containing 0.05% Tween 20 (PBST) [Sigma-Aldrich] three times using a 3-D rotator (Labline Instrument Inc., Melrose Park, IL, Model No. 4630). Bacterial cells attached to cellulose acetate were stained with 150 µl of 0.5% (w/v) crystal violet solution (BD Biosciences, Sparks, MD), incubated for 5 min at RT, and washed with PBST three times. The absorbance (OD) of each well was then measured at 590 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The difference in OD values was analyzed by ANOVA.

5.3.11 Statistical Analysis

All data were analyzed using SAS version 9.1.3 program (SAS Institute, Cary, NC). Data were obtained from three independent experiments using duplicate or triplicate bacterial samples per each experiment for the gene transcription level ($n = 9$), bacterial cell growth ($n = 6$), attachment assay ($n = 9$), and binding assay ($n = 9$). Data from real time RT-PCR to determine differential gene expression were analyzed using Student's *t*-test. Other data were analyzed using the procedures of analysis of variance (ANOVA). The statistical difference was considered significant at $P < 0.05$.

5.4 Results

5.4.1 The growth of listerial cells on lettuce leaves

The growth of F2365 on lettuce leaves or in PBS as a control was determined at 0, 8, 16, 24, 48, 72, and 96 h after incubation at 4°C. The growth curve demonstrated that the number of the bacterial cells from lettuce leaves and PBS were maintained without an exponential increase throughout the incubation periods (Figure 5.1), suggesting that *Listeria* can survive or colonize lettuce leaf at refrigerated temperature.

5.4.2 The transcription levels of genes encoding surface proteins and lipases and *in silico* analysis

To identify transcriptional changes in response to an attachment of *L. monocytogenes* to vegetables, the transcription levels of 32 genes encoding listerial surface proteins and lipases were measured at 8 and 16 h after incubation using real-time qRT-PCR. Data showed that transcription of five genes (LMOf2365_0413, LMOf2365_0498, LMOf2365_2052, LMOf2365_0859, and LMOf2365_2812) was up-regulated at 8 and 16 h time points (Figure 5.2).

In silico analysis based on the database of molecular 3D structures provided by the NCBI demonstrated that LCP (2027 aa), one of the up-regulated genes, contains a putative CBD (at position 20-144 aa), 7 bacterial Ig-like domains (Big_3), and LPXTG motif (a conserved sorting signal domain at carboxyl-terminal) [Figure 5.3a]. The surface protein encoded by *lcp* has amino acid sequences similar to a CBD of Endoglucanase D in *Clostridium cellulovorans,* which bound to cellotriose. Cellotriose is a derivative of cellulose degraded by cellulase. The structure prediction of the protein is available at http://www.ncbi.nlm.nih.gov/Structure/cblast/cblast.cgi? and the protein GenInfo Identifier (GI) number is 46907073. The amino acid sequence alignment was

generated from ClustalW2 (EMBL-EBI) and ESPript 2.2 softwares (Figure 5.3b). With these results, we hypothesized LMOf2365_0859 is involved in the interaction with cellulose and name this protein as a putative LCP. In addition, LCP of 4b strains has been shown to be a similar homology in other *Listeria* spp. (Figure 5.4).

5.4.3 Growth of F2365 Δ*lcp* strain

The candidate gene, *lcp*, was selected based on the real time qRT-PCR data (Figure 5.2) and *in silico* analysis (Figure 5.3). In-frame *lcp* deletion mutant (Δ*lcp*) was constructed (Figure 5.5) to determine the role of LCP in attachment to vegetables. Δ*lcp* was confirmed using PCR with 0859F and 0859R primers (Table 5.2). The PCR product (180 bp) was not amplified from Δ*lcp* (Figure 5.6a), indicating the success of in-frame deletion of LMOf2365_0859. First, we determined if Δ*lcp* or Δ*lcp*+pMAD_*lcp* was physiologically affected by the deletion of the target gene or the incorporation of the recombinant plasmid into F2365 via bacterial cell growth. Results showed that the growth kinetics for bacterial strains was not different (Figure 5.6b). Therefore, Δ*lcp* or Δ*lcp*+pMAD_*lcp* strains seemed not to be affected by the deletion of target gene or incorporation of pMAD containing the recombinant plasmid.

5.4.4 The role of LCP in an attachment to lettuce leaves

The most abundant components of plant cell walls are cellulose and pectin. We hypothesized that LCP might play an important role in attachment to vegetables due to the possession of a putative CBD. To test this possibility, fresh iceberg lettuce leaves were inoculated with 1×10^5 CFU/cm^2 of WT, Δ*lcp*, or Δ*lcp*+pMAD_*lcp* strain. The result showed that % adherence by the WT strain (2.97% ± 0.37) was significantly higher than that by Δ*lcp* (0.3% ± 0.05) [Figure 5.7a, $P < 0.0001$]. Δ*lcp*+pMAD_*lcp* showed

similar % adherence (3.03% ± 0.14) to the WT strain. Consistent with bacterial count data, fluorescence microscope analysis using CFSE labeled bacteria showed an increased CFSE signal (green dots) in the WT strain, compared to the *lcp* deletion mutant strain. The complement strain showed a similar CFSE signal to a WT strain (Figure 5.7b). FESEM analysis showed that most bacteria were evenly attached to the surface of leaves (Figure 5.7c). We were not able to observe any preferential attachment sites in the leaves. Combined, these results strongly suggest that LCP is an important adherence factor to vegetable leaves.

5.4.5 The role of LCP in an attachment to baby spinach and cantaloupe

In order to determine if LCP is a generalized attachment factor of *L. monocytogenes* to plants, we tested listerial attachment to baby spinach and cantaloupe. The WT strain showed the percentage (%) attachment to baby spinach leaves was approximately 2× higher than that to iceberg lettuce, showing 6.62% ± 2.59. By contrast, the numbers of Δ*lcp* attached was significantly lower than that of the WT strain, showing 0.64% ± 0.13 (Figure 5.8a). Of interest, overall attachment of WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp* strains to cantaloupe skins was much higher than other vegetables, showing 19.17% ± 4.05, 3.25% ± 0.88, and 17.12% ± 2.59, respectively (Figure 5.8b).

5.4.6 The role of LCP in an adherence to human cells

To study the interaction between LCP and animal host cells, we evaluated the functions of LCP involved in adherence to several human endothelial (hepatocellular liver carcinoma cell line; HepG-2) and epithelial cell (colorectal adenocarcinoma cell line; Caco-2) types. The data demonstrated that Δ*lcp* was significantly less attached to the host cells (decreased about 70%) when it was compared to the WT (Figrure 5.9).

5.4.7 The function of LCP involved in cellulose binding

To determine if there is an interaction between LCP and cellulose, we used a 96-well ELISA plate coated with 1% (w/v) cellulose acetate dissolved in glacial acetic acid. The WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp* strains were inoculated in each well, incubated at RT for 16 h, washed, and then stained with crystal violet. The OD for each plate was read at 590 nm. The OD values of F2365 WT (0.189 ± 0.014) and Δ*lcp*+pMAD_*lcp* strain were significantly increased compared to Δ*lcp* (0.110 ± 0.005) (Figure 5.10). These results suggest that LCP interacts with cellulose at acidic condition.

In conclusion, we have shown that LMOf2365_0859 (*lcp*) has a putative CBD and was highly expressed on lettuce leaf. Based on the attachment, fluorescence, and binding assays, data have demonstrated that *lcp* can be one of the adherence factors that contribute attachment of *L. monocytogenes* to vegetable and fruit surfaces.

