**Detection of *Listeria monocytogenes* in the Food Manufacturing
Environment**

by

John Xue

A thesis submitted to the Faculty of Graduate and Post Doctoral Affairs
in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Carleton University
Ottawa, Ontario

© 2011

John Xue

Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-83170-0
Our file Notre référence
ISBN: 978-0-494-83170-0

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

■ ♦ ■
Canada

ABSTRACT

Environmental contamination of food manufacturing environments by *Listeria monocytogenes* is a growing food safety concern. In this research, two new PCR-based methods: LmCHAS and LmVCHAS, were developed for the definitive identification of *L. monocytogenes* and the detection of high risk outbreak-related strains of the pathogen, respectively, from isolates obtained by standard enrichment culture techniques. Both methods were assayed for specificity and LmCHAS was found to be more specific and robust than the current standard detection method. LmVCHAS had poor exclusivity results and will need to be revised. In order to improve current surface sampling techniques, model single- and dual-species *L. monocytogenes* biofilms were developed on stainless steel surfaces and various non-bactericidal agents were tested for their ability to improve recovery of sessile cells. Pectinase treatment increased cell recovery over the control buffer by approximately 2-fold and when combined 1:1 with Tween 20, an enhanced 4-fold increase over the control occurred.

ACKNOWLEDGEMENTS

Working in the Research and Development Section at the Canadian Food Inspection Agency was a great learning experience. I would like to thank my supervisor, Dr. Burton Blais, for the opportunity to work on such a high profile and exciting project. Dr. Blais' scientific acumen and insight in applied food microbiology research has been invaluable and I appreciate his guidance as a supervisor and mentor. I also wish to thank Amalia Martinez, Neil Vary, Martine Gauthier, Johanna Murphy, Leah Isaac, and Mylène Deschênes for all their help. Thanks to the Food, Feed and Fertilizer Section at Ottawa Laboratory Carling for letting me use some of their reagents and equipment. Thanks to Dr. Iain Lambert and Dr. John Vierula at Carleton and Dr. Paul White at Health Canada for their assistance and advice as members of my supervisory committee. I also owe a debt of gratitude to Dr. Franco Pagotto from the Listeriosis Reference Center at Health Canada for the provision of key *Listeria monocytogenes* strains used in this research. Thanks to Dr. Milos Kalab at Agriculture and Agri-Food Canada for performing the scanning electron imaging of biofilm samples. My sincerest thank you to my parents, Allen Xue and Dawn Chi, my brother, Mark Xue, my fiancée, Emily Xu, and all my friends for their love and support throughout this journey.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF APPENDICES	xi
GLOSSARY OF TERMS AND ABBREVIATIONS.....	xii
STATEMENT OF CONTRIBUTIONS.....	xv
CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW.....	1
1.1 FOODBORNE DISEASES AND FOOD SAFETY IN CANADA	1
1.2 <i>LISTERIA MONOCYTOGENES</i> AND HUMAN LISTERIOSIS	4
<i>CHARACTERISTICS AND CLASSIFICATION</i>	4
<i>FOODBORNE TRANSMISSION, DISTRIBUTION AND PERSISTENCE</i>	5
<i>VIRULENCE AND PATHOGENESIS</i>	6
<i>LISTERIOSIS SYMPTOMS AND EPIDEMIOLOGY</i>	7
1.3 CANADIAN POLICY ON <i>L. MONOCYTOGENES</i>: THE RISK-BASED INSPECTION	
APPROACH	8
1.4 CURRENT STANDARD SAMPLING METHOD FOR THE RECOVERY OF <i>L.</i>	
<i>MONOCYTOGENES</i> ON SURFACES IN THE FOOD MANUFACTURING ENVIRONMENT.....	10

TABLE OF CONTENTS (CONT'D)

1.5	CURRENT STANDARD METHODS FOR <i>L. MONOCYTOGENES</i> DETECTION AND IDENTIFICATION.....	11
1.6	MICROBIAL BIOFILM FORMATION AND CONTROL IN FOOD MANUFACTURING ENVIRONMENTS	14
1.7	CHALLENGES ENCOUNTERED IN THE RECOVERY AND DETECTION OF <i>L. MONOCYTOGENES</i> IN BIOFILMS.....	17
1.8	CURRENT METHODS FOR CREATING SINGLE- AND DUAL-SPECIES <i>L. MONOCYTOGENES</i> BIOFILMS IN THE LABORATORY	18
1.9	JUSTIFICATION FOR RESEARCH, OBJECTIVES AND HYPOTHESES.....	21

CHAPTER 2: IMPROVED METHODS FOR THE CHARACTERIZATION AND IDENTIFICATION

	OF <i>LISTERIA MONOCYTOGENES</i> ISOLATES FROM FOODS AND RELATED SAMPLES.....	24
2.1	INTRODUCTION	24
2.2	MATERIALS AND METHODS.....	27
	<i>BACTERIAL STRAINS AND CULTURE CONDITIONS</i>	27
	<i>PREPARATION OF BACTERIAL LYSATES</i>	28
	<i>L. MONOCYTOGENES</i> MULTIPLEX PCRS	28
	<i>L. MONOCYTOGENES</i> CHAS PROCEDURES	31
	<i>LIMIT OF DETECTION/DETECTABILITY</i>	35
	<i>SPECIFICITY PANELS</i>	35

TABLE OF CONTENTS (CONT'D)

2.3	RESULTS AND DISCUSSION.....	41
	<i>INCLUSIVITY/EXCLUSIVITY AND SPECIFICITY OF LM^VCHAS</i>	43
	<i>INCLUSIVITY/EXCLUSIVITY AND SPECIFICITY OF LMCHAS</i>	45
	<i>SENSITIVITY AND LOWER LIMIT OF DETECTION</i>	47
	<i>BENEFITS OF LMCHAS OVER THE EXISTING STANDARD METHOD</i>	49
2.4	CONCLUSIONS	53
 CHAPTER 3: CREATION OF MODEL <i>LISTERIA MONOCYTOGENES</i> SINGLE- AND DUAL-SPECIES FOOD MANUFACTURING ENVIRONMENT BIOFILMS AND USE OF POTENTIAL BIOFILM- DISPERSING AGENTS FOR THE IMPROVED RECOVERY OF THE PATHOGEN FROM MODEL BIOFILMS.....		
3.1	INTRODUCTION	55
3.2	MATERIALS AND METHODS	57
	<i>CREATION OF SINGLE-SPECIES <i>L. MONOCYTOGENES</i> BIOFILMS</i>	57
	<i>IDENTIFICATION OF BACTERIAL ISOLATES FROM THE FOOD MANUFACTURING ENVIRONMENT</i>	58
	<i>CREATION OF DUAL-SPECIES BIOFILMS</i>	60
	<i>VISUALIZATION OF BIOFILMS</i>	61
	<i>PRELIMINARY EXPERIMENTS EVALUATING POTENTIAL BIOFILM-DISPERSAL AGENTS</i>	61
	<i>RECOVERY OF <i>L. MONOCYTOGENES</i> FROM STAINLESS STEEL CHIPS</i>	62
	<i>RECOVERY OF <i>L. MONOCYTOGENES</i> FROM STAINLESS STEEL SQUARES BY SWABBING</i>	62

TABLE OF CONTENTS (CONT'D)

3.3	RESULTS.....	63
	<i>IDENTIFICATION OF BACTERIAL ISOLATES FROM THE FOOD MANUFACTURING</i>	
	<i>ENVIRONMENT</i>	63
	<i>VISUALIZATION OF BIOFILMS</i>	67
	<i>EFFECT OF POTENTIAL BIOFILM-DISPERSAL AGENTS ON CELL VIABILITY</i>	69
	<i>EFFECT OF POTENTIAL BIOFILM-DISPERSAL AGENTS ON THE RECOVERY OF</i>	
	<i>L. MONOCYTOGENES CELLS FROM BIOFILMS</i>	73
	<i>EFFECT OF VARIOUS PECTINASE CONCENTRATIONS ON THE RECOVERY OF</i>	
	<i>L. MONOCYTOGENES CELLS FROM BIOFILMS</i>	75
	<i>EFFECT OF PECTINASE COMBINED WITH TWEEN 20 ON THE RECOVERY OF</i>	
	<i>L. MONOCYTOGENES CELLS FROM BIOFILMS</i>	77
3.4	DISCUSSION.....	80
	<i>DEVELOPMENT OF A MODEL L. MONOCYTOGENES BIOFILM.....</i>	80
	<i>IDENTIFICATION OF BACTERIAL ISOLATES FROM THE FOOD MANUFACTURING</i>	
	<i>ENVIRONMENT</i>	81
	<i>INCREASED RECOVERY OF L. MONOCYTOGENES CELLS FROM BIOFILMS.....</i>	82
3.5	CONCLUSIONS	84
	CHAPTER 4: CONCLUSIONS AND FUTURE PROSPECTS	85
	APPENDICES	90
	REFERENCES.....	91

LIST OF TABLES

CHAPTER 2

Table 1. Oligonucleotide primer sequences used in the newly devised LmCHAS and LmVCHAS procedures	30
Table 2. Oligonucleotide probe sequences used in the newly devised LmCHAS and LmVCHAS procedures	32
Table 3. LmVCHAS specificity assays against 16 selected non- <i>Listeria</i> and <i>Listeria</i> spp. (excluding <i>L. monocytogenes</i>) bacterial strains deposited in the CFIA Ottawa Laboratory Carling culture collection	36
Table 4. LmVCHAS specificity assays against 35 selected serotyped <i>L. monocytogenes</i> strains deposited in the CFIA Ottawa Laboratory Carling culture collection	37
Table 5. LmCHAS specificity assays against 16 selected non- <i>Listeria</i> and <i>Listeria</i> spp. (excluding <i>L. monocytogenes</i>) bacterial strains deposited in the CFIA Ottawa Laboratory Carling culture collection	38
Table 6. LmCHAS specificity assays against 35 selected serotyped <i>L. monocytogenes</i> strains deposited in the CFIA Ottawa Laboratory Carling culture collection.....	39
Table 7. LmCHAS specificity assay against 24 selected non-serotyped <i>L. monocytogenes</i> strains recovered from the food manufacturing environment and related foods	40

CHAPTER 3

Table 8. List of 38 bacterial isolates recovered from four food manufacturing environment samples obtained in 2010 in Canada	65
--	----

LIST OF FIGURES

CHAPTER 2

- Figure 1. Detection of LmCHAS multiplex PCR amplicons by colorimetric reaction on cloth-based hybridization array system42
- Figure 2. Detection of LmVCHAS multiplex PCR amplicons by colorimetric reaction on cloth-based hybridization array system42
- Figure 3. Analysis of *L. monocytogenes* grown on different plating media by LmVCHAS ...48
- Figure 4. Diagnostic testing scheme for *L. monocytogenes*51

CHAPTER 3

- Figure 5. SEM photograph of *L. monocytogenes* and *S. epidermidis* subject to dual-species biofilm forming protocol with 2 days incubation and 1:10 dilution of BHI in Milli-Q water68
- Figure 6. Effect of potential biofilm-dispersal agents (aPBS, Dispersin B, non specific protein, and mild detergent) on *L. monocytogenes* cell viability as determined by aerobic plate counts70
- Figure 7. Effect of potential biofilm-dispersal agents (aPBS, pectinase, and sponge extract) on *L. monocytogenes* cell viability as determined by aerobic plate counts71
- Figure 8. Effect of potential biofilm-dispersal agents (aPBS, D-Tyrosine, D-Tryptophan, D-Alanine, D-Leucine, D-Methionine, DNase 1) on *L. monocytogenes* cell viability as determined by aerobic plate counts72
- Figure 9. Effect of potential biofilm-dispersing agents on *L. monocytogenes* cell recovery from 2-day old single-species biofilms as determined by aerobic plate counts.....74

LIST OF FIGURES (CONT'D)

CHAPTER 3

- Figure 10. Effect of various concentrations of Pectinex Ultra SP-L (pectinase) on the recovery of *Listeria monocytogenes* cells from 2-day old single-species biofilms as determined by aerobic plate counts.....76
- Figure 11. Effect of Pectinex Ultra SP-L (pectinase), Tween 20 and a combination of both treatments on the recovery of *Listeria monocytogenes* KB cells from 2-day old single-species biofilms as determined by aerobic plate counts.....78
- Figure 12. Effect of Pectinex Ultra SP-L (pectinase) combined with Tween 20 on the recovery of *L. monocytogenes* cells from 2-day old single-species biofilms formed on 4 cm square stainless steel chips as determined by aerobic cell counts. Cells were recovered by swabbing with a commercial sponge.....79

LIST OF APPENDICES

Appendix 1. Sample calculation of CFU/sample values	90
---	----

GLOSSARY OF TERMS AND ABBREVIATIONS

acyl-HSLs: acyl-homoserine lactones

ANOVA: Analysis of variance

aPBS: acidic Phosphate Buffered Saline

ATCC: American Type Culture Collection

BHI: Brain Heart Infusion

CFIA: Canadian Food Inspection Agency

CFU: Colony Forming Unit

CHAS: Cloth-based Hybridization Array System

ELISA: Enzyme-linked Immunosorbent Assay

EPS: Extracellular Polymeric Substances

Exclusivity: percentage of non-target analyte excluded/not detected

HACCP: Hazard Analysis Critical Control Point

HSB: High Salt Buffer

Inclusivity: percentage of target analyte included/detected

LEB: *Listeria* Enrichment Broth

LmKB: *Listeria monocytogenes* strain from Kane Biotech

Lower Limit of Detection /Detectability (LOD): The lowest number of CFU that can elicit a positive result

MBECTM assay: Minimum Biofilm Eradication Concentration assay

MFB: Modified Fraser Broth

NCBI: National Center for Biotechnology Information

OLC: Ottawa Laboratory Carling

PAI: Pathogenicity island

PBS: Phosphate Buffered Saline

PBST: Phosphate Buffered Saline with 0.05% v/v Tween 20

PCR: Polymerase Chain Reaction

PrfA: Positive regulatory factor

PVC: polyvinyl chloride

Q-PCR: Quantitative Polymerase Chain Reaction

RH : Relative Humidity

RODACTM: Replicate Organism Detection and Counting

RTE foods: ready-to-eat foods

SDS: Sodium Dodecyl Sulfate

Selectivity (Efficiency): percentage of samples rightfully assigned

SEM: scanning electron microscopy

Sensitivity: percentage of positive results correctly assigned (ability to detect all the analyte targeted)

Specificity: number of negative results properly assigned

s.s. chips: stainless steel chips

TMB: tetramethylbenzidine

TSA: Trypticase Soy Agar

TSB-YE: Trypticase Soy Broth with Yeast Extract

TSB: Trypticase Soy Broth

STATEMENT OF CONTRIBUTIONS

A portion of Chapter 2 was published in Xue et al., 2010. The contributions of the authors are listed below.

Conception and experimental design: Burton Blais

Provision of characterized bacterial strains: Franco Pagotto

Culturing and preparation of bacteria for molecular diagnostics: John Xue

Primer and probe design: Burton Blais and John Xue

Performing multiplex-PCR and CHAS procedures: John Xue

Recording observations and preparation of tables and figures: John Xue

Analysis of results: Burton Blais, John Xue and Franco Pagotto

Preparation of manuscript: Burton Blais, John Xue and Franco Pagotto

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Foodborne diseases and food safety in Canada

Foodborne illness has become a major concern in modern society due to a variety of means in spreading foodborne pathogens and an increased number of susceptible individuals to those pathogens (Ray and Bhunia, 2008). The public has also become more dependent on new products to make cooking and meal preparation more efficient and convenient. As a result, there has been increased consumer demand for ready-to-eat (RTE) meat products. These RTE meat products may become contaminated with pathogenic strains of bacteria that have become attached to surfaces and form biofilms in the food manufacturing environment (Lianou and Sofos, 2007). The biofilm structure provides protection from disinfectants and sanitizers and allows the pathogen to escape detection by current pathogen sampling methods (Wong, 1998; Kang et al., 2007). Thus, biofilms on food contact surfaces may infest RTE food products by persistent low-level contamination (Annous et al., 2009). In addition to contamination from food manufacturing plants, post-processing contamination of RTE foods commonly occurs. These factors, coupled with *L. monocytogenes*' robust nature and ability to grow at refrigeration temperatures, may lead to high enough levels of the pathogen on RTE foods to cause foodborne illness. As a result, there has been a significant emergence of the foodborne pathogen, *Listeria monocytogenes*, the causative agent of the deadly foodborne disease known as listeriosis (Mead et al., 1999; Swaminathan and Gerner-Smidt, 2007; Weatherill, 2009). Listeriosis is one of the deadliest foodborne diseases because it has

the highest hospitalization rate (92%) and the second highest case-fatality rate (25%) of any foodborne disease caused by pathogens (Mead et al., 1999).