5.5 Discussion

Vegetables have been considered as vehicles to transmit *L. monocytogenes* to human because the presence of *L. monocytogenes* in vegetables is much higher than in other RTE food (*7*). Nevertheless, studies for the attachment or colonization of *L. monocytogenes* on RTE vegetable leaves at molecular level have been less studied. In the current study we hypothesized that a listerial surface protein or lipase is involved in attachment to leafy vegetables for their colonization. First, we selected 32 genes encoding surface proteins and lipases provided from the JCVI annotation. F2365 grown in PBS and on lettuce leaves for 8 and 16 h at 4°C was used to determine the transcription levels of the genes using real-time qRT-PCR. Five up-regualted genes generated from the real-time qRT-PCR were used for *in silico* analysis to select a candidate gene involved in the interaction with a component (i.e., cellulose or pectin) of lettuce leaf. The Kyoto

Encyclopedia of Genes and Genomes (KEGG) database provides that *lcp* has 7 bacterial Ig-like domains (Big_3) and LPXTG motif (a conserved sorting signal domain at carboxyl-terminal). Many listerial surface proteins containing LPXTG motif have Big_3 domains which may be involved in an interaction with carbohydrates on the surface of host cells (*8*). However, the role of Big_3 domain associated with the interaction with carbohydrates is still unclear. Interestingly, using the NCBI CBLAST module, we found that LCP has a putative CBD. Therefore, this study characterized the function of LCP associated with attachment to vegetables or fruits.

The growth kinetics of the WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp* strains was evaluated. Data presented that the growth of Δ*lcp* or Δ*lcp*+pMAD_*lcp* strain was not different from WT (Figure 5.6b), suggesting that LCP is not be associated with listerial survival or growth under normal growth conditions. Attachment data showed that the percent attachment of all strains was much higher in cantaloupes than in leafy vegetables. It may be related to a physical difference by a rough surface or a component of cantaloupe skin. A recent study has demonstrated that the numbers of *Salmonella typhimurium* attached on romaine lettuce varied in the different regions and with ages of the leaves (*23*). In addition, bacterial attachment on spinach was higher than on lettuce. It could be caused by a different content of dietary fiber including cellulose in iceberg lettuce (1.25 g fiber/100 g leaf) and spinach (2.33 g fiber/100 g leaf) (*27*). A relatively more amount of dietary fiber in spinach than in iceberg lettuce may contribute the higher % of the pathogen attached to spinach than lettuce. Data from other studies and the current study involved in attachment to leafy vegetables may imply that bacterial attachment or colonization can be changed by different compositions of plant tissue or cell wall components according to various vegetables, sites, or ages. Therefore, further study for

the attachment of *L. monocytogenes* to various leafy vegetables is needed for the risk assessment of the food born-pathogen. In addition, we showed the role of LCP in an adherence to human cells (Figrure 5.9). Using the recombinant proteins with different domains of LCP can be utilized to study the functional interactions between LCP and human cells.

For cellulose binding assay we coated 96-well plates with commercially available cellulose or its derivatives such as cellulose acetate, methyl cellulose, and cellulose microcrystalline. Except cellulose acetate, data from other cellulose derivatives were not consistent due to a high background of non-specific staining or poor coating on plates. Thus, we coated the ELISA plate with 1% (w/v) cellulose acetate dissolved in glacial acetic acid followed by Wierzba et al (*38*). This suggests that the binding between *L. monocytogenes* and cellulose acetate is pH dependent. We also confirmed the effect of cellulose acetate coating via the measurement of the OD values of all the strains from coated or uncoated wells. Differences between wild type and mutant in attachment assay (10-fold) and cellulose binding assay (1.72-fold) were presented. The explanations could be : 1) a synthetic cellulose compound is different from natural cellulose; 2) *L. monocytogenes* may bind to more than one component of plant cell walls, such as pectin or hemicelluloses; 3) Besides LCP, there may be other bacterial attachment-inducing ligand (an adhesin) to the surface of vegetables leaf.

In conclusion, this is the first study to identify LCP of *L. monocytogenes,* a listerial surface protein that interacts with cellulose in vegetables or fruit component. To best out knowledge, LCP in serotype 4b strains contains higher homology to the CBD of Endoglucanase D in *Clostridium cellulovorans* than other *Listeria* ssp. strains (Figure 5.4). Further studies for *lcp* mutant strain with various RTE edible vegetables and fruits

or different serotype strains (i.e., 1/2a and 1/2b) for risk assessment as well as under different environmental stress conditions are required to find effective strategies for the prevention of human listeriosis. In addition, a mechanistic study on the relationship between listerial surface proteins and biofilm formation on a biotic (plant or animal host) or an abiotic surface can be further conducted. Consequently, results from this study may provide a better understanding of the adherence of *L. monocytogenes* in leafy vegetables and fruit. This study may also contribute to the development of novel strategies for decontamination, preservation, or storage of RTE edible vegetables.

5.6 Acknowledgments

This work was supported by grants from USDA/ARS (#58-6402-7-230) and the Mississippi Agriculture and Forestry Experiment Station. We thank Drs. Stephen Pruett and Janet R. Donaldson for their valuable review and suggestions. We also thank Ms. Amanda Lawrence for the preparation of specimens, FESEM imaging, and manuscript editing.

Table 5.1 Pirmers sequences used in vegetables study

Gene	Locus	Forward Primer	Reverse Primer
gap	LMOf2365_2432	GCTCAACGTGTTCCAGTTCCA	GCTGCTTCCATAGCTGCATTTA
	LMOf2365_0128	ATTTAGCTCCCGAATTCCCG	CGAAGCGTCGATACCGAGTT
	LMOf2365_0174	GGATTTCACACTGCCAAGCC	CCCTTTTTACAGTGGCCGTTC
	LMOf2365_0186	TTCATGTAGCAACTCCGGCA	TCCCGTCAATGTGTCTGATTCT
plcB	LMOf2365_0216	TGGCTGATTACCGAGAAGGG	ATTATTGGCGTGCATAGGTTGA
	LMOf2365_0338	TTACTGCCTTACCTGCGATGG	TTTCGTGTCACCAACATGGG
	LMOf2365_0347	TGCAAGCGTAACTCCAGCAA	CTGCCACGCTTAGTTTTACAACAT
	LMOf2365_0349	TTTTACAACAGATAAAATGCCAGCA	TTGAGGGCTTATCTCCACCG
	LMOf2365_0350	CAAGTTGCAGCATTACCAGTGTT	AGTTCTGGCAAAATAGCGCC
	LMOf2365_0413	GGAGCAGGTTCTGTTCTTCCAA	ACTGGAGCCTCCGCTTGTAC
	LMOf2365_0498	TCAGCTTTTGTGGGATGTTCTTC	TTCACTGACATCCAAATCATCCTC
	LMOf2365_0524	GCGGATGCAATTTCACTTCA	GCCGTCACCGAAGTATCTTCC
	LMOf2365_0656	TGGGCAAGATTACGGTTTCC	TGTAAGCCGCTCCATCATTG
	LMOf2365_0693	TGGGACGAGCAGTGTGGA	TCGCATGGTATAAATCGCCA
	LMOf2365_0694	GCCTGATGCGCCTACTCTTT	GCCCAACTGCCTCTTCAAATT
	LMOf2365_0768	TCCAGCACCACCAGTAACACC	TTCCAGGTTTTGCAACGTGA
	LMOf2365_0805	ATGGTCGTGTGGGAGAGGC	TTCCGCCGAAAACACACC
	LMOf2365_0852	CAGTAGGATTGGCGAGTGGG	CTCGGCTCCGTCTGCATC
lcp	LMOf2365_0859	AGTGAAGCATTTAAGTGGGACATG	TCATTGGCTTGCGTTGCATA
	LMOf2365_1144	CGCTTACAGATTCAAAGCCGAT	AACTGGGCTAGTTGCAGAGGG
	LMOf2365_1254	GCTGGCCATAAGCGAGGTATT	CATTAGCGGGCTTTTCCACTAG
	LMOf2365_1432	ACGTCCGACACATTATCCGG	CCTGTTGCGTTTGTTGGTGTT
	LMOf2365_1974	AGATACAGCACTCGCAAACGAA	TAACGCTGACTGGCTCACCAT
	LMOf2365_2052	AGAACTGGCAGCCTCTTCAGAT	GGATGAGCACTAAACCCAACAAG
	LMOf2365_2117	AGGAGATAAGGCGCTGAGTGG	CCGCTGTCCCTGTACCATTT
	LMOf2365_2121	CACTTCACCGTTATCCGACGT	TTTCAACATCGCCAATTTCG
	LMOf2365_2210	GTATCAAGTTACCTATGAGCGTTCCA	TTGATAACATGCCAGAACTTCCA
	LMOf2365_2211	AAATATTAAAACAGTTGTAAGCGGCA	CCCCGACTAAACCTGCATGA
	LMOf2365_2212	GCAAGTGACCCAGCGACAAT	CCGGTCACAGTAGCGATTCC
	LMOf2365_2638	GTGATTTCATCCGAAGCAACAA	CTTCCTAGTGCAAAAAAGAGAGCAT
	LMOf2365_2694	CAAGAAGTCACGCTCCTTGGA	TCTGTAATAGTCAGTTGGGCCGT
	LMOf2365_2812	TTTGTCTTGCGTAAATGCTCACA	GCATCGTGATTGTTTGCCCT