There are several foodborne pathogens that pose a risk to food safety and human health: *Salmonella*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli*, *Clostridium perfringens* and *Staphylococcus aureus*. These pathogens can be present on foods, but normally at innocuously low levels. On processed food products, the initial bacterial level normally does not exceed 10^2 CFU/g; however, psychrotrophic facultative anaerobic bacteria (*Lactobacillus*, *Leuconostoc*, some coliforms, *Serratia*, *Listeria*, *Clostridium* spp.) are capable of multiplying quickly at low temperatures (4°C) and pose increased food safety risks compared to other pathogens even at innocuously low levels (Ray and Bhunia, 2008). Therefore, widespread contamination of *L. monocytogenes* in food manufacturing environments and related foods is a growing concern as currently no control measures can entirely eliminate the risk associated with this foodborne pathogenic bacterium (Bell and Kyriakides, 2005; Peccio et al., 2003; Lianou and Sofos, 2007). Risk of human illness varies with individual susceptibility and with type of food product. RTE meats that are consumed without further processing are the greatest concern (Pradhan et al., 2009; Ingham et al., 2010). These RTE meat products may become contaminated post manufacturing and the pathogen may grow to high numbers on the finished product before being consumed.

In order to mitigate contamination in the food manufacturing plants, the food industry is highly regulated and follows good manufacturing practices, as well as the Hazard Analysis Critical Control Point (HACCP) system of prevention (Ray and Bhunia,

2008). Moreover, a variety of physical, chemical and biological methods are followed to clean both food contact and non-food contact surfaces in the food manufacturing environment. Periodic inspections of the facility are also performed by inspectors from the Canadian Food Inspection Agency (CFIA) in addition to end product testing by both the food manufacturer and by the CFIA. Food safety policies are developed and maintained by Health Canada and are generally risk-based to utilize resources effectively (Health Canada, 2010).

Despite the efforts to combat foodborne pathogens, microbial foodborne diseases still occur, with many self-limiting cases going unreported. The types of foodborne diseases that may occur fall into the main categories of: food intoxication, food infection, food toxicoinfection and gastroenteritis by opportunistic pathogens (Ray and Bhunia, 2008). Many cases are self-limiting; however, a few cases may be fatal (CFIA and PHAC, 2008; CBC, 2009). The general population is resilient to many foodborne diseases that occur, but high risk groups such as pregnant women, immunocompromised individuals and the elderly are more susceptible. Demographics, culture and eating habits are also major factors in altering susceptibility. Therefore, the amount of contamination needed to elicit a foodborne disease varies with each individual.

With over 100 million meals eaten every day by Canadians, foodborne pathogens pose a real risk to public health. In order to safeguard the public from this risk, the Canadian government develops and enforces food safety measures that target high risk areas and minimize the contamination of food manufacturing environments and related foods by pathogens.

1.2 *Listeria monocytogenes* and human listeriosis

Characteristics and classification. *Listeria monocytogenes* is a bacterial pathogen and causative agent for listeriosis, a deadly foodborne disease (Mead et al., 1999; Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* is ubiquitous in the environment and frequently contaminates food manufacturing plants, posing a threat to food safety, especially RTE meat safety. *L. monocytogenes* is Gram positive, psychrotrophic (grows between 1 and 44 °C), facultative anaerobic, non-sporulating, motile rod-shaped bacterium. Motility is temperature dependent, with high flagellar expression and motility at 20-30 °C. *L. monocytogenes* is extremely hardy (resistant to desiccation, and resistant to high and low temperatures), highly acid tolerant and heat resistant, surviving stomach acid and up to 76 °C in vivo (McClure et al., 1991).

L. monocytogenes is one of six species in the genus *Listeria* (*L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*), all of which are present in soil, water, and mammals and widespread in the environment (Vasquez-Boland et al., 2001). *L. monocytogenes* and *L. ivanovii* are the only two pathogenic species in the genus; however, *L. ivanovii* is limited to ungulate animals while *L. monocytogenes* can infect both animals and humans (Vazquez-Boland et al., 2001). In addition, 13 different serotypes of *L. monocytogenes* have been characterized, based on somatic (O) and flagellar (H) antigens (Graves et al., 2007). The 13 serotypes are 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Serotype 1/2a isolates are predominately obtained from food and the environment, while serotype 4b isolates are mostly found in human clinical samples (Gilot et al., 1996). Three serotypes: 1/2a, 1/2b and 4b, account for over 95% of all cases of listeriosis documented, with serotype 4b being most commonly implicated in

listeriosis outbreaks (Graves et al., 2007; Gorski, 2008). Within serotype 4b, a variant named serotype 4bx was implicated in a listeriosis outbreak in the United Kingdom (McLauchlin et al., 1991). Furthermore, each serotype can be grouped into one of three lineages based on genomic divisions (Cheng et al., 2008). Lineage I consists of the majority of human clinical isolates and includes serotypes 1/2b, 3b, 4d, 4e, and most 4b strains (Jeffers et al., 2001). Lineage II consists of most strains implicated in animal cases as well as some strains implicated in human clinical cases and strains prevalent in food and the environment, and includes serotypes 1/2a, 1/2c, 3a and 3c. Lineage III strains are rare and found mostly in animal isolates and include serotypes 4a, 4c and certain 4b strains. Lineage I is the most significant in terms of disease-causing potential in humans, followed by lineage II and lineage III is the least significant.

Foodborne transmission, distribution and persistence. *L. monocytogenes* can be transmitted to raw foods in the food manufacturing plant through unsafe practices that lead to contamination (Weatherill, 2009). The main vehicles for introduction of *L. monocytogenes* in a food manufacturing plant are contaminated workers, carcasses, silage and feeds (Swaminathan et al., 2007). Foods that are consumed without post manufacture processing are at higher risk of harbouring pathogens and thus are higher risk foods (Health Canada, 2010). These foods are mainly RTE products such as deli meats; however, soft cheeses, pâtés and meat spreads, raw or lightly cooked eggs and dairy products, seafood, unpasteurized juices, and raw sprouts are also foods that are higher risk (McLauchlin et al., 1991; Pradham et al., 2009). It is recommended that meat and eggs be thoroughly cooked, sprouts be washed and cooked, and pasteurized milk and juices consumed as safer alternatives (Health Canada, 2010). *L. monocytogenes* can

grow and persist on contaminated foods even while they are refrigerated or when raw contaminated foods are only lightly cooked (Chasseignauz et al., 2001; Ingham et al., 2010). The psychrotrophic nature of *L. monocytogenes* is an important risk factor in food safety. Certain strains may be more likely to cause sporadic contamination, where contamination occasionally occurs, while other strains can cause persistent contamination, where contamination constantly occurs despite efforts to remove it (Peccio et al., 2003). The difference in strains causing sporadic versus persistent contamination may be a result of the strain's ability to form biofilms and survive in unfavourable environmental conditions (Kalmokoff et al., 2001). The robust nature of the *L. monocytogenes* means diligent food manufacturing, processing, and handling measures should be undertaken to avoid foodborne spread of the pathogen.

Virulence and pathogenesis. The virulence factors present in *L. monocytogenes* that are essential for pathogenesis are located primarily in a 9.0 kb region of the chromosome known as a pathogenicity island (PAI) (Johnson et al., 2004). Most genes in the PAI are regulated by positive regulatory factor (PrfA) and sigma B. PrfA is also present in *L. ivanovii*, a pathogen of ungulate animals. Environmental factors such as temperature, pH, and nutrient availability can also regulate the expression of virulence genes and thus infection in a host (Roche et al., 2008).

The mode of *L. monocytogenes* pathogenesis starts with oral entry through ingestion of contaminated foods, usually RTE food products. The pathogen survives transport into the gastro-intestinal tract and adheres, invades and translocates through the walls of the intestine. Growth and lysis of host cells occur followed by spread to

neighbouring cells. If the pathogen reaches the blood stream, it circulates to the liver, spleen, lymph nodes, placenta or brain where *L. monocytogenes* can cause invasive systemic diseases such as abscess of the liver, meningitis, encephalitis and abortion or still birth in pregnant women (McLauchin, 1997).

Listeriosis symptoms and epidemiology. Human listeriosis is an opportunistic disease that is rare and highly fatal (approximately 25% mortality) to infants, newborns, pregnant women and immunocompromised persons (McLauchin, 1997; Mead et al., 1999). It is estimated that only 10^2 - 10^3 cells are required for infection and disease in an immunocompromised host (Swaminathan and Gerner-Smidt, 2007). In these individuals, *Listeria monocytogenes* may survive in the macrophage in the bloodstream and be transported to the liver, spleen and lymphnodes within 24 h of ingestion and may cause invasive systemic disease. Persistent infection can allow the pathogen to infect the central nervous system, causing inflammation of the meninges and brain stem. In pregnant women, it can cross the placental barrier and infect the fetus. Symptoms of an invasive disease occur after 2-3 weeks of incubation and involve bacteremia (septicemia) resulting in fever and headache, meningitis, encephalitis, endocarditis and liver abscess among others. In healthy individuals, the infectious dose is 10^8 - 10^{10} cells. The disease is less severe and is manifested within 7 days following infection as febrile gastroenteritis. Symptoms include mild flu-like symptoms, slight fever, abdominal cramps and diarrhea. Most cases are self-limiting in a few days; however, the individual sheds *L. monocytogenes* in their feces for some time after symptoms subside.

1.3 Canadian policy on *Listeria monocytogenes*: the risk-based inspection approach.

The current Canadian policy on *L. monocytogenes* in RTE foods was established by Health Canada and CFIA in 2004, and was revised in 2010 to reflect new research and significant lessons learned in the aftermath of the listeriosis outbreak of 2008 (Health Canada, 2010).

The opportunistic pathogen, *L. monocytogenes*, is ubiquitous in the environment and thus difficult to eliminate entirely from food products and the food manufacturing environment (HCMMC, 2010). In addition, the ability of *L. monocytogenes* to grow at refrigeration temperatures is a concern because a low number of cells on food products could potentially grow to an infection level of approximately 1000 CFU/g by the time the product is consumed (Lianou and Sofos, 2007). In light of this, Health Canada has adopted a risk-based assessment approach where specific RTE foods are labelled into categories based on their potential to support *L. monocytogenes* growth and thus their risk for human infection (Health Canada, 2010). This approach takes into account HACCP principles.

Foods that are causally linked to listeriosis and that can support the growth of *L. monocytogenes* are deemed Category 1 and have the highest priority for inspection under the new Canadian policy on *L. monocytogenes* (Health Canada, 2010). Foods in category 1 may not have any *L. monocytogenes* cells present in an analytical sample size of 5 x 25 g of product. RTE foods that support limited growth of *L. monocytogenes* to levels no greater than 100 CFU/g throughout their refrigerated shelf life are considered “high risk”

and labelled Category 2A. These foods receive medium to low inspection priority and may not have *L. monocytogenes* above 100 CFU/g in an analytical sample size of 5 x 10 g of product (Health Canada, 2010). RTE foods that do not support *L. monocytogenes* growth throughout the expected shelf life of that food are labelled Category 2B.

Category 2B foods are considered low risk to consumers but are analyzed the same as Category 2A foods. These Category 2 foods receive low oversight priority; however, they may be assessed as “high risk” depending on the circumstances, such as a frozen RTE food product kept at room temperature (Health Canada, 2010). RTE foods intended to be produced for high risk population groups (pregnant women, elderly and/or immunocompromised individuals) receive the highest priority for regulatory oversight and compliance (Health Canada, 2010). The Canadian risk-based assessment approach is more practical for the food industry than eliminating all *L. monocytogenes* from RTE foods, as would be the case in a “zero tolerance” policy.

Based on epidemiological data, 100 CFU/g is considered to be the threshold below which, contaminated foods pose a low risk to most consumers (Lianou and Sofos, 2007). It is known that different serotypes of *L. monocytogenes* have different levels of virulence and risk to human infection (Graves et al., 2007; Gorski, 2008). Definitive identification of *L. monocytogenes* and detection of clinically relevant outbreak-related strains would help analysts to better gauge the risk that a particular sample poses to food safety. If it were possible to routinely differentiate virulent from avirulent strains, then the approach of quantifying the pathogen in the food manufacturing environment under the present policy may need to be re-examined because 100 CFU of an avirulent strain of *L. monocytogenes* would not pose the same risk to public health and food safety as, say

10 CFU of a highly virulent strain known to occur in epidemics. There is a need for more informative detection methods so that strains can be definitively identified and characterized so that their virulence is appropriately assessed. The detection of the same virulent strains over time may be of concern because persistent low-level contamination, when unrecognized, can cause outbreaks similar to the listeriosis outbreak of 2008. The valuable contributions that these more informative methods will provide to the *Listeria* risk-based assessment model will make future policy more rational and scientifically justified.

1.4 Current standard sampling method for the recovery of *L. monocytogenes* from surfaces in the food manufacturing environment

Environmental sampling is the first step in the diagnostic testing protocol for *L. monocytogenes*. The standard surface sampling procedure followed by CFIA inspectors is detailed in the Health Canada Compendium of Analytical Methods as MFLP-41 (HCCMM, 2010). MFLP-41 applies to both food contact and non-food contact surfaces in the food manufacturing environment. The procedure outlines using a commercial sampling kit to sample a non-sterile surface aseptically. The commercial kits utilize a bacterial carrier, typically a 4 x 8 cm moistened sponge (preferred) or 2 cm cotton tip swab (for access to smaller crevices) attached to an applicator stick 12-15 cm long. Each lot of bacterial carriers is tested for inhibitory properties against the targeted bacteria using the method described by Libras and Rose (1989) or another acceptable method (may be performed by the commercial suppliers). The sponge/swab is placed in 10-20 mL of sterile neutralizing buffer prior to sampling. The buffer moistens the sponge/swab

and acts to neutralize sanitizers applied in the food manufacturing environment. The swab sampling procedure is outlined in MFLP-41 and describes starting in finished product areas of a plant and proceeding into potentially contaminated processing areas, followed by raw product and receiving areas. After sampling, the collected samples are placed individually in sealable plastic bags (e.g. Whirl-pak), refrigerated (2-8°C), and analyzed as soon as possible. Sampling sites and frequency are at the discretion of the inspector and depend on the purpose and need for sampling as well as the regulatory frequency in the country the food products are exported to and the type of food product (HCCMMC, 2010). When sampling, two bacterial carriers are recommended as controls: one is left unopened and one is transferred to the media without being used to swab any surfaces. Larger sponges and swabs or swatches (J-cloths, gauze and cloths) are preferred for floors and walls larger than 1 m². All sampling activities and results are recorded for trend analysis. Although the swab method is the preferred method for sampling the food manufacturing environment, the use of Replicate Organism Detection and Counting (RODACTM) contact plates and air sampling has been used under appropriate circumstances.

1.5 Current standard methods for *L. monocytogenes* detection and identification

Following the environmental sampling of surfaces for *L. monocytogenes*, samples are sent to diagnostic laboratories for evaluation by microbiology analysts. The current methods for *L. monocytogenes* recovery and identification in foods and environmental samples relies on selective enrichment to grow a small quantity of the target pathogen originally present in the sample to enable isolation of colonies. Colonies are then characterized by polymerase chain reaction (PCR), an established rapid method to detect

foodborne pathogens, or alternatively, by biochemical tests or immunological tests for confirmation of positive result (Pagotto et al., 2001; Kang et al., 2007).

In the standard method, MFHPB-30 (Pagotto et al., 2001), a variety of selective media may be used to differentiate and grow *Listeria*. The standard is 24 and 48 h of *Listeria* enrichment broth (LEB) followed by secondary enrichment and screening with modified Fraser broth (MFB) for 24- 48 h (Pagotto et al., 2001). Both LEB and MFB are defined media that contain nutrients to promote the growth of injured and viable *Listeria*. LEB and MFB also contain selective agents: Nalidixic acid, which is bactericidal to Gram negative bacteria, and Acriflavin, which inhibits the growth of some bacteria and most fungi. A dark coloured MFB is indicative of a positive result which requires further isolation and identification. In contrast, a light straw color MFB is indicative of a negative result and no further steps are undertaken. Thus, the *L. monocytogenes* selective enrichment process screens for the presence of the target pathogen in a sample and allows for subsequent isolation and identification of presumptive positive samples. In MFHPB-30, samples are enriched in a primary broth and then a screening broth and plated and incubated on selective media for a specified period of time under specific conditions. Viable *L. monocytogenes* will grow in these conditions and give rise to visible colonies, which may be identified either biochemically, immunologically, or through rapid PCR methods targeting key genetic markers.

In the standard method, MFLP-78 (Blais et al., 2002), presumptive positive colonies detected from MFHPB-30 are confirmed as *L. monocytogenes* by subjecting them to a PCR procedure that targets a specific DNA sequence of the listeriolysin (*hlyA*)

gene. The resulting DNA fragment has a specific size that is identified by agarose gel electrophoresis. The MFLP-78 procedure is rapid (4 h), sensitive and specific for the target pathogen (Blais et al., 2002). The limitations to MFLP-78 are no quality control feature and no confirmation of amplicon.

An alternative to identification of *L. monocytogenes* by PCR is the identification of the pathogen using differential media such as Rapid L. mono agar, which contains aesculin, a substance that only *L. monocytogenes* can hydrolyze to produce black colonies, which are distinguishable from the white colonies produced by other *Listeria* spp. that do not hydrolyze aesculin. Used in conjunction with selective media, differential media can produce a positive identification of *L. monocytogenes*. The biochemical principle behind the identification of *L. monocytogenes* is also used in an API® *Listeria* test, where several characteristics of the unknown colony are tested so that its identity can be deduced. Similarly, analysis of an unknown sample with a Vitek machine follows the same principle whereby a series of biochemical tests screen out certain possibilities based on metabolic activities and/or morphological features, until one identity is reasonably deduced. The disadvantages of these biochemically based methods is that in addition to their slow turnaround time (days to weeks) and laborious nature, they may not produce results with high certainty (>85%) and often suggest 2 or more designations with comparable probability levels. Other challenges facing biochemically-based methods are that different strains in one species may not exhibit a specific characteristic, the same strain may not yield the same results in repeated tests, and the identification system databases have data on a limited number of species. There are several commercial kits available for the specific detection and identification of *L.*

monocytogenes, such as BAX® and VIDAS® (Janzen et al., 2006). These kits come with ready-to-use reagents, are simple to use and maintain and have rapid turnaround times for identification of isolates, but are costly. BAX® relies on molecular determination using real-time PCR to identify *L. monocytogenes*, while VIDAS® relies on an immunoassay method using an enzyme-linked fluorescent assay for identification. The drawback of immunoassay-based methods is their relatively low sensitivity compared to PCR-based methods (Janzen et al., 2006). Enzyme-linked immunosorbent assays (ELISA) have a sensitivity of approximately 10^5 - 10^6 CFU/mL compared to 10 - 10^2 CFU/mL for PCR-based methods. The expression of antigens may vary under different culture conditions and under environmental stress leading to substantial cross-reactivity for antibody-based immunoassays. Furthermore, real-time PCR is more expensive and less robust compared to conventional PCR. There is also a limited degree of multiplexing due to limited detector channels for fluorescence markers. Microarrays and biosensors have been developed by researchers as alternative detection methods to PCR-based methods, but these technologies have not proven themselves to be fast, simple, sensitive, accurate and inexpensive. Due to the drawbacks of biochemical and immunological methods, rapid PCR methods are often preferred for identification of presumptive positive results (Liu et al., 2003; 2007).

1.6 Microbial biofilm formation and control in food manufacturing environments

It is well known that microorganisms, including pathogens, establish on surfaces in biofilms in order to gain selective advantages when environmental conditions are not

optimal for survival (Donlan, 2002). In a nutrient-limiting environment, bacteria will concentrate on surfaces and liquid-air interfaces and adapt physiologically to a sessile state for survival (Watnick and Kolter, 2000; Annous et al., 2009). Adhesion of cells to surfaces consists of the hydrophobic link and the ionic interactions of the cells to the surface to overcome inherent electrostatic repulsion forces (Di Bonaventura, 2007). Adhesion of cells to surfaces depends on physiochemical factors involving the environment such as temperature, pH, surface hydrophobicity and microorganism motility. Quorum sensing also plays a role in adhesion of cells to a surface and the formation of extracellular polymeric substances (EPS) (Anous et al., 2009).

While in a sessile state, microorganisms produce a protective matrix of EPS consisting mainly of sugars (eg. glucose, galactose, mannose, fructose, rhamnose, *N*-acetylglucosamine, glucuronic acid, galacturonic acid, mannuronic acid and guluronic acid) and some nucleic acids, proteins, and lipids (Wimpenny, 1993; Vu et al., 2009). In a mature biofilm, energy for cell division is allocated to EPS production, which can provide nutrients and increase the binding of water and prevent desiccation (Watnick and Kolter, 2000). Although biofilms may consist of a single species, in nature they are commonly a mixed population of microorganisms (Watnick and Kolter, 2000; Donlan, 2002; Annous et al., 2009). Furthermore, the EPS of biofilms are species-specific and differ depending on bacterial composition of the biofilm.

Biofilm development occurs in an ordered temporal sequence that starts with reversible surface attachment, then growth and irreversible attachment mediated by EPS production that may contribute to 90% of the biofilm, then the formation of

microcolonies and finally the beginning of maturation. The maturation of a biofilm is characterized by a 3-dimensional structure containing channels for transport and incorporation of other components, followed by detachment and dispersion where the cells slough off the substratum surface and turn into a planktonic form from which initiation of new biofilm formation begins (Wimpenny, 1993; Deibel, 2003; Annous et al., 2009).

The control of biofilms in the food manufacturing environment is an ongoing challenge for the food industry (Frank and Koffi, 1990, Pan et al., 2006; Bell and Kyriakides, 2005). Industry employs their own in house testing and cleaning regimes, which mainly use physical, chemical and biological methods or a combination of these methods to combat biofilms (Huigens et al., 2008; Janseens et al., 2008). Physical cleaning by scrubbing is the most effective method; however, it is time consuming and labour-intensive. Chemical sanitizers may be effective; but, biofilms confer a degree of protection to these due to a physical barrier formed by the matrix of EPS. In addition, pathogens may develop antimicrobial resistance as a result of continued exposure to sanitizers (Lourenço et al., 2011). Biological compounds such as bacteriocins, enzymes, amino acids and bacteriophages have also shown some effectiveness; however, they tend to be expensive and not always highly bactericidal (Kaplan et al., 2004; Kolodkin-Gal et al., 2010; Lequette et al., 2010; Soni and Nannapaneni, 2010). Thus, the impractical nature of a lot of these techniques make combating biofilms in the food manufacturing environment challenging for the food industry. Since routine cleaning may not eliminate biofilms entirely, it is important to detect pathogenic cells harboured in biofilm before they cause persistent low level contamination (Guilband et al., 2005).

1.7 Challenges encountered in the recovery and detection of *Listeria monocytogenes* in biofilms.

Current pathogen detection methods rely on swabbing to recover cells from surfaces. However, swabbing may be ineffective at liberating cells in a sessile form in biofilms (Jay et al., 2005; Kang et al., 2007). Bacterial EPS may decrease the efficacy of current detection methods by forming a physical barrier, thus making it difficult for target cells to be liberated from the layered matrix of a biofilm (Wong, 1998). In addition, there is an increased risk of lateral gene transfer in biofilms than in a free-floating planktonic state, increasing the potential for microbes to acquire antimicrobial resistance and be resistant to antibiotics, disinfectants and sanitizers (Frank and Kofi, 1990; Annous et al., 2009). It is hypothesized that *L. monocytogenes* forms both heterogeneous and homogenous biofilms on surfaces in the food manufacturing environment for better survival (Carpentier and Chassaing, 2004; Di Bonaventura et al., 2007). This would explain the persistence of various strains despite efforts to eradicate the pathogen from these environments. The development of different characteristics for *L. monocytogenes* while in sessile form compared to a planktonic form, merit further research on the pathogen's biofilm formation and survival in order to be able to combat the pathogen. Novel agents from natural compounds such as secondary metabolites of marine sponges and algae (*Delisea pulchra*), enzymes (Dispersin B and pectinase) and lactic acid bacteriocins, have been reported to combat pathogenic biofilms (Garcia-Almendarez et al., 2007; Janssens et al., 2008; Lequette et al., 2010; Huigens et al., 2008). These potential biofilm-dispersal agents may be incorporated as part of an EPS-lysing technique in an improved surface sampling method to recover *L. monocytogenes* in biofilms on

condition that they conserve the viability of the cells for subsequent detection. It is hereby hypothesized that if the EPS matrix, holding *L. monocytogenes* cells together in a food manufacturing environment biofilm, is disrupted, more cells will be released and recovered by swabbing, assuming the potential biofilm-dispersal agent is not bactericidal to *L. monocytogenes*.

1.8 Current methods for creating single- and dual-species *L. monocytogenes* biofilms in the laboratory

In order to study the efficacy of potential biofilm-dispersal agents, it is first necessary to create a model biofilm system. Several methods have been investigated for creating *L. monocytogenes* biofilms in the laboratory, but no single method is currently considered a “gold standard” model. The microtitre plate method (Harvey et al., 2007), Leriche and Carpentier method (1-step and 2-step) (Leriche and Carpentier, 2000) and the MBECTM (minimum biofilm eradication concentration) assay (Ceri et al., 1999) are the three commonly used methods for creating biofilms. All three methods are primarily used for creating single-species biofilms on polystyrene surfaces. *L. monocytogenes* biofilm formation occurs on almost all food contact and non-food contact surfaces in the food manufacturing environment (Wong, 1998). These surfaces are mainly stainless steel, polyvinyl chloride (PVC), and rubber. Stainless steel is by far the most common surface used for food processing due to its durability, ease to clean and maintain, and resistance to sanitizers and disinfectants. Due to a prevalence of stainless steel in the food manufacturing environment, a model of *L. monocytogenes* biofilms developed in the laboratory should focus on producing biofilms on stainless steel. Moreover, several microbes have been identified to engage in synergistic relationships with *L.*

monocytogenes to form multi-species biofilms in the food manufacturing environment. *Hafnia alvei* and *Staphylococcus epidermidis* have been reported to form biofilms (Vivas et al., 2008; Zameer et al., 2010) and *S. epidermidis* has been found to partner with *L. monocytogenes* (Zameer et al., 2010) to form biofilms in the food manufacturing environment. In the “real world”, biofilms are rarely homogeneous monocultures, but rather a mixture of different microbes present in the food manufacturing environment. A model dual-species biofilm developed in the laboratory should incorporate these microbes to better resemble the mixed biofilms occurring in the food manufacturing environment.

The microtitre plate method is the most commonly used method for *Listeria* biofilm formation because it is relatively easy to work with in the laboratory (Harvey et al., 2007). Generally, standard cultures are prepared and inoculum is transferred to each desired well of a sterile polystyrene microtitre plate. The plate is incubated at room temperature for a desired amount of time followed by the removal of broth and washing of wells by rinsing three times per well with sterile water to remove loosely adhered bacteria. The wells are then air-dried, followed by addition of a 1% crystal violet solution to the wells and incubation for a specified time. After incubation, the crystal violet solution is removed and the wells washed and dried as before. A volume of 95% ethanol is then added to each well to destain the biofilms and the concentration of crystal violet is determined by measuring the optical density with a spectrophotometer at 595 nm. To correct for background staining, controls are performed and values obtained are subtracted from those of the treatments.

In the Leriche and Carpentier method for creating *L. monocytogenes* biofilms on stainless steel chips, a standard culture inoculum of *L. monocytogenes* is centrifuged and cells washed twice with saline (Leriche and Carpentier, 2000). Suspensions of 10^5 CFU/mL in saline are prepared and an aliquot is deposited on the chips which are incubated at room temperature, and high relative humidity (RH) for 3 h to allow for adhesion of the *L. monocytogenes* cells, followed by washing off the non-adherent bacteria by rinsing. An aliquot of trypticase soy broth with yeast extract (TSB-YE) is then deposited on the adhering bacteria before incubation for one day at room temperature under high RH. Finally, rinsing with sterilized pure water is performed to eliminate non-biofilm bacteria (Leriche and Carpentier, 2000).

In addition to single-species *Listeria* biofilms, dual-species biofilms may also be created (Leriche and Carpentier, 2000). Dual-species *Listeria* biofilms may be formed in two different ways. In the 1-step method, both species of bacteria are mixed together (usually in a specific ratio) before depositing on the chip for adhesion. In the 2-step method, one bacterial species is used to form biofilms for 1 day on the chip, then the other species (usually *Listeria*) is added in the same manner, followed by incubation for two more days.

The MBECTM assay, formally known as the Calgary Biofilm Device, was developed for high-throughput antimicrobial susceptibility testing of biofilms (Ceri et al., 1999). The assay relies on simple batch culture technique to grow up to 96 equivalent biofilms at the same time. Biofilms are grown by incubating an aliquot of a 300 fold dilution of 1.0 McFarland standard inoculum (approximately 10^7 CFU/mL) in each well

of a microtitre plate covered with a lid with 96 identical polystyrene pegs protruding down from the top. This device is placed on a shaker and incubated for a period of time depending on the bacteria. In the presence of shear force from shaking at 100-150 rpm, the bacteria become irreversibly attached and grow to form biofilms on the plastic pegs. These biofilms are rinsed and may be exposed to various levels of antimicrobials for a set period of time, recovered by sonication and quantified to determine the effect of the antimicrobial agents. Several bacterial species have been grown using this assay, including *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus* spp. (Ceri et al., 1999).

1.9 Justification for research, objectives and hypotheses

A recent outbreak of listeriosis traced to meat contamination in Canada occurred in 2008, and was commonly referred to as the Maple Leaf Foods listeriosis outbreak. This outbreak resulted in 22 deaths in Canada and made *L. monocytogenes* a high profile pathogen (CBC, 2009). The listeriosis outbreak gave the Canadian public a sense of the importance of food safety and pathogen detection, something many people might have previously taken for granted. It has also shed light on an ongoing major problem of environmental contamination of food manufacturing plants. Since the listeriosis outbreak, regulatory and public health agencies, along with the Canadian food industry, have implemented new measures and policies regarding *L. monocytogenes* in RTE foods to improve food safety (Weatherill, 2009). In addition, there have been increased efforts by the research community to develop more rapid, accurate, robust, highly sensitive and

selective assays to detect pathogens in foods, especially *L. monocytogenes* in RTE foods (Xue et al., 2010).

The current rapid detection and identification protocol for *L. monocytogenes*, MFLP-78, relies on PCR targeting the presence of only the *hlyA* gene marker and agarose gel electrophoresis for identification of the amplicon (Blais et al., 2002). Recently, several strains known to be *L. monocytogenes* have escaped detection by MFLP-78, indicating the method does not provide unequivocal identification of the pathogen. In addition, MFLP-78 lacks an internal control feature in its PCR. Therefore, the first aim of this research was to develop a novel multiplex PCR- based *L. monocytogenes* detection method that is more specific and sensitive than MFLP-78 by allowing microbiology analysts at CFIA to unequivocally identify *L. monocytogenes* and determine whether it is a clinically relevant strain. It is hypothesized that conserved species-specific gene markers and virulence genes specific to high-risk serotypes may be incorporated into a multiplex PCR procedure and coupled to a cloth-based hybridization array system for improved detection of the amplicons.

The strains implicated in the recent listeriosis outbreak were able to persist in the food manufacturing environment for a long period of time (Weatherill, 2009). It is believed that biofilm formation aids in *L. monocytogenes* survival and persistence in this environment (Wong, 1998; Chae and Schraft, 2001; Harvey et al., 2007). Thus, the second aim of this research was to develop model *L. monocytogenes* biofilms on stainless steel surfaces and evaluate the efficacy of potential biofilm-dispersal agents to release an increased quantity of cells from the EPS matrix of biofilms for subsequent recovery and

detection. These potential biofilm-dispersal agents would not be bactericidal to *L. monocytogenes* and the best agent may be used to improve the current surface sampling method, MFLP-41. It is hypothesized that using enzymes that break apart the carbohydrate bonds in EPS will release an increased number of target cells from biofilms and thus improve recovery and detection of persistent *L. monocytogenes* strains implicated in pre- and post-processing contamination.

The results of the present research will provide an immediate impact to RTE food safety and public health. The use of improved methods and procedures devised from this research will mitigate problems caused by *L. monocytogenes* contamination of food manufacturing environments and help safeguard the public from future listeriosis outbreaks.