Table 5.2 Strains, plasmids, and primers used in vegetables study

Strain or plasmid	Description	Referecne or source
Strains		
E. coli DH5α	Cloning host	Invitrogen
L. monocytogenes F2365	Wild type of serotype 4b strain	this study
Δ*lcp*	LMOf2365_0859 deletion mutant strain, Cm^r	this study
Δ*lcp*+pMAD_*lcp*	Complementation of LMOf2365_0859 deletion mutant strain, Er^r, Cm^r	this study
Plasmids		
pMK4	Shuttle vector (5.585 kb) harboring *bla* and *cat*, Ap^r, Cm^r	(*34*)
pMAD	Temperature sensitive shuttle vector (9.666 kb), Er^r	(*3*)
pMAD_*cat*	pMAD derivative containing *cat*, Er^r, Cm^r	this study
pMAD_*lcp*	pMAD_*cat* derivative allowing deletion of LMOf2365_0859, Er^r , Cm^r	this study
Primers		
0859UF	5'- GCGCGGATCCCAATTGCTTATCTATTTGCA -3'	this study
0859UR	5'- GCGCGTCGACCATTGCAACTCTTATATTAC -3'	this study
0859DF	5'- GCGCGAATTCGGAAAGACAATAAGAAGGTA -3'	this study
0859DR	5'- GCGCAGATCTACCTGCTGGATTTGGCACCG -3'	this study
0859F	5'- ACGCATCTAATGGGGAAGCAACCA -3'	this study
0859R	5'- TGGCTTGCGTTGCATAGCTCAC -3'	this study
CatF	5'- GCGCGTCGACTCTAGAGCGCTTAAAACCAGTCATACCAA -3'	this study
CatR	5'- GCGCGAATTCCTCGAGTCACCTAGATCTGGAGCTGTAAT -3'	this study

* Underlines indicate restriction enzyme sites: GGATCC (*Bam*HI), GTCGAC (*Sal*I), GAATTC (*Eco*RI), AGATCT (*Bgl*II).

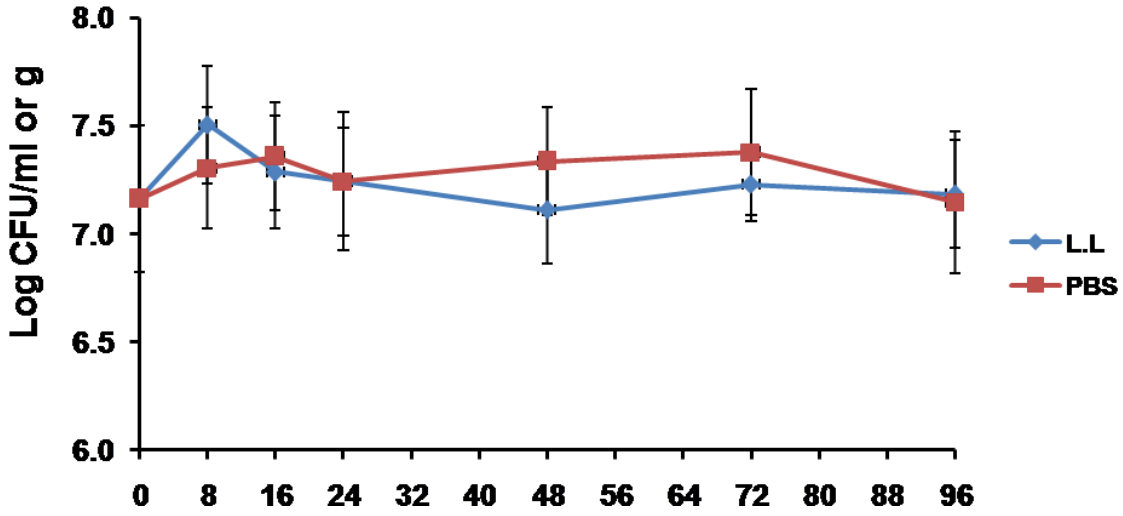

Figure 5.1 The growth of F2365 on lettuce leaves (L.L) or in PBS.

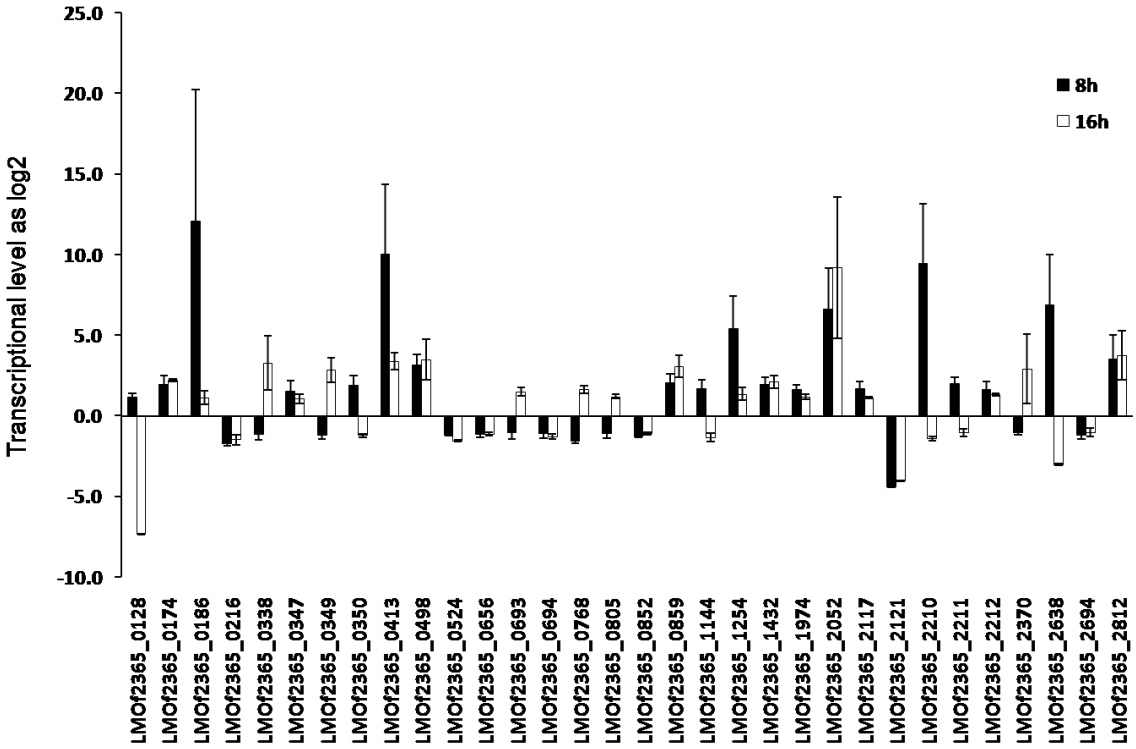

Figure 5.2 The transcription levels of genes encoding listerial surface proteins and lipases.

The transcription levels of 32 genes encoding surface proteins and lipases in *L. monocytogenes* colonized on lettuce leaf was measured at 8 and 16 h after incubation. *gap* was used as an internal control. Transcription levels were expressed as log2. Data were obtained from three independent experiments using three biological RNA samples per each experiment (n = 9). Data were analyzed using Student's t-test. Bars represent SEM.