**CHAPTER 2: IMPROVED METHODS FOR THE CHARACTERIZATION AND
IDENTIFICATION OF *LISTERIA MONOCYTOGENES* ISOLATES FROM FOODS AND RELATED
SAMPLES**

2.1 Introduction

Based on public health data, only a limited subset of *L. monocytogenes* serotypes (1/2a, 1/2b and 4b) and strains have been implicated in foodborne outbreaks of listeriosis. As a result, we know that some *L. monocytogenes* strains are more virulent than others. Several studies have been undertaken to identify different genetic markers that can be used for characterizing strains of *L. monocytogenes* with respect to their potential to cause foodborne illness. Researchers have been especially interested in finding genes present only in virulent strains of *L. monocytogenes*. Nishibori et al. (1995) reported that PCR detection of established *L. monocytogenes* virulence genes: *inlA* and *inlB* (internalins involved in colonization *in vivo*), *actA* (actin assembly), *hlyA* (listeriolysin), *plcA* and *plcB* (lipid production for escape from phagosomes of macrophages), was not suitable for differentiation between virulent and avirulent isolates since these genes were found in both clinical and non-clinical strains. Promadej et al. (1999) reported that a novel gene *gtcA* is conserved among *L. monocytogenes* serotype 4 strains and is expressed for cell wall teichoic acid glycosylation. While not a virulence marker per se, sequences from the *gtcA* locus may be a useful marker for serotype 4b strains associated with human illness. Also noteworthy is the *inlJ* gene which reportedly differentiates the avirulent serotype 4a strains from virulent strains of the pathogen (Liu et al., 2003; 2007). Liu et al., (2007) reported that an intact *inlJ* gene was necessary for optimal expression of

virulence. *InlJ* encodes a LPXTG protein (internalin) and allows the pathogen to invade mammalian cells (Sabet et al., 2005). In addition, Zhang and Knabel (2005) demonstrated that *lmo*, a gene encoding a putative peptidoglycan-bound protein, was specific for serotype 1/2a and 1/2c strains.

Current methods for the identification of *L. monocytogenes* recovered from foods using standard culture techniques (MFLP-78) employed by CFIA determine the presence or absence of *L. monocytogenes* by targeting the species-specific marker *hlyA* using PCR (Blais et al., 2002). The *hlyA* gene has been shown to be specific for *L. monocytogenes* (Mengaud et al., 1988); however aberrant strains of *L. innocua* have recently been found to be *hlyA* positive and haemolytic (Johnson et al., 2004). In addition, four *L. monocytogenes* strains isolated from CFIA laboratories in Toronto were biochemically and physiologically identified as *L. monocytogenes*, but failed to be detected using MFLP-78 (Burton Blais, personal communication). The MFLP-78 results were credible because all standard tests were performed according to quality assurance requirements and positive and negative controls as well as replicates were used. The results were also verified in separate experiments. Thus, there is an urgent need for improved pathogen detection methods to unequivocally identify *L. monocytogenes* isolated from foods using standard culture techniques. Furthermore, the risk-based system might be better served by the availability of methods capable of distinguishing strains highly associated with foodborne human illness (eg. strains from serotypes 1/2a and 4b) from those rarely associated with human infection (eg. avirulent *L. monocytogenes* strains).

The objective of this project was to investigate the utility of different gene markers in the identification and differentiation of *L. monocytogenes* strains based on their association with virulent serotypes. Also, the aim was to improve PCR for *L. monocytogenes* identification by redesigning *hlyA* primers and developing a cloth-based hybridization array system (CHAS) format for detection of the amplicon. An original CHAS method was developed that targeted *hlyA*, *inlJ*, *inlC* (secreted virulence factor involved in dissemination of infection) and *gctA*. The *inlC* marker was dropped after preliminary experiments, and new *hlyA* primers were designed, producing a smaller sized amplicon, for better optimization of the multiplex-PCR. In preliminary experiments, the original CHAS method was split into two methods and additional features were added. The two methods developed by this research were LmCHAS (*Listeria monocytogenes* cloth-based hybridization array system) and LmVCHAS (*Listeria monocytogenes* virulence cloth-based hybridization array system). These methods utilized multiplex-PCR to target key markers for species identification and detection of high risk-outbreak related strains, respectively. LmCHAS targeted conserved virulence markers: *hlyA* and *inlJ* and LmVCHAS targeted high risk serotype-specific markers: *gtcA* and *lmo*. An internal control feature consisting of a primer pair with complementary 3' ends, *IAC*, was used to validate the process and eliminate false negatives in both methods (Leggate and Blais, 2006). A 16S/23S rRNA intergenic sequence *IGS* marker, specific for all *Listeria* spp. except *L. grayi*, was also used as a positive control in the LmCHAS (Graham et al., 1996), while the *hlyA* marker was also used in LmVCHAS as a positive control to verify the presence of *L. monocytogenes*. The multiplex-PCRs of both LmCHAS and LmVCHAS incorporate digoxigenin (DIG) –dUTP to simultaneously amplify target gene

sequences, with subsequent rapid detection of the amplicons in CHAS by hybridization with an array of probes immobilized on polyester cloth and immunoenzymatic assay of the bound label (Gauthier and Blais, 2004; Blais et al., 2007). A distinct colorimetric reaction to a positive result will indicate the presence of the amplicon. In contrast to gel electrophoresis, the CHAS format allows similar sized amplicons from a multiplex-PCR to be definitively and accurately detected (Gauthier and Blais, 2004; Blais et al., 2007). The devised LmCHAS and LmVCHAS methods will be able to detect one colony of cells cultured using standard enrichment techniques. The use of these two newly devised methods will lead to a more rational assessment of the risk a contaminated sample poses to public health and food safety so that appropriate control measures can be undertaken. The results from the LmCHAS procedure developed from the present study have been published (Xue et al., 2010).

2.2 Materials and Methods

Bacterial strains and culture conditions. A total of 75 different non-*Listeria*, *Listeria* spp. and *L. monocytogenes* strains were obtained from the Ottawa Laboratory Carling (OLC) culture collection at the Canadian Food Inspection Agency. Of these strains, 5 were non-*Listeria* spp.: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Staphylococcus epidermidis*, 11 were *Listeria* spp. other than *L. monocytogenes*: *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria grayi*, 35 were various serotyped *L. monocytogenes*, and 24 were non-serotyped *L. monocytogenes* strains. All non-*Listeria* bacteria were grown on Trypticase Soy agar (Sigma Aldrich Co., St. Louis, MO, USA) at 37°C for 24 h. All

Listeria bacteria were grown on Brain Heart Infusion (BHI) agar (Sigma Aldrich) at 30°C for 24 h.

Preparation of bacterial lysates. All bacterial colony lysates were prepared for molecular diagnostics by picking a single colony (approximately 10^8 CFU) and suspending in 100 μ L of 1% v/v Triton X-100 (Sigma Aldrich) followed by heating at 80°C for 5 min. This method was compared to the gold standard Wizard Genomic DNA extraction protocol for Gram positive bacteria to determine the effectiveness of the Triton X-100 lysis procedure. *L. monocytogenes* strains 519 and 524 were grown in BHI overnight and serially diluted 10-fold to 10^{-8} . Both protocols were followed and run with the same PCR mastermix and program. Endpoint detection was performed on CHAS and subsequent lower limit of detection (LOD) was calculated for comparison.

***Listeria monocytogenes* multiplex PCRs.** Sequences for oligonucleotide primers (Sigma-Genosys, Oakville, ON, Canada) targeting *hlyA*, *inlJ*, *IGS*, *IAC*, *gtcA* and *lmo* are listed in Table 1. All primers were rehydrated to 100 μ M and aliquoted for storage at -20°C. Primers were diluted to working concentrations as needed. The concentrations of primers used in the LmCHAS and LmVCHAS methods were optimized. For the LmCHAS multiplex PCR, 5 μ L of bacterial lysate was added to 45 μ L of multiplex PCR mixture containing 2.5 units HotStar Taq and 1X HotStar PCR buffer (Qiagen Inc., Mississauga, ON, Canada), 2.5 mM $MgCl_2$, 200 μ M of each dNTP, 10 μ M alkali-stable digoxigenin (DIG)-11-deoxyuridine triphosphate (dUTP) (Roche Diagnostics, Laval, QC, Canada), 25 μ g non-acetylated bovine serum albumin (B8667, Sigma Aldrich Co.), 0.4 μ M *IGS* primers, 0.1 μ M *IAC* primers and 0.2 μ M for both sets of *hlyA* and *inlJ* primers.

For the LmVCHAS multiplex PCR, the same mastermix as the LmCHAS method was used except instead of *inlJ* and *IGS* primers, 0.4 μ M for both sets of *gtcA* and *lmo* primers were used. Also, 0.4 μ M instead of 0.2 μ M of *hlyA* primers were used in the mastermix. Both multiplex PCRs were carried out in a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) using the following conditions: an initial denaturation cycle at 94°C for 15 min, followed by 34 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, and primer extension at 72°C for 1 min 30 s, with an additional extension at 72°C for 2 min following the last cycle.

Table 1. Oligonucleotide primer sequences used in the newly devised *Listeria monocytogenes* Cloth-based Hybridization Array System (LmCHAS) and *Listeria monocytogenes* Virulence Cloth-based Hybridization Array System (LmVCHAS) procedures.

Primer name	Sequence (5'→3')	Product size (bp)	Reference
<i>IGS-F</i>	CTA TAG CTC AGC TGG TTA GAG	275	Graham et al., 1996
<i>IGS-R</i>	TTC TCG GTT ACT TGT GTC A		
<i>hlyA-F</i>	GAA CCT ACA AGA CCT TCC AG	193	This study, based on <i>hlyA</i> gene sequence in Mengaud et al., 1988
<i>hlyA-R</i>	TTT CCG CTT ACG GCA GCA TC		
<i>lmo-F</i>	TTG CAA CTC CGG GAG ATT AT	199	Zhang and Knabel, 2005
<i>lmo-R</i>	GTA ACT ACC GCG CCA GAT TT		
<i>inlJ -F</i>	TGT AAC CCC GCT TAC ACA GTT	238	Liu et al., 2007
<i>inlJ -R</i>	AGC GGC TTG GCA GTC TAA TA		
<i>gtcA -F</i>	TCC GTG GTA CAC AGA TCA GG	238	This study, NCBI ^a accession number AF072894
<i>gtcA -R</i>	ATG AAC TCG CTT CAC GGA GG		
<i>IAC-1</i>	CAT AAT ATC ACT CGC GTC CGT TGA AGC TTA	40	Leggate and Blais, 2006
<i>IAC-2</i>	GAC GAA ATC GTA AGC TTC AA		

^aNCBI = National Center for Biotechnology Information

***Listeria monocytogenes* CHAS procedures.** All probes were checked for cross-reactivity in CHAS. Sequences for the oligonucleotide probes (Sigma-Genosys) specific for *hlyA*, *inlJ*, *IGS*, *IAC*, *gtcA* and *lmo* are listed in Table 2. The CHAS procedure involves preparation of cloth strips, application of probe to the strips followed by hybridization and colorimetric detection.

Table 2. Oligonucleotide probe sequences used in the newly devised *Listeria monocytogenes* Cloth-based Hybridization Array System (LmCHAS) and *Listeria monocytogenes* Virulence Cloth-based Hybridization Array System (LmVCHAS) procedures.

Oligonucleotide probe	sequence (5'→3')	Reference
<i>hlyA</i> probe	ATC CTC CTG CAT ATA TCT CAA GTG TGG CGT ATG GCC GTC AAG TTT ATT TG	This study, designed from Mengaud et al., 1988
<i>IGS</i> probe	GCA CGC CTG ATA AGC GTG AGG TCG ATG GTT CGA GTC CAT TTA GGC CCA CT	This study, designed from sequence in Graham et al., 1997
<i>inlJ</i> probe	GAC CTA ACA CAC AAC ACA CAA TTA ATA TAT TTT CAA GCT GAA GGA TGT AG	This study, NCBI ^a accession number FM242711
<i>lmo</i> probe	CCA CAA GTC GCG GTT AAC TCG AAC GAT ATT TCA TTT GAA GTA GGA ACA GA	This study from sequence in NCBI ^a
<i>gtcA</i> probe	ATG GGT GGA TTT ACT ACC ATT ATT AAT ATA GTA ACA TTT TGG CTA TGT AC	This study from NCBI ^a accession number AF072894
<i>IAC</i> probe	CAT AAT ATC ACT CGC GTC CGT TGA AGC TTA CGA TTT CGT C	Leggate and Blais, 2006

^aNCBI = National Center for Biotechnology Information

1. Preparation of cloth strips

Polyester cloth (DuPont Sontara 8100) strips (2×6 cm), bearing a printed sample location grid with 5 cells, were manufactured by contract with Apace Screen Printing & Enterprises Ltd (Ottawa, Ontario, Canada). These were washed with 95% v/v ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The strips were placed in an uncovered petri dish and air dried, stored in a drawer or cupboard, overnight at room temperature.

2. Application of probe to cloth strips

The probes were separately diluted to $14.6 \mu\text{M}$ in high salt buffer (HSB) (0.1 M Tris/HCl [pH 8], 0.01 M MgCl_2 and 0.15 M NaCl), then heated for 10 min at 100°C (to disrupt any possible secondary structures), and snap chilled on ice. The probe solutions were diluted with a sufficient volume of 95% v/v ethanol to give final coating solutions containing $10 \mu\text{M}$ of probe in HSB containing 30% v/v ethanol. Probes ($7 \mu\text{L}$) were pipetted in discrete spots on the cloth strip, followed by incubation in an uncovered petri dish at 37°C for 30 min. Probes were cross-linked to the cloth by exposing the strips to UV light (254 nm , 120 mJ/cm^2) using a Stratalinker® UVcross-linker (Stratagene, La Jolla, CA, USA). The strips were blocked by incubating for 1 h at 37°C with 1 mL hybridization solution (5 x SSC [1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.02% w/v SDS, 0.1% w/v N-lauroyl sarcosine and 1% w/v protein blocking reagent [Bio-Rad, Hercules, CA, USA]), after which they were washed five times by saturating strips with 0.01 M phosphate-buffered saline (pH 7.2, 0.15 M NaCl) (PBS) containing 0.05% v/v Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-

coated cloth strips were air-dried and stored in a closed container at 4 °C until use. The probe-coated cloth strips were stable for at least 4 months when stored dry at 4 °C.

3. Hybridization and colorimetric detection

For the assay of DIG-labelled amplicons, 40 µL of multiplex PCR product was denatured by heating at 100 °C for 10 min, snap chilled on ice, and mixed with 960 µL of ice-cold hybridization solution containing 50% v/v formamide. The entire mixture (1 ml) was pipetted onto a strip of probe-coated cloth and incubated for 10 min (optimized time) at 45 °C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Strips were saturated with 1 ml of anti-digoxigenin Fab fragments-peroxidase conjugate (Roche) diluted 1:2000 in PBST containing 0.5% w/v protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips were saturated with 1 ml tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), and incubated for 10 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

Limit of detection/detectability. Preliminary lower limit of detection (LOD) experiments of the original CHAS method used *L. monocytogenes* strains 519 (serotype 4b) and 524 (serotype 1/2a). An inoculum of each strain was grown in BHI and incubated for 24 h at 30°C and 100 rpm. The inoculated broths were then diluted 10-fold to 10⁻⁸ and 5 µL was added for PCR. Dilutions were also plated on BHI plates for MPN calculations. The CHAS protocol was then followed and the results analyzed to determine the highest dilution necessary to elicit a positive result. Based on enumerated colonies, and the dilution factor, the LOD was calculated. The LOD of the LmCHAS and LmVCHAS was not calculated. Instead, a single colony of approximately 10⁸ CFU was used for the purposes of each method.

Specificity panels. The devised LmCHAS and LmVCHAS methods were assayed for specificity against a panel of five different non-*Listeria*, 11 *Listeria* spp. (Tables 3 and 5), and 35 serotyped *L. monocytogenes* strains including strains that have been implicated in listeriosis outbreaks (Tables 4 and 6). The LmCHAS method was assayed for specificity against an additional panel of 24 *L. monocytogenes* strains isolated from various food manufacturing environment and related food samples (Table 7).

Table 3. *L. monocytogenes* Virulence Cloth-based Hybridization Array System (LmVCHAS) specificity assays against 16 selected non-*Listeria* and *Listeria* spp. (excluding *L. monocytogenes*) bacterial strains^a deposited in the CFIA Ottawa Laboratory Carling (OLC) culture collection.