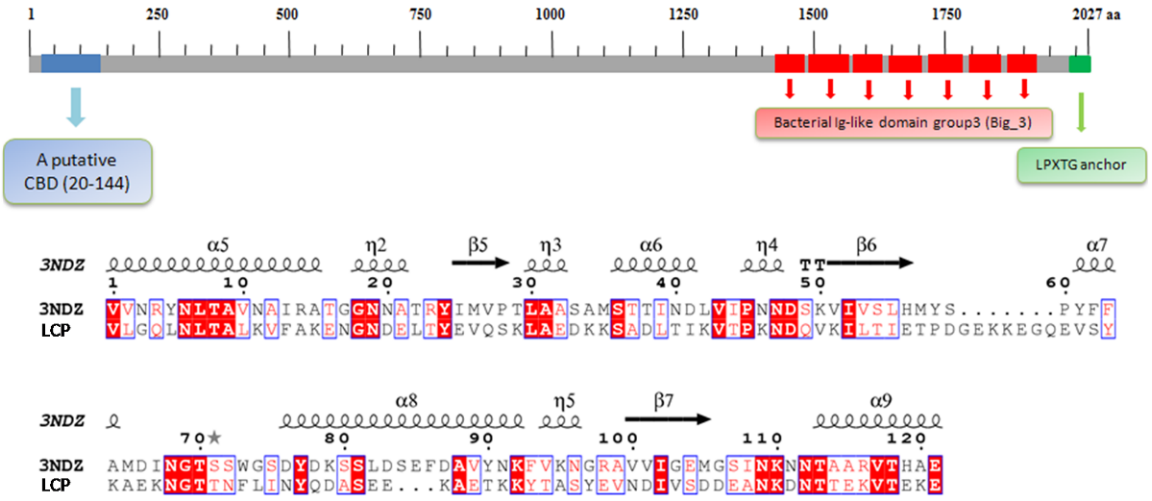

Figure 5.3 *In silico* analysis of a putative *Listeria* cellulose binding protein.

J. Craig Venter Institute (JCVI) annotation file, KEGG database, and NCBI CBLAST module were used to select a candidate gene for the listerial attachment on lettuce leaf. The NCBI database showed that LCP (2027 aa) contains a putative cellulose binding domain (CBD, at position 20-144 aa), 7 bacterial Ig-like domains (Big_3), and LPXTG motif. The surface protein (LCP) has amino acid sequences similar to a CBD of Endoglucanase D from *Clostridium cellulovorans* (3NDZ). ClustalW2 and ESPript 2.2 softwares were used to generate the sequence alignment.

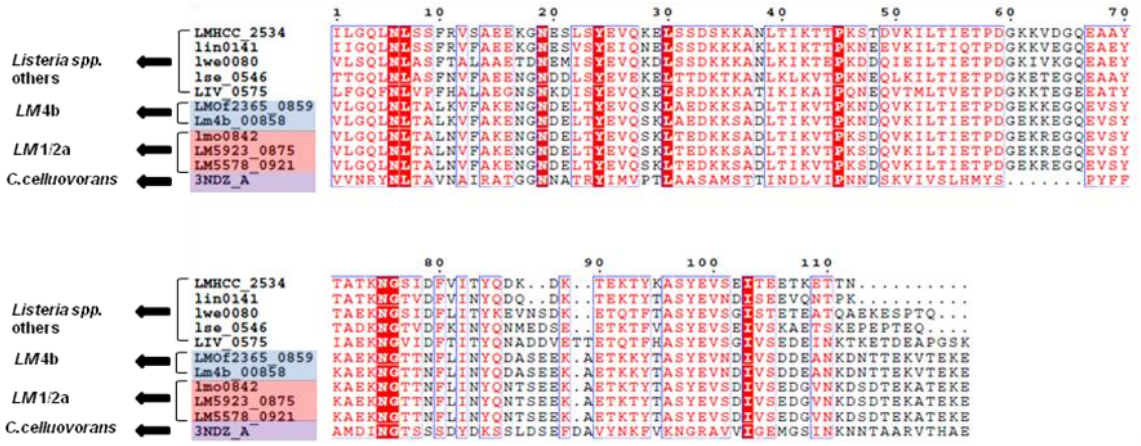

Figure 5.4 Orthologous sequences of LCP in *Listeria* spp. strains.

Clostridium cellulovorans retains a cellulose binding domain (accession number: 3NDZ_A). Mutiple sequence alignment was done using ClustalW2 and ESPript 2.2 softwares.

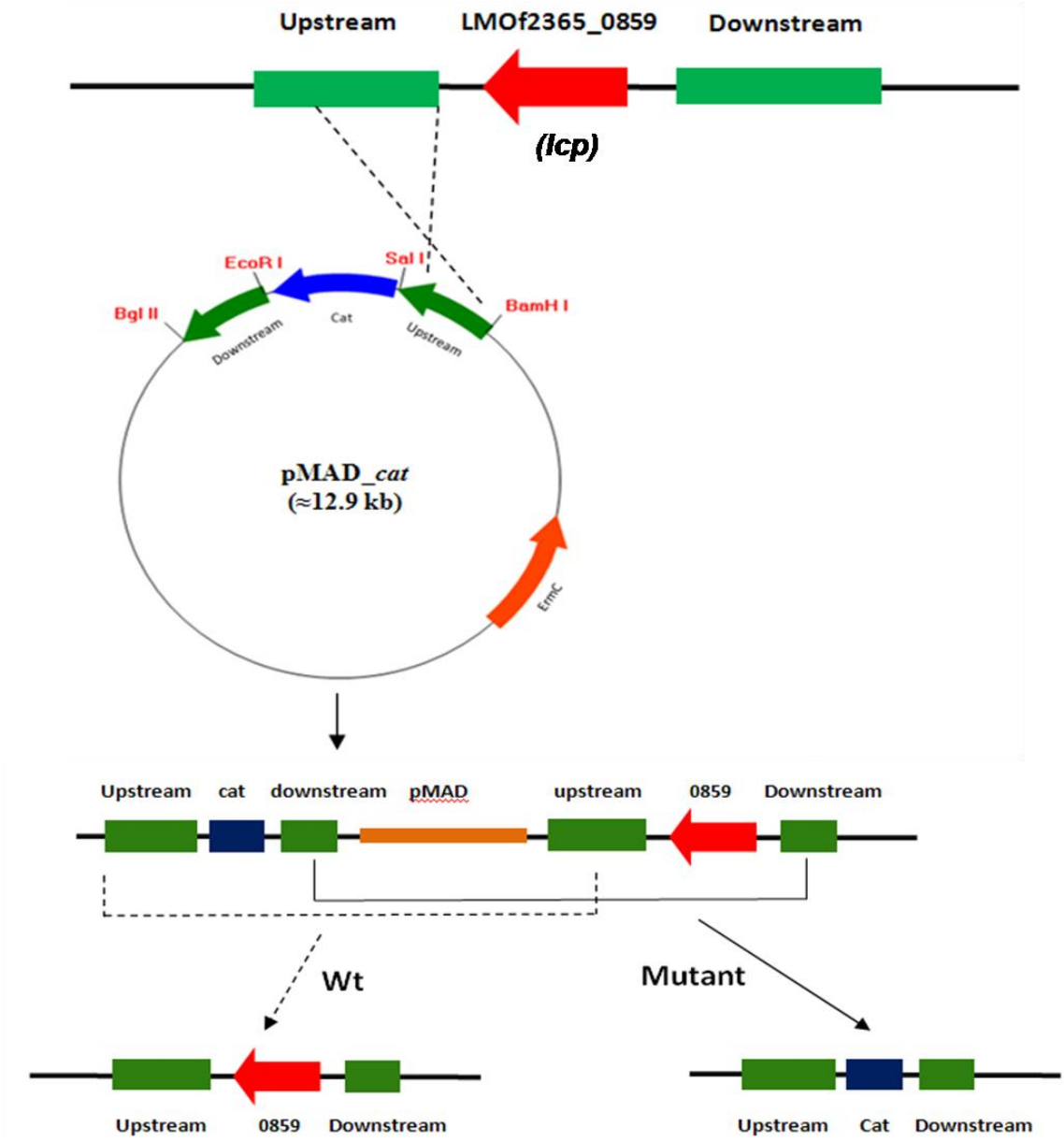

Figure 5.5 Construction of in-frame deletion *lcp* mutant.

The PCR products from adjacent the 5′ and 3′ flanking regions of LMOf2365_0859 were amplified and the generated up- and down-DNA fragments were digested with *Bam*HI and *Sal*I and *Eco*RI and *Bgl*II, respectively. The digested each DNA fragment with pMAD_*cat* digested with the same restriction enzymes was ligated. pMAD_*lcp* was transformed into *L. monocytogenes* F2365. The recombinant plasmid was incorporated into the chromosome of F2365 by 1st homologous recombination at 43°C. Δ*lcp*+pMAD_*lcp* was subcultured at 30°C to select deletion mutant mediated through 2nd homologous recombination. The complementation of Δ*lcp*+pMAD_*lcp* was generated after first incorporation of the recombinant plasmid into the chromosome of F2365.

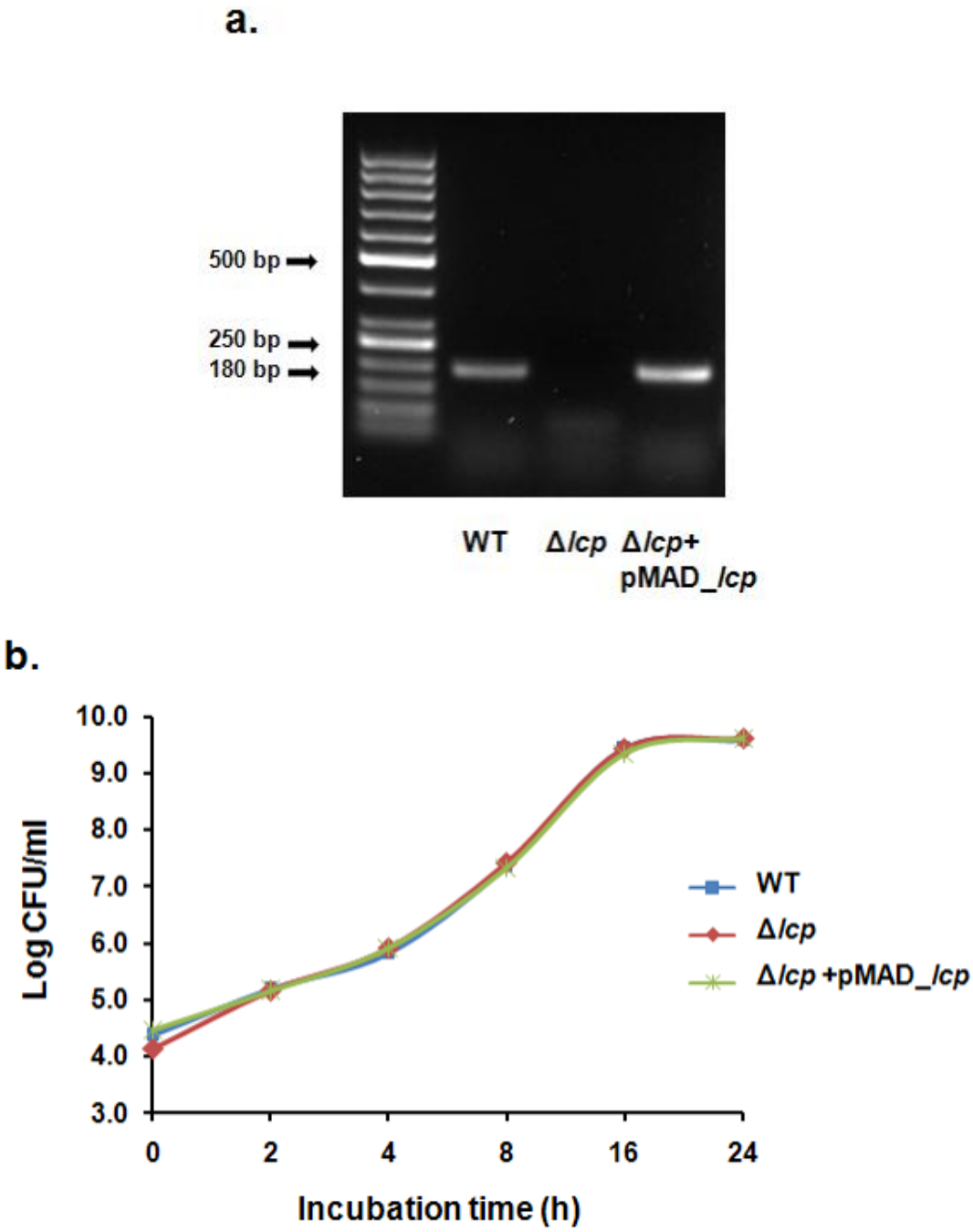

Figure 5.6 Confirmation of Δ*lcp* mutant strain and the cell growth.

Δ*lcp* and complemented strains were confirmed by PCR using primers (0859F/R) designed from a region of deleted gene. The size of PCR product is 180 bp (a). The growth of WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp*. The growth kinetics for bacterial strains was measured at 2, 4, 8, 16, and 24 h by a standard plate count. Data were obtained from three independent experiments using duplicate bacterial samples per each experiment (n = 6). Data were analyzed by ANOVA. Bars represent SEM (b).

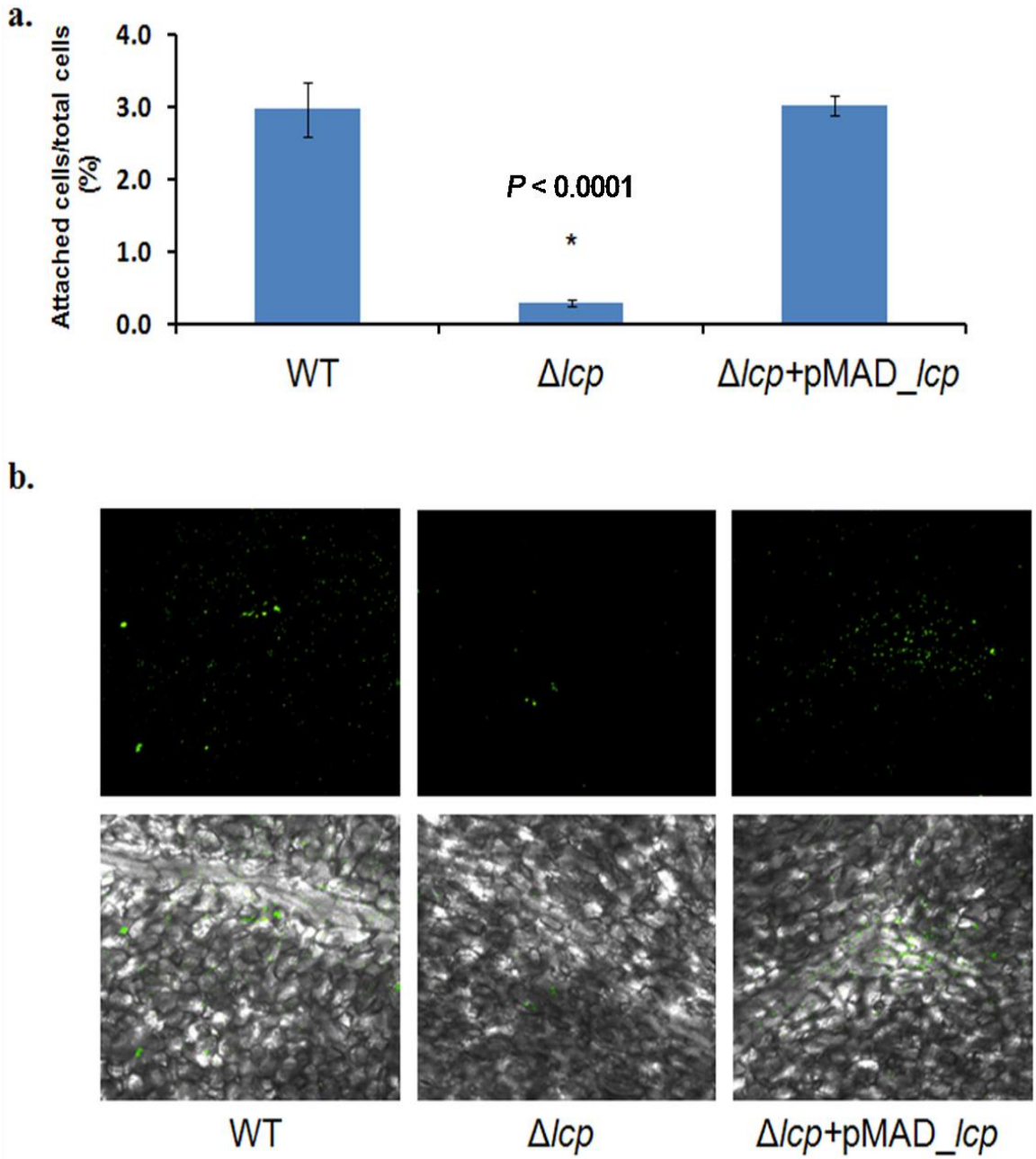

Figure 5.7 Attachment assay in WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp* strains to lettuce leaves.

c.