OLC no.	Bacterial strain	LmVCHAS ^b			
		<i>hlyA</i>	<i>lmo</i>	<i>gtcA</i>	<i>IAC</i>
23	<i>Escherichia coli</i> ATCC 11775	-	-	-	+
32	<i>Pseudomonas aeruginosa</i> ATCC 10145	-	-	-	+
59	<i>Salmonella typhimurium</i> ATCC 14028	-	-	-	+
161	<i>Bacillus subtilis</i> ATCC 6051	-	-	-	+
646	<i>Staphylococcus epidermidis</i> ATCC 14990	-	-	-	+
4	<i>Listeria innocua</i> ATCC 33090	-	-	-	+
9	<i>Listeria innocua</i>	-	-	-	+
267	<i>Listeria innocua</i>	-	-	-	+
5	<i>Listeria ivanovii</i> ATCC 19119	-	-	-	+
10	<i>Listeria ivanovii</i>	-	-	-	+
287	<i>Listeria ivanovii</i>	-	-	-	+
249	<i>Listeria seeligeri</i>	-	-	-	+
11	<i>Listeria seeligeri</i>	-	-	-	+
544	<i>Listeria welshimeri</i>	-	-	-	+
12	<i>Listeria welshimeri</i>	-	-	-	+
13	<i>Listeria grayi</i>	-	-	-	+

^aAn isolated colony from a pure culture of each strain grown on Typticase Soy Agar (Sigma Aldrich) was lysed and then subjected to the Lm CHAS procedure as described in Methods. ATCC = American Type Culture Collection.

^bReactivity in the CHAS for each amplicon-specific probe feature: +, blue spot; -, no spot.

Table 4. *L. monocytogenes* Virulence Cloth-based Hybridization Array System (LmVCHAS) specificity assays against 35 selected serotyped *L. monocytogenes* strains^a deposited in the CFIA Ottawa Laboratory Carling (OLC) culture collection.

OLC no.	Serotype	LmVCHAS ^b			
		<i>hlyA</i>	<i>lmo</i>	<i>gtcA</i>	<i>IAC</i>
2 ^c	1/2a	+	+	-	+
535	1/2a	+	+	-	+
949	1/2a	+	+	-	+
950	1/2a	+	+	-	+
951	1/2a	+	+	-	+
952	1/2a	+	+	-	+
533	1/2b	+	-	-	+
523	1/2c	+	+	-	+
524	1/2c	+	+	-	+
529	3a	+	+	-	+
530	3b	+	-	-	+
531	3c	+	+	-	+
964	4a	+	-	-	+
965	4a	+	-	+	+
966	4a	+	-	+	+
967	4a	+	-	+	+
968	4a	+	-	-	+
521	4b	+	-	+	+
953	4b	+	-	+	+
954	4b	+	-	+	+
955	4b	+	-	+	+
956	4b	+	-	+	+
538 ^d	4bx	+	-	+	+
525	4c	+	-	-	+
846	4c	+	-	-	+
847	4c	+	-	-	+
848	4c	+	-	-	+
849	4c	+	-	-	+
850	4c	+	-	-	+
942	4c	+	-	+	+
943	4c	+	-	-	+
944	4c	+	-	-	+
527	4d	+	-	+	+
526	4e	+	-	+	+
532	7	+	-	-	+

^a An isolated colony from a pure culture of each strain grown on Trypticase Soy Agar was lysed and then subjected to the LmVCHAS procedure

^b Reactivity in the Cloth-based Hybridization Array System for each amplicon-specific probe feature: +, blue spot; -, no spot.

^c American Type Culture Collection (ATCC) 15313.

^d Implicated in a listeriosis outbreak in the UK between 1987 and 1989, traced to contaminated pâté (McLaughlin et al. 1991).

Table 5. *Listeria monocytogenes* Cloth-based Hybridization Array System (LmCHAS) specificity assays against 16 selected non-*Listeria* and *Listeria* spp. (excluding *L. monocytogenes*) bacterial strains^a deposited in the CFIA Ottawa Laboratory Carling (OLC) culture collection.

OLC no.	Bacterial strain	LmCHAS ^b			
		<i>hlyA</i>	<i>inlJ</i>	<i>IGS</i>	<i>IAC</i>
23	<i>Escherichia coli</i> ATCC 11775	-	-	-	+
32	<i>Pseudomonas aeruginosa</i> ATCC 10145	-	-	-	+
59	<i>Salmonella typhimurium</i> ATCC 14028	-	-	-	+
161	<i>Bacillus subtilis</i> ATCC 6051	-	-	-	+
646	<i>Staphylococcus epidermidis</i> ATCC 14990	-	-	-	+
4	<i>Listeria innocua</i> ATCC 33090	-	-	+	+
9	<i>Listeria innocua</i>	-	-	+	+
267	<i>Listeria innocua</i>	-	-	+	+
5	<i>Listeria ivanovii</i> ATCC 19119	-	-	+	+
10	<i>Listeria ivanovii</i>	-	-	+	+
287	<i>Listeria ivanovii</i>	-	-	+	+
249	<i>Listeria seeligeri</i>	-	-	+	+
11	<i>Listeria seeligeri</i>	-	-	+	+
544	<i>Listeria welshimeri</i>	-	-	+	+
12	<i>Listeria welshimeri</i>	-	-	+	+
13	<i>Listeria grayi</i>	-	-	-	+

^aAn isolated colony from a pure culture of each strain grown on Typticase Soy Agar (Sigma Aldrich) was lysed and then subjected to the Lm CHAS procedure as described in Methods. ATCC = American Type Culture Collection.

^bReactivity in the CHAS for each amplicon-specific probe feature: +, blue spot; -, no spot.

Table 6. *Listeria monocytogenes* Cloth-based Hybridization Array System (LmCHAS) specificity assays against 35 selected serotyped *L. monocytogenes* strains^a deposited in the CFIA Ottawa Laboratory Carling (OLC) culture collection.

OLC no.	Serotype	LmCHAS ^b			
		<i>hlyA</i>	<i>inlJ</i>	<i>IGS</i>	<i>IAC</i>
2 ^c	1/2a	+	+	+	+
535	1/2a	+	+	+	+
949	1/2a	+	+	+	+
950	1/2a	+	+	+	+
951	1/2a	+	+	+	+
952	1/2a	+	+	+	+
533	1/2b	+	+	+	+
523	1/2c	+	+	+	+
524	1/2c	+	+	+	+
529	3a	+	+	+	+
530	3b	+	+	+	+
531	3c	+	+	+	+
964	4a	+	+	+	+
965	4a	+	+	+	+
966	4a	+	+	+	+
967	4a	+	+	+	+
968	4a	+	+	+	+
521	4b	+	+	+	+
953	4b	+	+	+	+
954	4b	+	+	+	+
955	4b	+	+	+	+
956	4b	+	+	+	+
538 ^d	4bx	+	+	+	+
525	4c	+	+	+	+
846	4c	+	+	+	+
847	4c	+	+	+	+
848	4c	+	+	+	+
849	4c	+	+	+	+
850	4c	+	+	+	+
942	4c	+	+	+	+
943	4c	+	+	+	+
944	4c	+	+	+	+
527	4d	+	+	+	+
526	4e	+	+	+	+
532	7	+	+	+	+

^a An isolated colony from a pure culture of each strain grown on Trypticase Soy Agar was lysed and then subjected to the LmVCHAS procedure

^b Reactivity in the Cloth-based Hybridization Array System for each amplicon-specific probe feature: +, blue spot; -, no spot.

^c American Type Culture Collection (ATCC) 15313.

^d Implicated in a listeriosis outbreak in the UK between 1987 and 1989, traced to contaminated pâté (McLaughlin et al. 1991).

Table 7. *Listeria monocytogenes* Cloth-based Hybridization Array System (LmCHAS) specificity assay against 24 selected non-serotyped *L. monocytogenes* strains recovered from the food manufacturing environment and related foods.

OLC no.	Environment/food source	LmCHAS ^b			
		<i>hlyA</i>	<i>inlJ</i>	<i>IGS</i>	<i>IAC</i>
6	Scott A strain, unknown source	+	+	+	+
17	Environmental sample, egg	+	+	+	+
19	Environmental sample, cheese	+	+	+	+
65	Environmental sample, meat	+	+	+	+
66	Environmental sample, meat	+	+	+	+
67	Environmental sample, meat	+	+	+	+
68	Pizza, Italian sausage	+	+	+	+
69	Chicken parmigiana	+	+	+	+
70	Chicken breast, bacon, processed cheese	+	+	+	+
71	Veal, bacon, processed cheese	+	+	+	+
75	Environmental sample, meat	+	+	+	+
78	Pork spareribs	+	+	+	+
79	Environmental sample, meat	+	+	+	+
81	Polish sausage	+	+	+	+
82	Environmental sample, cheese	+	+	+	+
84	Environmental sample, ice cream	+	+	+	+
85	Environmental sample, dairy	+	+	+	+
86	Environmental sample, dairy	+	+	+	+
99	Pouligny cheese, raw milk	+	+	+	+
179	Ground beef	+	+	+	+
211	Cheese	+	+	+	+
223	Le Bougon cheese; Fromagerie du Nord	+	+	+	+
230	Cour de Brie; raw milk cheese	+	+	+	+
925 ^a	Roast beef	+	+	+	+

^aImplicated in a listeriosis outbreak in Canada in 2008, traced to contaminated ready-to-eat meats from a Maple Leaf Foods manufacturing plant

^bReactivity in the Cloth-based Hybridization Array System for each amplicon-specific probe feature: +, blue spot; -, no spot.

OLC = Ottawa Laboratory Carling culture collection. All strains were initially obtained from the Listeriosis Reference Center, Bureau of Microbial Hazards, Health Canada, Ottawa, Ontario, Canada.

2.3 Results and discussion

Typical results of the LmCHAS and LmVCHAS using *L. monocytogenes* strain 519 are shown in Figures 1 and 2, respectively. For quality control, all the primer sets were verified to work properly to produce appropriate amplicons in multiplex-PCR and all probes were verified in CHAS with no cross-reactivity. In addition, all tested strains produced a positive result for the internal control (*IAC*) in the LmCHAS and LmVCHAS methods indicating no interference or inhibition in the multiplex-PCRs.

Figure 1. Detection of *Listeria monocytogenes* Cloth-based Hybridization Array System (LmCHAS) multiplex PCR amplicons by colorimetric reaction (blue spots) on cloth-based hybridization array system. Columns 1-5: *hlyA*, *inlJ*, *IGS*, *IAC*, n/a. This exemplifies a typical result using *Listeria monocytogenes* strain 519 (serotype 4b).

Figure 2. Detection of *Listeria monocytogenes* Virulence Cloth-based Hybridization Array System (LmVCHAS) multiplex PCR amplicons by colorimetric reaction (blue spots) on cloth-based hybridization array system. Columns 1-5: *hlyA*, *lmo*, *gtcA*, *IAC*, n/a. A: this exemplifies a typical result using *Listeria monocytogenes* strain 519 (serotype 4b). B: this exemplifies a typical result using *Listeria monocytogenes* strain 535 (serotype 1/2a).

A comparison of the LODs of the Triton X-100 cell lysis procedure (described in preparation of bacterial lysates section of Methods) to the Wizard genomic DNA extraction procedure for Gram positive bacteria (commercially available DNA extraction kit), was performed to determine which method was more effective at lysing cells for PCR. Serial dilutions were performed for the end products of each protocol and 5 μ L was added to the multiplex-PCR CHAS procedure. The highest dilution that produced a positive CHAS result was the LOD. A LOD of 90 CFU was calculated for the Triton X-100 lysis procedure (18 CFU/ μ L in 200 000-fold dilution X 5 μ L for PCR) compared to 180 CFU for the Wizard Genomic procedure for detection of *L. monocytogenes* strain 519 (36 CFU/ μ L in 100 000-fold dilution X 5 μ L for PCR). Similarly, LODs of 118 CFU and 235 CFU were observed for the Triton X-100 lysis procedure and Wizard genomic DNA extraction procedure, respectively for *L. monocytogenes* strain 524. These results confirm that Triton X-100 is a better method than the Wizard kit to use for DNA extraction from *L. monocytogenes*. In addition, the Triton X-100 lysis procedure is simpler and more rapid compared to the Wizard genomic DNA extraction procedure. The Triton X-100 lysis procedure was subsequently used in all other experiments to prepare DNA for molecular diagnostics.

Inclusivity/exclusivity and specificity of LmVCHAS. The LmVCHAS exhibited 100% exclusivity in the analysis of pure cultures of five non-*Listeria* spp. and 11 *Listeria* spp. (excluding *L. monocytogenes*) strains (Table 3). Using a panel of 35 different serotyped *L. monocytogenes* strains, the LmVCHAS displayed 100% inclusivity for all serotype 1/2a and 4b high risk strains assayed that were characterized by

researchers as having the *lmo* and *gtcA* gene targets (Table 4). However, the LmVCHAS had poor exclusivity and specificity overall, with several strains that have been rarely implicated in human infection getting positive results (Table 4). In LmVCHAS, the *gtcA* gene target was positive for all serotype group 4b (d,e) strains, one serotype 4c strain (OLC no. 942), and three serotype 4a strains (OLC no. 958, 959, 960) (Table 4), suggesting that a sample positive for *gtcA* is most likely in serotype 4 (based on the strains tested in the present study). Within serotype 4, serotype 4b is predominantly isolated from humans and poses a high risk of human infection while serotypes 4a, 4c, 4d, and 4e are rare and pose little risk (Bell and Kyriakides, 2005; McLauchlin, 1997). The *lmo* gene target was positive for all serotype group 1/2a (3a) and 1/2c (3c) strains, which are all in lineage II and are prevalent in foods and the environment (Table 4). Most lineage II strains are implicated in animal cases of listeriosis; however, some serotype 1/2a strains have been implicated in human clinical cases, making this lineage moderately significant in terms of risk for human infection. The presence of *lmo* in serotype 3c strains contrasts results by Zhang and Knabel (2005), where *lmo* was negative for serotype 3c strains. Serotype groups 1/2a (3a) and 1/2c (3c) have a common somatic type antigen ancestor (Zhang and Knabel, 2005), which may explain the presence of the conserved *lmo* gene in these serotypes. Furthermore, serotype 3 strains are rare and pose little risk for human infection (Bell and Kyriakides, 2005; McLauchlin, 1997). The avirulent and rare strains that were positive for *lmo* and *gtcA*, may have the gene sequence present but not transcribed. It is possible that these are false positives but highly unlikely given the robustness of the multiplex-PCR (primer specificity tested by other researchers) and the CHAS system (hybridization to specific probe). The only way

to know for sure is to sequence the strains. The results obtained with all the purported virulence markers largely indicate that they are not good indicators of pathogenic potential. However, *inlJ* was discovered to be a good species-specific marker.

Inclusivity/exclusivity and specificity of LmCHAS. In the present study, the *inlJ* gene was found in all *L. monocytogenes* strains tested, which span a variety of different serotypes and sample origins. In contrast to Liu et al. (2007), the present research found that some avirulent strains had the *inlJ* marker as all five avirulent serotype 4a strains tested were positive for *inlJ* (Table 6). Liu et al. (2003) used comparative genomics to screen clones from virulent and avirulent strains to discover the *inlJ* gene; however, only three avirulent serotype 4a strains were assayed. Also, the detection of amplicons by hybridization with a probe could have been a factor in this discrepancy as Liu et al. (2003) detected PCR products by agarose gel electrophoresis. Results of this research suggest that the use of *inlJ* is better suited as a species-specific marker to complement *hlyA*, than as a virulence determinant (*inlJ* may still be a virulence determinant). The use of two markers in LmCHAS for species identification reduces the chance of false positives as both markers would have to be present to indicate a true positive result.