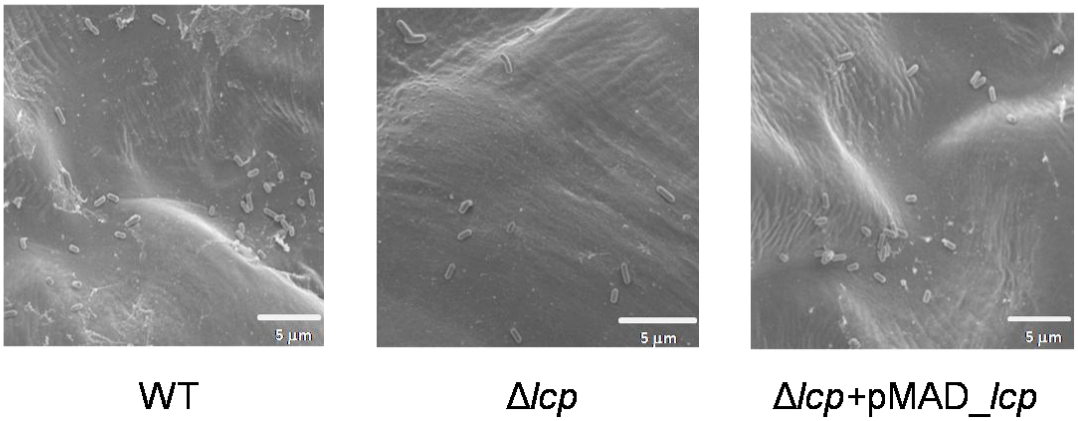

Figure 5.7 (Continued)

Data were obtained from three independent experiments using triplicate biological samples per each experiment ($n = 9$). The difference in the percentage of attached bacteria to total bacterial numbers inoculated on lettuce leaves was analyzed by ANOVA. Bars represent SEM. The symbol (*) represents a significant difference ($P < 0.0001$) between WT/complement and Δ*lcp* (a). All strains labeled with CFSE (5 nM) revealed under fluorescent microscope (Nickon, Tokyo, Japan) at 20 × magnification with FITC filter (b). Attached WT, Δ*lcp,* and Δ*lcp*+pMAD_*lcp* strains were observed under a JEOL JSM-6500F scanning electron microscope (JEOL USA, Peabody, MA) at 5kv (c).

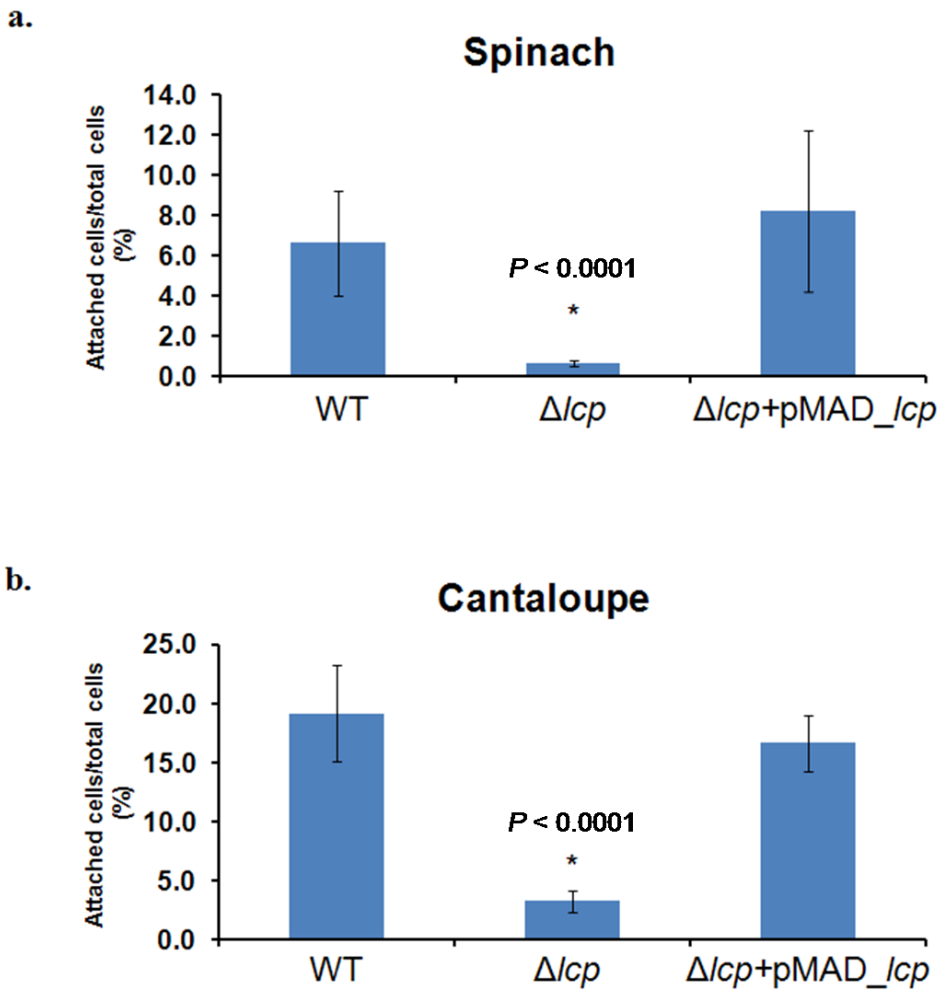

Figure 5.8 Attachment of WT, Δ*lcp*, and Δ*lcp*+pMAD_*lcp* strains to spinach and cantaloupe.

Data were obtained from three independent experiments using triplicate bacterial samples per each experiment ($n = 9$). The difference in the percentage of attached bacteria to total bacterial numbers inoculated on spinach leaves (a) and cantaloupe skins (b) was analyzed using ANOVA. Bars represent SEM. The symbol (*) represents a significant difference ($P < 0.0001$) between WT/complement and Δ*lcp*.

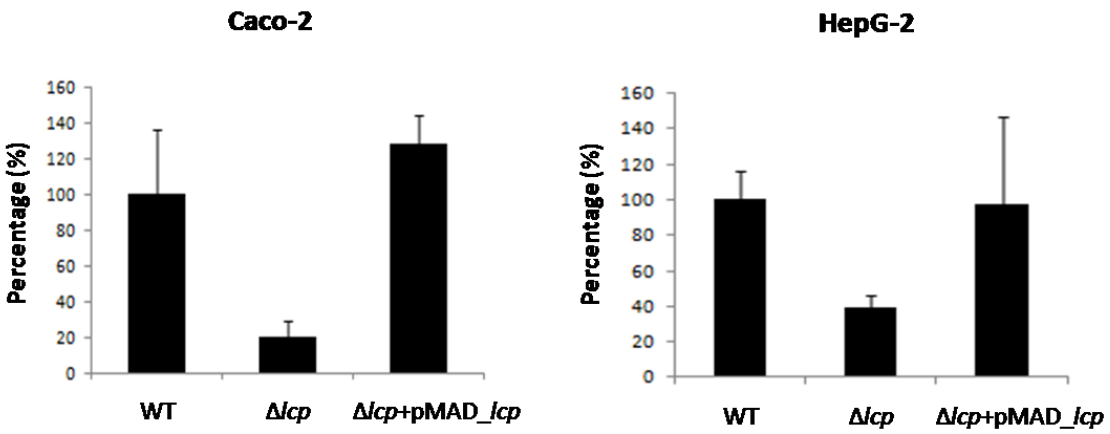

Figure 5.9 The adhesion of *L. monocytogenes* to Caco-2 and HepG-2.