The LmCHAS exhibited 100% exclusivity/inclusivity and specificity in the analysis of pure cultures of five non-*Listeria* spp. and 11 *Listeria* spp. (excluding *L. monocytogenes*) strains (Table 5), 35 different serotyped *L. monocytogenes* strains (Table 6), and 24 non-serotyped *L. monocytogenes* strains obtained from foods and their manufacturing environments (Table 7). In LmCHAS, the original *hlyA* primers taken

from MFLP-78 produced a 730 bp amplicon with a faint reaction with agarose gel electrophoresis when used in a multiplex-PCR procedure targeting *hlyA*, *inlJ*, and *IGS* (not shown). After adjusting primer, buffer and MgCl₂ concentrations, and changing annealing temperatures to optimize the PCR and not finding success, primers were redesigned to produce a smaller 193 bp amplicon that was able to yield a stronger reaction under the same conditions. The advantages of having smaller amplicons in a multiplex-PCR are that smaller amplicons are preferentially amplified. Furthermore, having all the amplicons a similar size (approximately 200 bp) ensures there is a more balanced amplification. The redesigned *hlyA* primers produced amplification with 100% inclusivity and 100% exclusivity for species-specific detection in subsequent trials. *IAC* was also positive for all samples tested. *IAC* is used as an internal control for PCR because it forms a primer dimer from 3' overlaps of the forward and reverse primers (Leggate and Blais, 2006). *IGS* is the 16S/23S rRNA intergenic spacer region common in *Listeria* spp. *IGS* was specific for all *Listeria* spp. except for *L. grayi*, a rare species in the *Listeria* genus from a closely related but distinct line of descent from the other *Listeria* spp. (Graham et al. 1996; 1997) (Table 5). The non-detection of *L. grayi* by *IGS* will not be a problem from a food safety perspective as this species is non-pathogenic to both humans and animals (Vasquez-Boland, 2001). An *IGS*-negative, *hlyA*- and *inlJ*-positive result can be confirmed as *L. grayi* by performing biochemical tests. The inclusion of *IGS* adds a second control feature to the LmCHAS method by confirming the identification of *Listeria* spp., with the exception of *L. grayi*.

Sensitivity and limit of detection. All *hlyA*, *inlJ* and *IGS* positive results were correctly assigned for LmCHAS, indicating 100% sensitivity (ability to detect all the analyte targeted) (Tables 6 and 7). Surprisingly, the *lmo* and *gtcA* markers were positive for several serotype 3a and 3c strains reported by researchers in previous studies as negative for the markers, making the LmVCHAS sensitivity incalculable (Table 4). In preliminary experiments, the LOD for the original CHAS was approximately 90 CFU for *L. monocytogenes* strain 519. After splitting the original CHAS method into LmCHAS and LmVCHAS, both methods required just a single colony (approximately 10^8 CFU) isolated from standard culture on agar plates to effectively produce a definitive colorimetric reaction. Additional experiments to determine a more accurate LOD for LmCHAS and LmVCHAS were not performed because the purpose of these methods was to perform single colony testing of a presumptive positive colony isolated by standard culture techniques. As part of the guidelines for producing a new method for detecting a pathogen of interest, various plating media typically used to grow of the pathogen of interest were assayed. Results from testing the LmCHAS method using a positive control *L. monocytogenes* strain to grow colonies on the common media used by microbiology analysts to culture the pathogen, revealed that no reagent in the media interferes with the procedure (Figure 3).

Figure 3. Analysis of *Listeria monocytogenes* grown on different plating media by *Listeria monocytogenes* Cloth-based Hybridization Array System. LSA = Listeria Selective Agar. PAL = Palcam. RLM = Rapid L. mono chromogenic media. TSA + 7% HB = Trypticase Soy Agar + 7% horse blood. BHI = Brain Heart Infusion media. Columns 1-5: *hlyA*, *inlJ*, *IGS*, *IAC*, n/a.

Benefits of LmCHAS over the existing standard method. The newly devised LmCHAS and LmVCHAS methods are PCR-based rapid methods for identification of *L. monocytogenes*. PCR amplification is recognized as an effective approach for the identification of *L. monocytogenes* (Vazquez-Boland et al., 2001). LmCHAS is more specific and robust than the current rapid method used for identification, MFLP-78, as it supplements the species-specific *hlyA* gene marker with the addition of a second species-specific marker, *inlJ*, which will strengthen the identification of isolates as well as provide for the identification of atypical strains lacking *hlyA*. By using a multiplex approach, *hlyA* and *inlJ* are used for unequivocal detection of the pathogen.

LmCHAS and LmVCHAS contrast MFLP-78 in endpoint detection of PCR products by using CHAS instead of agarose gel electrophoresis. CHAS is reported to be better suited than gel electrophoresis for end-point detection of multiplex-PCR amplicons (Gauthier and Blais, 2004). Advantages of CHAS compared to a gel are that CHAS can differentiate amplicons unambiguously by using a probe immobilized on polyester cloth that binds a specific DIG-labelled amplicon to produce a colorimetric reaction that can be easily detected (Gauthier and Blais, 2004). This also ensures specificity by hybridization.

The use of LmCHAS provides more definitive identification of *L. monocytogenes* compared to MFLP-78. This rapid method only takes half a day for an analyst to perform compared to 1-2 days for biochemical tests that may not provide definitive identification (Figure 4). Microbiology analysts will have the flexibility to assay presumptive colonies at different junctures during the enrichment culture process (i.e., identification of *L. monocytogenes* directly on selective agars, haemolysis plates and non-selective media).

Thus, analysts can perform diagnostic tests on a greater number of samples and have more time to analyse the results. This leads to better testing efficiency and increased food safety. Furthermore, the use of a colony allows for downstream analyses such as epidemiological investigations using, for example, pulsed-field gel electrophoresis, which can be readily accomplished in the context of the present approach since only a portion of the colony is required for the CHAS procedure. The use of two species-specific markers, *inlJ* and *hlyA*, and the inclusion of two quality control features (*IGS* detection as a control for DNA extraction, amplification and detection, and *IAC* as a control for PCR inhibition) enhance the reliability of the method as a confirmatory tool. In this assay the genes acting as the basis for the species-specific markers are key elements in the pathogenic mechanism of *L. monocytogenes*, and their discernment in food-borne isolates will contribute significantly to risk-based regulatory decision making.

Figure 4. Diagnostic testing scheme for *Listeria monocytogenes*. Timeframe indicated is approximate for processing 20 samples by one analyst. MFLP-41, MFHPB-30 and MFLP-78 are standard methods used and described in the Health Canada Compendium of Analytical Methods. LmCHAS = *Listeria monocytogenes* cloth-based hybridization system. LmVCHAS = *Listeria monocytogenes* virulence cloth-based hybridization system.

Several strains biochemically identified as *L. monocytogenes* have been noted to be negative by the current PCR-based standard detection protocol (MFLP-78); however, they have been detected by the improved methods developed in the present study, making these new methods more robust than MFLP-78 (Xue et al., 2010). In addition, these new methods are more informative, with the option to determine if the detected strain is clinically relevant and of high concern to public health and food safety.

By definitively identifying the presence of *L. monocytogenes* in a sample using the PCR-based rapid method developed by the present research, samples can be processed more efficiently, thereby reducing costs, and allowing more samples to be tested and analysed. This new method has recently been validated for definitive identification of *L. monocytogenes* isolated from food and environmental samples by standard culture techniques and it may be implemented by CFIA in the near future for use as a standard diagnostic method.

2.4 Conclusions

In conclusion, microbiology analysts cannot use LmVCHAS to determine that a strain is unequivocally high risk. A strain positive for *lmo* and *gtcA* is likely a high risk serotype 1/2a or 4b strain; however, it may be a rare serotype: 3a, 3c, 4a, 4c, 4d, or 4e strain not usually implicated in human illness (Graves et al., 2007; Gorski, 2008).

LmVCHAS may be improved in the future by revising the key genetic markers targeted as more information on the differences between serotypes becomes available. With the recent advances in comparative genomics and bioinformatics, housekeeping genes that are exclusive to high risk outbreak-related strains may be characterized soon. Moreover, researchers may identify marker genes associated with virulence to develop a suitable test differentiating virulent from avirulent *L. monocytogenes* strains. Armed with more informative methods, food industry inspectors and food safety regulators may impose appropriate and effective control measures to combat *L. monocytogenes* contamination in food manufacturing environments and related foods.

The devised LmCHAS method constitutes an improvement over the existing pathogen detection method. LmCHAS can detect one colony of cells and can be used to meet regulatory needs by providing rapid definitive identification of *L. monocytogenes*. The *inlJ* gene was found to be present in all *L. monocytogenes* tested and acts as a second species-specific marker, increasing the sensitivity of the method and allowing it to provide definitive identification. The results obtained in the present research from LmCHAS support its use as a reliable tool for the identification of food borne *L. monocytogenes*. In addition, this method significantly reduced the turnaround time for

reporting results by eliminating the need for time-consuming biochemical tests. In LmCHAS, the genes acting as the basis for the species-specific markers are key elements in the pathogenic mechanism of *L. monocytogenes*, and their discernment in food-borne isolates will contribute significantly to risk-based regulatory decision making. The LmCHAS was validated by separate CFIA laboratories in Toronto, Ontario and Dartmouth, Nova Scotia. Thus, the LmCHAS protocol may be implemented by CFIA in the near future to replace the current CFIA standard method, MFLP-78, for identification of presumptive positive *L. monocytogenes* from foods and environmental samples.

**CHAPTER 3: CREATION OF MODEL *LISTERIA MONOCYTOGENES* SINGLE- AND DUAL-
SPECIES FOOD MANUFACTURING ENVIRONMENT BIOFILMS AND USE OF POTENTIAL
BIOFILM-DISPERSING AGENTS FOR THE IMPROVED RECOVERY OF THE PATHOGEN FROM
MODEL BIOFILMS**

3.1 Introduction

Biofilms afford food pathogens, such as *Listeria monocytogenes*, an increased ability to survive under unfavourable environmental conditions on food contact surfaces in the food manufacturing plant (Wong, 1998). The ability of *L. monocytogenes* to form heterogeneous biofilms with other microbes allows it increased rates of genetic transfer and subsequently increased antimicrobial resistance (Watnick and Kolter, 2000). Furthermore, the extracellular matrix formed while in sessile state allows for increased protection from detergents and sanitizers (Wong, 1998; Pan et al., 2006). This all means that biofilms allow *L. monocytogenes* to persist in the food manufacturing environment.

Biofilm formation is ubiquitous in the food manufacturing environment and it is not practical to completely prevent or eliminate biofilms (Lourenco et al., 2011). Thus, inspectors have to be able to detect foodborne pathogens in biofilms on food contact surfaces. The current sponging and swabbing methods of pathogen detection may not be effective in liberating *L. monocytogenes* cells in sessile form in a biofilm (Jay et al., 2005; Kang et al., 2007). As a result, *L. monocytogenes* may cause persistent low level of contamination in a food manufacturing environment without being detected. The ineffective sampling that failed to detect the persistent and virulent *L. monocytogenes*

strain established in biofilms in the Maple Leaf Foods manufacturing plant was the main cause for the recent listeriosis outbreak in Canada in 2008.

The primary objective of this research was to devise treatments releasing biofilm bacteria from food contact surfaces to improve the efficacy of sampling for detection. A sub-objective of this research was to create a model system of single- and dual-species *L. monocytogenes* biofilms on stainless steel surfaces in order to test the potential biofilm-dispersal agents. Most biofilms in the natural environment are heterogeneous (Deibel, 2003). Thus, in order to devise a method that would detect *L. monocytogenes* in a heterogeneous biofilm, the key microbes present in the food manufacturing environment must be identified first and used to create multi-species biofilms with *L. monocytogenes*. These key microbial inhabitants are more likely to engage in synergistic interactions with *L. monocytogenes* and form biofilms for survival and persistence under harsh environmental conditions.

Several agents have recently been reported to disperse biofilms from surfaces: Dispersin B, pectinase, D-amino acids, DNase 1 and marine algal and sponge extracts. Dispersin B is an enzyme that is marketed by biotechnology companies to disperse and prevent biofilm formation on medical devices. Researchers have found that Dispersin B hydrolyzes the $\beta(1,6)$ -linked N-acetylglucosamine oligomers found in the extracellular matrix of biofilms comprised of bacterial pathogens such as *Staphylococcus epidermidis* (Kaplan et al., 2004; Ramasubbu et al., 2005; Kerrigan et al., 2008). Pectinase, D-amino acids, DNase, and natural sponge extracts have also been reported to combat biofilms formed by pathogenic bacteria. (Lequette et al., 2010; Kolodkin-Gal et al., 2010; Hall-

Stoodley et al., 2008; Huigens et al., 2008). The mode of action of pectinase and DNase has not been elucidated; however, it is believed that these agents remove biofilms in a similar manner as Dispersin B by degrading the extracellular matrix of biofilms (Lequette et al., 2010; Hall-Stoodley et al., 2008). Specific D-amino acids, such as D-Tyrosine, D-Leucine, D-Tryptophan, D-Methionine and a mixture of all 4 D-amino acids, have been reported by Kolodkin-Gal et al. (2010) to replace the D-Alanine in the peptide side chains in the EPS matrix to trigger biofilm disassembly. Natural marine sponge and algae extracts are known to remove biofouling in the marine environment and their anti-biofilm properties have been traced to the release of brominated furanones, which are known to interfere with biofilm formation in several pathogens (Janssens et al., 2008). Annous et al. (2009) reported that brominated furanones released by the marine algae, *Delisea pulchra*, interfere with the quorum sensing required for biofilm formation. If these agents are not bactericidal to *L. monocytogenes*, then incorporating a potential biofilm-dispersal agent into a conventional sampling process for foodborne pathogens would increase the recovery of *L. monocytogenes* cells in biofilms. Thus, sponges and swabs used by inspectors for sampling may be moistened with a biofilm-dispersal agent to improve recovery of *L. monocytogenes* in biofilms. This novel strategy may be validated in the food manufacturing plant in the future and adopted by CFIA for increased food safety to Canadians.

3.2 Materials and Methods

Creation of single-species *L. monocytogenes* biofilms. The method of Leriche and Carpentier (2000) was adapted for the preparation of *L. monocytogenes* biofilms on

circular stainless steel confetti chips (5 mm in diameter, 2 mm thickness, type 304, no. 4 finish) and stainless steel square plates (4 cm x 4 cm, 2 mm thickness, type 304, no. 4 finish). Prior to use the stainless steel surfaces were washed in 95% ethanol for 10 min then rinsed 5 times with sterile distilled water and autoclaved at 121°C for 10 min. Biofilms were prepared using *L. monocytogenes* (biofilm forming) strain KB (Kane Biotech, Winnipeg, Manitoba) grown in Brain Heart Infusion (BHI) broth at 30°C for 24 h. A 100 µL portion of enrichment broth culture was transferred to a 1.5 mL microfuge tube and centrifuged at 10 000 g for 10 min. The pellet was re-suspended in 1.0 mL of sterile 0.01 mol L⁻¹ phosphate-buffered (pH 7.2)/0.85% (w/v) NaCl (PBS), followed by centrifugation and re-suspension in sterile PBS as above. A stainless chip was placed smooth side up in a 2 mL-capacity microfuge tube and covered with 100 µL of washed cell suspension, then incubated at room temperature for 3 h in the dark. The chips were washed 10 times by gentle vortexing in 500 µL of PBS, and the washed chips were then overlaid with 100 µL of BHI diluted 1:10 in sterile distilled water, followed by incubation at room temperature for 2 days in the dark. The chips were then washed by holding with forceps over a receptacle and squirting a gentle stream of sterile PBS on each side for 20 s. These biofilm-coated chips were used immediately in the recovery experiments described below. For biofilms formed on stainless steel squares, the preceding protocol was used with the exception that 1.0 mL of washed cell suspension was used to overcoat the squares placed in a sterile petri dish, and all washes were carried out under a gentle stream of sterile PBS over a receptacle.

Identification of bacterial isolates from the food manufacturing environment.

Four different environmental samples were obtained by swabbing food contact surfaces

in a food manufacturing environment in Canada. Sample 1 was obtained from a cutting board, sample 2 was from a knife, sample 3 was from a conveyor belt and sample 4 was from a slicer blade. To recover bacterial isolates from the samples, 100 mL of Trypticase Soy broth (TSB) (Sigma Aldrich) was added to each of the four environmental sample swabs and they were incubated at 35°C for 24 h then streaked on BHI agar plates and then incubated at 35°C for another 24 h. A total of 38 bacterial isolates as judged by morphology were obtained from plating the four samples.