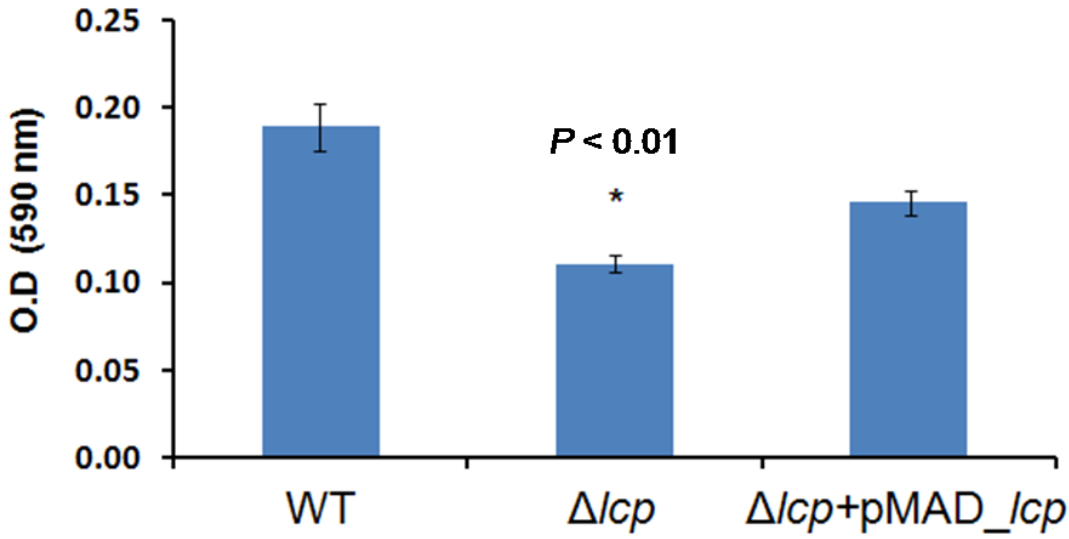

Figure 5.10 Cellulose binding assay.

A 96-well plates were coated with 1% (w/v) cellulose acetate. *L. monocytogenes* bound to cellulose acetate using 0.5% (w/v) crystal violet was measured using a microplate reader at OD590. Three independent experiments with triplicate biological samples per each experiment were used. The difference in OD values was analyzed using ANOVA. The symbol (*) represents a significant difference ($P < 0.01$) between wild type/complement and Δ*lcp*.

5.7 References

1. Abadias, M., J. Usall, M. Anguera, C. Solsona, and I. Vinas. 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *Int J Food Microbiol.* 123:121-9.

2. Ananth, V., J. S. Dickson, D. G. Olson, and E. A. Murano. 1998. Shelf life extension, safety, and quality of fresh pork loin treated with high hydrostatic pressure. *J Food Prot.* 61:1649-56.

3. Arnaud, M., A. Chastanet, and M. Debarbouille. 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol.* 70:6887-91.

4. Bae, D., C. Liu, T. Zhang, M. Jones, S. N. Peterson, and C. Wang. 2012. Global Gene Expression of *Listeria monocytogenes* to Salt Stress. *J Food Prot*, in press.

5. Bae, D., M. R. Crowley, and C. Wang. 2011. Transcriptome analysis of *Listeria monocytogenes* grown on a ready-to-eat meat matrix. *J Food Prot.* 74:1104-11.

6. Bergmann, B., D.Raffelsbauer, M.Kuhn, M. Goetz, S.Hom, and W. Goebel. 2002. InlA- but not InlB-mediated internalization of *Listeria monocytogenes* by non-phagocytic mammalian cells needs the support of other internalins. *Mol Microbiol.* 43:557-570.

7. Beuchat, L. R. 1996. Pathogenic Microorganisms Associated with Fresh Produce. *J Food Prot.* 59:204-216.

8. Bierne, H., and P. Cossart. 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. *Microbiol Mol Biol Rev.* 71:377-97.

9. Bubert, A., Z. Sokolovic, S. K. Chun, L. Papatheodorou, A. Simm, and W. Goebel. 1999. Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. *Mol Gen Genet.* 261:323-36.

10. Burnett, S. L., E. L. Mertz, B. Bennie, T. Ford, and A. Starobin. 2005. Growth or Survival of *Listeria monocytogenes* in Ready-to-Eat Meat Products and Combination Deli Salads During Refrigerated Storage. *J Food Sci.* 70:M301-M304.

11. Buzby, J. C. 2001. Children and microbial foodborne illness. *Food Review.* 24:32-37.

12. Chaturongakul, S., and K. J. Boor. 2006. SigmaB activation under environmental and energy stress conditions in *Listeria monocytogenes*. *Appl Environ Microbiol.* 72:5197-203.

13. Chaturongakul, S., S. Raengpradub, M. Wiedmann, and K. J. Boor. 2008. Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol.* 16:388-96.

14. Chen, H. 2007. Temperature-assisted pressure inactivation of *Listeria monocytogenes* in turkey breast meat. *Int J Food Microbiol.* 117:55-60.

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

16. Desvaux, M., and M. Hebraud. 2006. The protein secretion systems in *Listeria*: inside out bacterial virulence. *FEMS Microbiol Rev.* 30:774-805.

17. Drevets, D. A., M. J. Dillon, J. S. Schawang, N. Van Rooijen, J. Ehrchen, C. Sunderkotter, and P. J. Leenen. 2004. The Ly-6Chigh monocyte subpopulation transports *Listeria monocytogenes* into the brain during systemic infection of mice. *J Immunol.* 172:4418-24.

18. Ells, T. C., and L. Truelstrup Hansen. 2006. Strain and growth temperature influence *Listeria* spp. attachment to intact and cut cabbage. *Int J Food Microbiol.* 111:34-42.

19. Farber, J. M. 2000. Present situation in Canada regarding *Listeria monocytogenes* and ready-to-eat seafood products. *Int J Food Microbiol.* 62:247-51.

20. Ho, J. L., K. N. Shands, G. Friedland, P. Eckind, and D. W. Fraser. 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. *Arch Intern Med.* 146:520-4.

21. Johnston, L. M., L. A. Jaykus, D. Moll, J. Anciso, B. Mora, and C. L. Moe. 2006. A field study of the microbiological quality of fresh produce of domestic and Mexican origin. *Int J Food Microbiol.* 112:83-95.

22. Kazmierczak, M. J., M. Wiedmann, and K. J. Boor. 2006. Contributions of *Listeria monocytogenes* sigmaB and PrfA to expression of virulence and stress response genes during extra- and intracellular growth. *Microbiology.* 152:1827-38.

23. Kroupitski, Y., R. Pinto, E. Belausov, and S. Sela. Distribution of *Salmonella typhimurium* in romaine lettuce leaves. *Food Microbiol.* 28:990-7.

24. Marino, M., M. Banerjee, R. Jonquieres, P. Cossart, and P. Ghosh. 2002. GW domains of the *Listeria monocytogenes* invasion protein InlB are SH3-like and mediate binding to host ligands. *EMBO J.* 21:5623-34.

25. McLauchlin, J. 1990. Human listeriosis in Britain, 1967-85, a summary of 722 cases. 2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence. *Epidemiol Infect.* 104:191-201.

26. Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Bayles, J. B. Luchansky, and C. M. Fraser. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res.* 32:2386-95.

27. Nutrient Data Laboratory, Agricultural Research Service, US Department of Agriculture, USDA Nutrient Database for Standard Reference, release 24. Washinton, DC: US Department of Agriculture, 2011. www.ars.usda.gov/Services/docs.htm?docid=22114.

28. Patel, J. and M. Sharma. 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *Int J Food Microbiol.* 139:41-47.

29. Pomerleau, J., K. Lock, C. Knai, and M. McKee. 2005. Interventions designed to increase adult fruit and vegetable intake can be effective: a systematic review of the literature. *J Nutr.* 135:2486-95.

30. Roberts, A. J., S. K. Williams, M. Wiedmann, and K. K. Nightingale. 2009. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, *inlA* transcript levels, and swarming motility in vitro. *Appl Environ Microbiol.* 75:5647-58.

31. Sabet, C., M. Lecuit, D. Cabanes, P. Cossart, and H. Bierne. 2005. LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence. *Infect Immun.* 73:6912-22.

32. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis.* 17:7-15.

33. Schlech, W. F., 3rd, P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome. 1983. Epidemic listeriosisevidence for transmission by food. *N Engl J Med.* 308:203-6.

34. Schwaiger, K., K. Helmke, C. S. Holzel, and J. Bauer. Comparative analysis of the bacterial flora of vegetables collected directly from farms and from supermarkets in Germany. *Int J Environ Health Res.* 21:161-72.

35. Suarez, M., B. Gonzalez-Zorn, Y. Vega, I. Chico-Calero, and J. A. Vazquez-Boland. 2001. A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. *Cell Microbiol.* 3:853-64.

36. Sullivan, M. A., R. E. Yasbin, and F. E. Young. 1984. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. *Gene.* 29:21-6.

37. Swaminathan, B., and P. Gerner-Smidt. 2007. The epidemiology of human listeriosis. *Microbes Infect.* 9:1236-43.

38. Wierzba, A., U. Reichl, R. F. Turner, R. A. Warren, and D. G. Kilburn. 1995. Adhesion of mammalian cells to a recombinant attachment factor, CBD/RGD, analyzed by image analysis. *Biotechnol Bioeng.* 46:185-93.

CHAPTER VI

CONCLUSION

The concern about the prevalence of *L. monocytogenes* in RTE foods has been escalated due to food-borne outbreaks and the pathogen's abilities in resistance to heat, antimicrobial agents, sanitizers or detergents, physical treatments during food processing. According to the USDA/ERS, the economic loss due to diseases caused by five major food-borne pathogens including *L. monocytogenes* and others (*Escherichia. coli* O157:H7, non-O157 Shiga-toxin producing *E. coli, Campylobactor*, and *Samonella*) approach 2.3 and 4.6 billion dollars per year. Particularly, food-borne pathogen contamination in RTE foods is a major challenge for the food industry. The FDA established a "zero tolerance" policy for *L. monocytogenes* in food as well as the European Union has a "zero tolerance" regulation for *L. monocytogenes* in infant and special diets for patients. The policy is based on data that even low concentration of *L. monocytogenes* exists in food products, the growth of *L. monocytogenes* during the packaging, distribution, or storage can increase and reach high numbers, to cause listeriosis. Currently, the consumption of RTE poultry deli meats and vegetables has annually increased, and the presence of *L. monocytogenes* in the RTE meat and fresh produce has been frequently reported. However, there is no clear understanding how *L. monocytogenes* attach, colonize, survive, and grow on the food matrices.

In 1998-1999 and 2002 several major outbreaks of human listeriosis were occurred due to ingestion of turkey deli meat contaminated with *L. monocytogenes*. Our

previous study showed that *L. monocytogenes* grown on a RTE turkey deli meat was more pathogenic to mouse macrophage cell line J774A.1 when compared to the bacteria grown in BHI. Thus, we examined which gene is involved in the virulence factors of *L. monocytogenes* accounting for the adaptation and survival ability of the pathogen in the food matrix using a microarray. However, the transcripts of genes encoding for known virulence factors such as *sigB, prfA, inlA, inlB, plcA, plcB,* and *hly* were not significantly changed in *L. monocytogenes* grown on the deli meat. The transcriptome data showed differentially expressed genes which are involved in energy metabolism, fatty acid and phospholipid metabolism, biosynthesis of proteins, transport and binding proteins, DNA metabolism, cellular processes, and regulatory functions. The growth curve of *L. monocytogenes* on a turkey deli meat showed a short exponential time, suggesting that deli meat products containing low concentration of *L. monocytogenes* can potentially be increased to high bacterial numbers in a short storage time.

For a better understanding how *L. monocytogenes* adapts to a high salt concentration contained in RTE deli meat products, the transcriptome of *L. monocytogenes* was profiled under a typical salt concentration used in RTE products. Microarray data presented that genes involved in the uptake of glycine betaine/L-proline were up-regulated, whereas genes associated with phosphotransferase system (PTS), metabolic enzymes, and virulence factor were down-regulated. Interestingly, the transcripts in the most of PTS transport genes involved in the uptake of sugars such as glucose, fructose, mannose, and cellobiose were decreased. A relationship between the down-regulated PTS genes and the decreased cell growth was further examined by various concentrations (2.5, 5, and 10%) of salt. Results showed that the transcript levels of PTS genes with listerial cell growth shown to be dependent on NaCl concentration,

suggesting that a decrease in the transcript levels of PTS genes may be caused by salt stress or inhibited cell growth through salt stress.

The consumption of RTE or minimally processed vegetables (low calorie diet) with poultry deli meat (white meat) has annually increased and the prevalence of *L. monocytogenes* in vegetables and fruits has also increased due to properties of environmental factors in harvest and distribution. Thus, the safety issues in vegetables and fruits are a major concern for the producers and regulatory agents. Unlike animal hosts or their derivative food products, few studies have been conducted on the adhesion, invasion, and/or virulence regulation of *L. monocytogenes* in RTE or minimally processed edible vegetables and fruits at molecular level. Therefore, one of the objectives in this dissertation was to understand the adherence and growth of *L. monocytogenes* on leafy vegetables and cantaloupe skins which can be vehicles cause listeriosis in humans. LMOf2365_0859 (*lcp*) was selected as a target gene among genes encoding surface proteins and lipases in *L. monocytogenes* via qRT-PCR and *in silico* analyses. *In silico* analysis revealed that LCP contains a putative CBD. We then hypothesized that LCP may be involved in attachment to lettuce leaf due to an interaction with a major component of plant cell walls, cellulose. *lcp* in-frame deletion mutant (Δ*lcp*) was constructed and tested using attachment assay on leafy vegetables and cantaloupe skins. Results showed that Δ*lcp* was less attached to lettuce, spinach, and cantaloupe than the WT. To further study an interaction between *L. monocytogenes* and cellulose, 96-well plate coated with 1% (w/v) cellulose acetate was used. Binding assay data showed that Δ*lcp* was less bound to cellulose acetate than the WT and the complemented strains, suggesting that Δ*lcp* lost its ability to bind to cellulose when compared to WT and the complemented strains. Results may imply that LCP is one of the factors that contribute to the attachment of *L.*

monocytogenes to vegetables and fruits. Additionally, the functions of LCP involved in adherence to several human endothelial and epithelial cell types were evaluated. Results showed that Δ*lcp* significantly less attached to the host cells than the WT.

Throughout my dissertation research the global transcriptome profiles of *L. monocytogenes* grown on a turkey deli meat revealed differentially expressed genes involved in the adaptation, attachment, or growth in the pathogen. Besides, the transcriptome profiles of *L. monocytogenes* in response to salt stress also showed transcriptional changes of genes associated with homeostasis, survival, or adaptation. Finally, the last study has identified a novel gene of *L. monocytogenes*, *lcp*, as a gene coding for attachment to vegetables and fruit as well as a virulence factor. In conclusion, the data from the current observational, mechanistic, and functional studies have demonstrated the biological properties of *L. monocytogenes* in RTE food products. The information generated from this dissertation research can help the development of strategies to prevent food contamination by *L. monocytogenes* during processing, storage and preservation.

APPENDIX A

PERMISSION TO PUBLISH

International Association for
Food Protection®
6200 Aurora Avenue, Suite 200W
Des Moines, Iowa 50322-2864, USA

100th Anniversary 1911 2011
International Association for Food Protection
Celebrating a Century of Advancing Food Safety

February 17, 2012

Dongryoul Bae
240 Wise Center Dr.
Mississippi State University
Department of Basic Sciences
College of Veterinary Medicine,
P.O. Box 6100
Mississippi State, MS 39762

E-mail: bae@cvm.msstate.edu

Dear Dongryoul,

I am writing in response to your request to include two articles, one that has already been published in the *Journal of Food Protection* and the other article, which is currently in press, in your Ph.D dissertation.

Permission to use this material as stated in your attached request, is granted provided the material includes the following:

> Reprinted with permission from the *Journal of Food Protection*. Copyright held by the International Association for Food Protection, Des Moines, Iowa, U.S.A. Additionally, please include the name and affiliation of the authors.

This material is to be used only for the purpose outlined in your letter. Permission is granted without a fee per David Tharp, Executive Director, International Association for Food Protection. Non-compliance with the requirements contained herein will result in suspension of the requestor's rights to reproduce information from *Journal of Food Protection*.

If you have any questions regarding the use of this information from the Association, please contact me at 515.276.3344 or fax me at 515.276.8655.

Sincerely,

Didi Loynachan

Didi Loynachan
JFP Administrative Editor