To identify the species of these bacteria, each different isolate was labelled and streaked on separate BHI plates to produce single colonies. Biomérieux Vitek GPI (Gram positive bacteria) and GNI+ (Gram negative bacteria) kits of 20 cards each were obtained for diagnostic identification of the isolates. First, a Gram test was performed for each isolate. A sample was smeared onto a microscope slide, heat fixed and subject to crystal violet for 60 s, rinsed with Milli-Q water, iodine 60 s, rinse, 95% ethanol 60 s, rinse, safranin 60 s, rinse. All isolates then underwent a catalase test with 20% v/v hydrogen peroxide in water. Isolates that produced bubbles were catalase positive (oxidase negative) and those that did not were catalase negative (oxidase positive). All Gram positive and catalase positive isolates were subjected to a coagulase test where 1 mL of BHI is inoculated with an isolate and incubated at 35°C for 24 h then 200 µL is transferred to coagulase test broth in a test tube. The inoculated test tubes were incubated at 35°C for 4 h and then checked for clumping (positive) and then placed at room temperature overnight and checked again. *Staphylococcus aureus* was used as the positive control. All Gram positive and catalase negative isolates underwent a beta-haemolysis test by picking a colony and placing on sheep blood agar and incubating 35°C

for 24 h to observe if there was a zone of haemolysis (positive). All Gram negative isolates were subject to the catalase test and the result is the opposite for the oxidase test. These results were entered on the GPI and GNI+ cards which were labelled and inoculated with a bacterial suspension of the isolates equivalent to the appropriate McFarland standard. The bacterial suspension was obtained from freshly grown culture, isolated and re-streaked 24 h beforehand. The inoculated cards were run on a Biomérieux Vitek Jr machine overnight and analysis was performed on the same machine to determine the identity of the isolates.

Creation of dual-species biofilms. Dual-species biofilms of *Staphylococcus epidermidis* and *L. monocytogenes* were created in the present study. To prepare for the dual-species biofilm, s.s. chips (5 mm in diameter, 2 mm thick, type 304, no. 4 finish, supplier unknown) were prepared by washing in 95% ethanol for 10 min, rinsing 5 times with milli-Q water followed by autoclaving at 121°C for 10 min. *L. monocytogenes* strain KB (Kane Biotech) was used to inoculate 10 mL of TSB, which was incubated at 30°C, 100 rpm for 24 h. An American Type Culture Collection (ATCC) strain of *S. epidermidis* (Ottawa Laboratory Carling culture collection #646) was used to inoculate 10 mL of TSB, which was incubated at 35°C, 190 rpm for 24 h. A 2:3 mix (400 µL: 600 µL) of *L. monocytogenes* to *S. epidermidis* was mixed together. A 100 µL aliquot of the mixed inoculum was used for the biofilm creation using the same protocol described above for single-species biofilm production.

Visualization of biofilms. Biofilms were visualized by scanning electron microscopy (SEM) performed by the Agriculture and Agri-food Canada Research Centre in Ottawa, Ontario. To fix biofilm samples and prepare them for imaging by SEM, the biofilm-coated chips were placed in 2 mL microfuge tubes containing 200 μ L of sterile PBS with 2.5% glutaraldehyde and incubated for 10 min, then dehydrated in ethanol, followed by critical point drying, gold coating and imaging using a Philips XL30 microscope (SEMTECH Solutions, Inc., North Billerica, Ma). Unseeded chips were also processed in like manner to account for artefacts in interpreting the SEM images.

Preliminary experiments evaluating potential biofilm-dispersal agents. Two controls had to be verified before testing the efficacy of the potential dispersal agents: impact of the agents on cell viability and sterility of the agents. The viability of *L. monocytogenes* cells in the various treatments was assayed by performing a serial dilution of an inoculum that was incubated for 24 h, adding an aliquot of the dilutions to the potential dispersal agents in a 1:1 ratio, incubating for 30 min and plating on BHI media in triplicate. Plates were incubated at 30°C and colony counts were performed after 48 h. As a control for sterility, an aliquot of the agents were plated on BHI media in triplicate.

The potential biofilm dispersal agents tested were 50 μ g/mL Dispersin B, undiluted, 3-fold and 10-fold diluted Pectinex Ultra SPL (pectinase from *Aspergillus aculeatus*, Sigma Aldrich), two units of DNase 1 (Sigma Aldrich), a mixture of 4 D-amino acids (Sigma Aldrich) (1 mM each of D-Tyrosine, D-Methionine, D-Tryptophan and D-Leucine), 1 mM D-Tyrosine (Sigma Aldrich) and 1 mM D-Alanine (Sigma Aldrich) as well as controls of 50 μ g/mL inactive Dispersin B (Kane Biotech), 50 μ g/mL

BioRad blocker (Bio Rad Laboratories) and 0.01% v/v Tween 20 (Sigma Aldrich).

Triplicates of these treatments were used in trials.

Recovery of *L. monocytogenes* from stainless steel chips. Biofilm coated stainless steel surfaces were subjected to a variety of treatments for recovery of *L. monocytogenes* cells, and the efficacy of the treatments was evaluated by performing aerobic plate counts of the eluates. Acidic phosphate buffered saline (aPBS; 0.05 mol L⁻¹ phosphate/0.85% (w/v), adjusted to pH 5 with HCl) was used as diluent in the preparation of all treatment solutions. The pectinase stock solution used in these experiments is commercially available as Pectinex Ultra SP-L (≥ 3800 units/mL)(Sigma Aldrich, St. Louis, MO). For each trial, one biofilm-coated chip was placed in the bottom of a 2 mL-capacity microfuge tube and covered with 200 μ L of treatment solution then incubated at room temperature for 30 minutes in the dark. After gently vortexing, the eluant was removed and serially diluted in sterile PBS followed by spread plating 100 μ L of each dilution on BHI agar. After incubation at 30°C for 48 h the number of colonies on each plate was recorded. Each treatment was repeated three times and the enumeration data subjected to statistical analysis using Microsoft Excel 2003.

Recovery of *L. monocytogenes* from stainless steel squares by swabbing. Each biofilm-coated stainless steel square was placed in a sterile petri plate and covered with 1.0 mL of treatment solution, then incubated at room temperature for 30 minutes in the dark.. A sterile SpongeSicle swab (3M Canada, London, Ontario) pre-moistened in 10 mL of peptone water was used to swab the surface of the square by rubbing each side of the sponge head for 10 seconds. The swab was then placed in a stomacher bag and

stomached in 20 mL of sterile PBS (30 seconds at speed 3) using an Interscience BagMixer (Saint Nom, France). The homogenate was serially diluted and plated on BHI agar as described above. Each treatment was repeated three times and the enumeration data subjected to statistical analysis using Microsoft Excel 2003.

3.3 Results

Identification of bacterial isolates from the food manufacturing environment.

Of the 38 bacterial isolates recovered from the four food manufacturing environment samples, 7 Gram positive isolates were identified with less than 85% confidence and another 7 Gram positive isolates were unidentified (Table 8). All 12 of the Gram negative isolates were *Hafnia alvei* and all 19 identified Gram positive isolates were *Staphylococcus* spp. or a mixture of *Staphylococcus* spp. with *Corynebacterium xerosis*.

Table 8. List of 38 bacterial isolates recovered from four food manufacturing environment samples obtained in 2010 in Canada.

Environmental Isolate*	Catalase	Oxidase	Gram stain	CoAg	β -hemolysis	Identification
1a	+	-	+	-	n/a	59% <i>Staphylococcus capitis</i> , 18% <i>S. hominis</i>
1b	+	-	+	-	n/a	<i>S. warneri</i>
1c	+	-	+	-	n/a	89% <i>S. saprophyticus</i> , 6% <i>S. simulans</i>
1d	+	-	+	-	n/a	<i>S. xylosus</i>
1e	+	-	+	-	n/a	89% <i>S. saprophyticus</i> , 6% <i>S. simulans</i>
1f	+	-	+	-	n/a	77% <i>S. epidermidis</i> , 12% <i>S. capitis</i>
1g	+	-	+	-	n/a	<i>S. xylosus</i>
1h	+	-	+	-	n/a	<i>S. xylosus</i>
1i	-	+	+	n/a	-	no identification
1j-1	+	-	+	-	n/a	<i>S. xylosus</i>
1j-2	+	-	+	-	n/a	<i>S. capitis</i>
1k	+	-	+	-	n/a	<i>S. xylosus</i>
1l	-	+	+	n/a	-	no identification
2a	+	-	+	-	n/a	60% <i>S. auricularis</i> , 31% <i>Corynebacterium xerosis</i>
2b	+	-	+	-	n/a	no identification
2c	+	-	+	-	n/a	88% <i>S. cohnii</i> , 8% <i>S. haemolyticus</i>
2d	+	-	+	-	n/a	89% <i>S. saprophyticus</i> , 6% <i>S. simulans</i>
2e	+	-	+	-	n/a	82% <i>S. xylosus</i> , 14% <i>S. capitis</i>
2f	+	-	+	-	n/a	60% <i>S. auricularis</i> , 31% <i>C. xerosis</i>
2g-1	+	-	+	-	n/a	no identification
2g-2	+	-	+	-	n/a	60% <i>S. auricularis</i> , 31% <i>C. xerosis</i>
2h	+	-	+	-	n/a	60% <i>S. auricularis</i> , 31% <i>C. xerosis</i>
2i	+	-	+	-	n/a	89% <i>S. saprophyticus</i> , 6% <i>S. simulans</i>
2j	+	-	+	-	n/a	no identification
2k	+	-	+	-	n/a	no identification
2l	+	-	+	-	n/a	no identification

Table 8. Continued

Environmental	Gram					Identification
Isolate*	Catalase	Oxidase	stain	CoAg	β -hemolysis	
4a	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4b	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4c	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4d	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4e	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4f	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4g	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4h	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4i	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4j	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4k	+	-	-	n/a	n/a	<i>Hafnia alvei</i>
4l	+	-	-	n/a	n/a	<i>Hafnia alvei</i>

n/a is not applicable

*Isolates were obtained from 4 different sources: Plate one contained environmental samples obtained from a cutting board, plate 2 was from a knife, plate 3 a conveyor belt and plate 4 a slicer blade. Plate 3 did not produce any bacterial growth. The bacteria were identified using various microbiological assays and Vitek analysis.

Visualization of biofilms. Imaging of the coated chips by scanning electron microscopy revealed the presence of surface-adhered cells exhibiting biofilm characteristics, such as the presence of extracellular fibrils (Figure 5) commonly associated with EPS formation.

Figure 5. Scanning electron photograph of *Listeria monocytogenes* (Kane Biotech strain) and *Staphylococcus epidermidis* subject to dual-species biofilm forming protocol with 2 days incubation and 1:10 dilution of Brain Heart Infusion broth in Milli-Q water. 7000 X magnification.

Effect of potential biofilm-dispersal agents on cell viability. From aerobic plate counts, it was determined that Dispersin B, Pectinex, 0.01% v/v Tween 20, 50 µg/mL BioRad blocker, and 50 µg/mL inactive Dispersin B did not affect *L. monocytogenes* cell viability (Figures 6 and 7). DNase 1 and D-amino acids also did not affect cell viability (Figure 8). In contrast, the natural sponge extract (University of Wisconsin) assayed did inhibit growth of *L. monocytogenes* compared to the control (Figure 7).

Figure 6. Effect of potential biofilm-dispersal agents (aPBS, Dispersin B, non specific protein, and mild detergent) on *L. monocytogenes* (Kane Biotech strain) cell viability as determined by aerobic plate counts. Treatments were acidic Phosphate Buffered Saline (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl), 50 µg/mL Dispersin B, non specific protein (50 µg/mL BioRad blocker) and mild detergent (0.01% v/v Tween 20). All treatments were buffered in aPBS and incubated at room temperature for 30 min. CFU/sample values are averages of duplicates of 50 µL. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

Figure 7. Effect of potential biofilm-dispersal agents (aPBS, pectinase and a natural sponge extract) on *Listeria monocytogenes* (Kane Biotech strain) cell viability as determined by aerobic plate counts. Treatments were acidic Phosphate Buffered Saline (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl), pectinase (Pectinex Ultra SP-L, ≥ 3800 units/mL), and a marine sponge extract obtained from the University of Wisconsin (concentration and origin unknown). Treatments were buffered in aPBS and incubated at room temperature for 30 min. CFU/sample values are averages of duplicates of 50 μ L. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

Figure 8. Effect of potential biofilm-dispersal agents (aPBS, D-Tyrosine, D-Tryptophan, D-Alanine, D-Leucine, D-Methionine and DNase 1) on *Listeria monocytogenes* (Kane Biotech strain) cell viability as determined by aerobic plate counts. Treatments were acidic Phosphate Buffered Saline (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl), 1 mM of D-Tyrosine, 1 mM of D-Tryptophan, 1 mM of D-Alanine, 1 mM of D-Leucine, 1 mM of D-Methionine and 2 units of DNase 1. Treatments were buffered in aPBS and incubated at room temperature for 30 min. CFU/sample values are averages of duplicates of 50 μ L. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

Effect of potential biofilm-dispersal agents on the recovery of *L.*

***monocytogenes* cells from biofilms.** DNase I (not shown), 50 µg/mL Dispersin B, inactive Dispersin B and 0.01% v/v Tween 20 were not better than the control buffer at liberating *L. monocytogenes* cells from biofilm (Figure 9). Greater quantities of cells were released by 50 µg/mL BioRad non-specific protein and the four D-amino acids (D-Tyrosine, D-Tryptophan, D-Methionine, and D-Leucine) compared to the control buffer; however, these results were not statistically significant. In contrast, the pectinase agent produced an approximately 2-fold increase in cell recovery compared to the control buffer. Pectinase was the only agent that had significantly greater cell recovery compared to the control on a consistent basis in subsequent trials of this experiment (not shown).

Figure 9. Effect of potential biofilm-dispersing agents on the recovery of *Listeria monocytogenes* (Kane Biotech strain) cells from 2-day old single-species biofilms as determined by aerobic plate counts. All treatments were buffered in acidic PBS (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl) and incubated at room temperature for 30 mins. Treatments were aPBS (control), 50 µg/mL Dispersin B, 50 µg/mL BioRad blocker, Pectinase (10-fold dilution with aPBS, ≥380 units/mL), 50 µg/mL inactive Dispersin B, 0.01% Tween 20, 4 D-amino acids (1 mM each of D-Tyrosine, D-Methionine, D-Tryptophan and D-Leucine), and 1 mM D-Tyrosine. CFU/sample values are averages of triplicates of 200 µL of eluant. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

Effect of different concentrations of pectinase on recovery of *L.*

***monocytogenes* cells from biofilms.** The effect of undiluted, 3-fold and 10-fold diluted pectinase was studied in Figure 10. The 10-fold diluted pectinase concentration tested produced the highest recovery of *L. monocytogenes* cells from the s.s. chips compared to the control buffer with an approximately two-fold increase in the recovery of viable cells. There was no statistical significance among the undiluted, 3-fold and 10-fold diluted pectinase treatments. The 0.01% v/v Tween 20 treatment was not better than the control.

Figure 10. Effect of various concentrations of Pectinex Ultra SP-L (pectinase) on the recovery of *Listeria monocytogenes* (Kane Biotech strain) cells from 2-day old single-species biofilms as determined by aerobic plate counts. All treatments were buffered in acidic PBS (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl) and incubated at room temperature for 30 mins. Treatments were aPBS (control), undiluted pectinase (≥ 3800 units/mL) and 3-fold (≥ 1267 units/mL) and 10-fold diluted pectinase (≥ 380 units/mL). CFU/sample values* were averages of triplicates of 200 μ L of eluant. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

* CFU/sample values are greater for all treatments in Figure 10 because a wash method with 10 exchanges of 0.5 mL of sterile PBS was used in biofilm formation. This wash method was a part of preliminary biofilm-formation experiments and was later replaced by flow-thru washing, which removed a greater number of loosely adhered bacteria and reduced the amount of cells recovered by the control buffer by one half (not shown).

Effect of pectinase combined with Tween 20 on recovery of *L. monocytogenes* cells from biofilms. Used alone, the 0.01% v/v Tween 20 detergent did not significantly improve the recovery of *L. monocytogenes* cells compared to the aPBS control buffer (Figure 11). However, when the pectinase stock was diluted 10-fold in aPBS and combined 1:1 with 0.02% Tween 20, a synergistic effect was observed whereby the recovery of *L. monocytogenes* increased 2-fold over treatment with pectinase alone and almost 4-fold over treatment with buffer (Figure 11). Using the swab method of recovery, the combination of pectinase and Tween 20 also produced an approximately 4-fold greater quantity of cells recovered from biofilm compared to the control buffer (Figure 12).

Figure 11. Effect of Pectinex Ultra SP-L (pectinase), Tween 20 and a combination of both treatments the on the recovery of *Listeria monocytogenes* (Kane Biotech strain) cells from 2-day old single-species biofilms as determined by aerobic plate counts. All treatments were buffered in acidic PBS (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl) and incubated at room temperature for 30 mins. Treatments were aPBS (control), 0.01% v/v Tween 20, 10-fold diluted pectinase (≥ 380 units/mL) and 1:1 ratio of Tween 20 and pectinase. CFU/sample values were averages of triplicates of 200 μ L of eluant. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

Figure 12. Effect of Pectinex Ultra SP-L (pectinase) combined with Tween 20 on the recovery of *L. monocytogenes* (Kane Biotech strain) cells from 2-day old single-species biofilms formed on 4 cm square stainless steel chips, as determined by aerobic cell counts. Cells were recovered by swabbing with a commercial sponge. All treatments were buffered in acidic PBS (aPBS = 50 mM sodium phosphate buffer pH 5, 100 mM NaCl) and incubated at room temperature for 30 mins. Treatments were aPBS (control), and a 1:1 mix of 10-fold diluted pectinase (≥ 380 units/mL) and 0.01% v/v Tween 20. CFU/sample values were averages of triplicates of 1 mL of eluant. Lines on top of bars represent standard deviations. Treatments with the same letter above bars are not significantly different at $P \leq 0.05$ (LSD).

3.4 Discussion

Development of a model *L. monocytogenes* biofilm. The LmKB strain used in this research was biochemically tested as a pure culture strain of *L. monocytogenes* and readily formed biofilms. Many researchers have reported that *L. monocytogenes*' biofilm-forming ability is strain-specific (Borucki et al., 2003; Djordjevic et al., 2002; Harvey et al., 2007). The ability to form biofilms may be related to presence and expression of specific genes. Lemon et al., 2010 reported that strains losing PrfA may not be able to form biofilms, as this transcriptional activator turns on several key biofilm-forming and virulence genes. The expression of these genes under stress-induced conditions, such as a nutrient limiting environment, prepares the free flowing bacteria for either eukaryotic cell invasion or surface-associated biofilm formation (Lemon et al., 2010).

The predominant surface of the food manufacturing environment is stainless steel, thus it was important to use this surface when creating model biofilms. It has been reported that biofilms may form more readily on stainless steel surfaces that have been conditioned with various media (Hood and Zottola, 1997). It is believed that conditioning may make the hydrophilic surface of stainless steel more hydrophobic. Several researchers have reported drastically higher adhesion of *L. monocytogenes* to hydrophobic surfaces such as polystyrene and polyvinyl chloride (PVC) compared to hydrophilic stainless steel surfaces (Djordjevic et al., 2002; Harvey et al., 2006). Food contact surfaces in the food manufacturing environment are more likely to be thoroughly cleaned due to strict safety and cleaning protocols and regulations. Thus non-preconditioned s.s. chips were used for creation of biofilms throughout this research,

which minimized the preparation time for the s.s. chips and better models the surfaces of the food manufacturing environment.

The model system for creating *L. monocytogenes* biofilms also incorporated a flow-thru wash method to utilize a greater volume of wash buffer to remove loosely adhered cells. The quantity of *L. monocytogenes* cells released by the control were expected because periodically, large particles of mature biofilm will slough off due to flow rate dynamics and the shear forces from the fluid as well as changing properties of the bacteria (Wimpenny, 1993; Deibel, 2003; Annous et al., 2009). The lack of a thorough wash method and the use of undiluted incubation media made adoption of another commonly used biofilm-formation method, the MBEC™ assay (Ceri et al., 1999), not suitable for development of a model biofilm for the purposes of this research.

Identification of bacterial isolates from the food manufacturing environment.

H. alvei and *S. epidermidis* have been reported in literature to be present in the food manufacturing environment (Zameer et al., 2010; Vivas et al., 2007). In this research, *H. alvei* and *Staphylococcus* spp. were the predominant bacteria isolated from the food manufacturing environment (Table 8). Thus, these bacterial species likely engage in synergistic relationships with *L. monocytogenes* in unfavourable environmental conditions to form multi-species biofilms. For research purposes, these bacterial species may be used to form dual-species biofilms with *L. monocytogenes*. This would greatly resemble the type of biofilms incorporating *L. monocytogenes* that occur in the food manufacturing environment. In the present research, only *S. epidermidis* was used for production of a dual-species *L. monocytogenes* biofilm and extracellular fibrils were

observed under SEM (Figure 5). These fibrils are indicative of enhanced attachment and the beginnings of an extracellular matrix (Kalmokoff et al., 2001).

Increased recovery of *L. monocytogenes* cells from biofilms. Increased cell recovery was observed for the BioRad blocker and the 4 D-amino acids treatments compared to the control buffer; however, these increases were not statistically significant and they were not consistent throughout repeated trials. Kolodkin-Gal et al. (2010) suggested that D-amino acids are incorporated into the peptide side chains of peptidoglycan in place of the terminal D-Alanine, triggering biofilm disassembly. In the Kolodkin-Gal study, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used to create pellicles on the surface of liquid media; however, in the present study, *L. monocytogenes* was used to form biofilms on stainless steel surfaces. Vu et al., 2009 reported that in Gram positive bacteria, communication is carried out with modified oligopeptides generating signals mediated by many phosphorylation steps. These signals may be blocked or prevented from reaching their receptors by the BioRad blocker protein. Quorum sensing molecules known as acyl-homoserine lactones (acyl-HSLs) have also been reported to separate the bacterial pillars in the three-dimensional structure of some single-species biofilms (Davies et al., 1998; Annous et al., 2009). The role of bacterial signalling in multi-species biofilms has not been studied extensively, but it is believed to be critical for expression of genes for biofilm diversity, distribution and maturation (Watnick and Kolter, 2000; Vu et al., 2009; Annous et al., 2009). Thus, interference with bacterial signalling in biofilms may cause an increased amount of cells to be released from the sessile state.

While previous work focused primarily on the use of polysaccharidases as a means of eliminating biofilms from surfaces in food processing environments (Lequette *et al.*, 2010), the results of the present study suggest that pectinase used in combination with a mild detergent is highly effective for the recovery of *L. monocytogenes* in viable form for subsequent microbiological analysis using standard culture techniques. Pectinase is a food grade mixture of enzymes with predominantly polygalacturonase activity which may act by breaking down the polysaccharide components of the EPS matrix. Tween 20 is a mild detergent which may contribute to destabilization of the EPS matrix by disrupting hydrophobic interactions between constituent lipid components, and also, perhaps, between cells and the adsorbent surface. The use of these agents in a food manufacturing setting in environmental sampling procedures is further supported by their low cost, ease of application and their innocuous nature (i.e., lack of toxicity). One suggested approach is to apply these agents on surfaces using a simple spray bottle, waiting 30 min, and then swabbing the treated area with a sampling sponge in the usual manner. Further work will be required to investigate whether other biofilm-dispersing agents can provide enhanced recovery of surface-adhered bacteria for food microbiology testing purposes. In this work stainless steel coupons were used as a model, though other types of surfaces made from different materials are also used in food manufacturing environments, and more work will be required to determine the efficacy of the present treatment regimen with a broader variety of surfaces.

3.5 Conclusions

A model system was developed for both single- and dual-species *L. monocytogenes* biofilms. This system may simulate the biofilm-forming conditions of the food manufacturing environment in the laboratory and may be used for future biofilm research. In these biofilms, cell aggregates and extracellular fibrils were observed, which indicate enhanced attachment and the beginnings of an EPS matrix, characteristic of biofilms.

From sponge samples taken from the food manufacturing environment, *H. alvei* and various *Staphylococcus* spp. including *S. epidermidis* were identified. These bacteria likely engage in synergistic relationships with *L. monocytogenes* to form biofilms.

All potential biofilm-dispersal agents were tested for any bactericidal effect to *L. monocytogenes*. No bactericidal effect was observed for the agents except for the natural sponge extract, which was not tested further. The pectinase agent was the best of all the agents tested at recovering *L. monocytogenes* cells from biofilms and this effect was enhanced when combined with Tween 20. Future research is needed to test the effect of these agents in the food manufacturing environment. Other factors such as the cost of using these agents, their availability and their safety may also be considered before implementation in a sampling scheme to recover *L. monocytogenes* from biofilms on food manufacturing environment surfaces.

CHAPTER 4: CONCLUSIONS AND FUTURE PROSPECTS

This study addressed a need for a more definitive *L. monocytogenes* identification method to improve upon the current detection method, MFLP-78. The two main drawbacks of MFLP-78 were that known strains of *L. monocytogenes* were escaping detection and that there was no internal control feature for the PCR procedure. The LmCHAS method improved upon MFLP-78 by having a more robust primer set targeting *hlyA*, where strains that escaped detection by MFLP-78 were properly identified by LmCHAS. In addition, the gene marker *inlJ*, was serendipitously discovered to be species-specific and was incorporated into a multiplex-CHAS procedure to help confer definitive identification of presumptive positive isolates. The *IGS* marker was added as a control for DNA extraction and conferred genus-specific detection along with an internal control for PCR amplification, *IAC*. The LmCHAS method was tested against a comprehensive specificity panel and had 100% inclusivity and 100% exclusivity. The use of two species-specific targets make LmCHAS more sensitive than MFLP-78, and the use of CHAS for endpoint detection of PCR products enabled the unambiguous identification of similar sized amplicons used in the multiplex-PCR, a feature that was not possible using agarose gel electrophoresis. The LmCHAS method performed as hypothesised and by using LmCHAS for definitive identification of *L. monocytogenes*, biochemical tests may be avoided, saving time and money. The LmCHAS method has been successfully validated and may be incorporated into the Health Canada Compendium of Analytical Methods in the near future to replace MFLP-78 for identification of *L. monocytogenes* isolated from food and environmental samples by standard culture techniques.

In contrast to the LmCHAS method, the LmVCHAS method that was developed by this research did not definitively identify high risk outbreak-related strains and will need to be revised in the future. The *lmo* and *gtcA* gene targets were not specific exclusively to serotype 1/2a and serotype 4b strains, respectively, as had been hypothesized. There is room for future improvements as genetic markers specific to these high risk outbreak-related strains are discovered as food microbiology becomes more genomics oriented. Researchers may undertake comparative genomic studies to compare sequences of high risk outbreak-related strains to avirulent strains in order to identify conserved markers that may be used to detect targeted virulent strains. More recent bioinformatics tools are able to perform full risk characterization of key isolates and identify useful diagnostic markers. As genomics research becomes less expensive, risk characterization may also target key antigens and proteins in addition to genes. Public health data, strain typing and epidemiological studies will also play a role in finding high risk outbreak-related isolates so researchers can procure them for characterization and analysis with genomic tools.

The high-profile Maple Leaf Foods listeriosis outbreak of 2008 put a spotlight on the challenges facing the Canadian food industry and its regulators to mitigate health risks associated with environmental contamination by *L. monocytogenes*. Ongoing environmental contamination of food manufacturing plants is a major concern and the presence of *L. monocytogenes* is a key problem for the food industry. *L. monocytogenes* cells can survive in biofilms in the food manufacturing environment and cause persistent low-level contamination, which is what transpired in the Maple Leaf Foods outbreak.

This research addressed the need for an improved technique to increase recovery of *L. monocytogenes* cells established in a biofilm in the food manufacturing environment. The current sampling protocol for surfaces, MFLP-41, relies on swabs moistened in neutralizing buffer and may not recover sessile cells due to the barrier formed by the EPS matrix. In this study, model single- and dual-species *L. monocytogenes* biofilms were successfully created on stainless steel surfaces and were used to evaluate the efficacy of various biofilm-dispersal agents to improve recovery of *L. monocytogenes* cells in biofilms. This research also confirmed the prevalence of *H. alvei* and *S. epidermidis* in the food manufacturing environment and the ability of *S. epidermidis* to engage in synergistic relationships with *L. monocytogenes* to form a dual-species biofilm. The results of this study lay the foundation for future research on *L. monocytogenes* cells in a sessile state. Relatively little is known about microbial interactions in biofilms and future research may target this area to gain a better understanding of how multi-species biofilms form. The ability of different bacteria to form biofilms may be linked to specific genes acquired in the environment (Watnick and Kolter, 2000; Jordon et al., 2008; Annous et al., 2009). Bacteria may lose these genes when continually cultured in the laboratory on rich undefined media. Future investigations may look at strains taken directly from the food manufacturing environment to confirm if they have increased biofilm-forming ability.

In this research, potential biofilm-dispersal agents were compared to a control buffer for their ability to increase recovery of *L. monocytogenes* cells from biofilms. All potential biofilm-dispersal agents that were evaluated were not bactericidal. In limited trials, Pectinex Ultra SPL (pectinase) was the most consistent agent at recovering a

significantly greater quantity of *L. monocytogenes* cells from biofilms than the control and this effect was enhanced when combined with Tween 20. Pectinase may break apart the carbohydrate bonds of EPS to release cells resulting in improved recovery, which is what was hypothesized. Further testing of selected agents is needed using multi-species biofilms and ultimately in a food manufacturing environment to determine their efficacy in the “real world”. As a next step, the concentrations of pectinase and Tween 20 may be optimized. The incubation time for application of these agents may be established at less than 30 mins, if this length of time is not practical for the food industry or inspectors. The future application of these agents to improve MFLP-41 would have to consider several additional factors including cost effectiveness, proprietary issues, quality assurance factors, safety and availability of the agents. Furthermore, the ability of potential biofilm-dispersal agents to be incorporated into the commercial kit, currently used by CFIA inspectors for surface sampling, may be explored. The application of a new agent to a sampling surface using a spray bottle and incubation for a period of time followed by swabbing using the commercial kit would not drastically change the current protocol and would keep the sampling procedure simple and efficient.

Though much remains to be understood about the detection of *L. monocytogenes* in the food manufacturing environment, this research yielded many promising and important results that may provide an immediate impact to food safety in Canada and worldwide. The new findings from this study may drive future research aimed at better understanding *L. monocytogenes* interactions with its environment while in a sessile state. With an improved understanding of *L. monocytogenes* genome variation, microbial ecology and synergistic interactions in biofilms, great potential exists to develop more

robust, highly sensitive and specific methods to recover and detect high risk outbreak-related strains of the pathogen.

APPENDICES

Appendix 1. Sample calculation of CFU/sample values

Formula: $(X \text{ CFU})(\text{dilution factor})/(1 \text{ mL}/\text{volume of sample in mL})$

Eg. For a 200 μL sample, if 30 CFU were found and there was a dilution factor of 10000:

$$(30)(10000)/5 = 60\,000 \text{ CFU/sample}$$

Standard deviation calculated using Microsoft Excel 2003

Significant difference calculated using one-way ANOVA in Microsoft Excel 2007

REFERENCES

- Annous, B.A., Fratamico, P.M and Smith, J.L. 2009. Quorum sensing in biofilms: why bacteria behave the way they do. *J. Food Sci.* 74: 24-37.
- Bell, C., and Kyriakides, A. 2005. *Listeria*: a practical approach to the organism and its control in foods. 2nd ed. Blackwell publishing, Oxford, UK.
- Blais, B.W., Phillippe, L.M ., Pagotto, F. and Corneau, N. 2002. Health Canada compendium of analytical methods, MFLP 78: Identification of presumptive positive *Listeria monocytogenes* from food and environmental samples by the polymerase chain reaction (PCR). Available at: http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/res-rech/mflp78-eng.pdf
- Blais, B., Martinez-Perez, A., Leggate, J., and Gauthier, M. 2007. Detection of multidrug-resistant *Salmonella typhimurium* DT104 in poultry by selective enrichment and cloth-based hybridization array system. *J. Rapid Methods Autom. Microbiol.* 15: 332-344.
- Boruki, M.K., Peppin, J.D., White, D., Loge, F., and Call, D.R. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 69: 7336-7342.
- CFIA and PHAC (Canadian Food Inspection Agency and Public Health Agency of Canada). 2008. News Release, August 23, 2008. Link between listeriosis outbreak strain and Maple Leaf Foods products confirmed. Available from http://www.phac-aspc.gc.ca/media/nr-rp/2008/2008_13-eng.php

- Carpentier, B., and Chassaing, D. 2004. Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. *Int. J. Food Microbiol.* 97: 111-122.
- CBC (Canadian Broadcasting Corporation). 2009. Maple Leaf apologizes again for listeriosis deaths. CBC News, August 24, 2009. Available at: <http://www.cbc.ca/consumer/story/2009/08/24/maple-leaf-anniversary-listeriosis.html>
- Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D.W., and Buret, A.G. 1999. The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities in bacterial biofilms. *J. Clin. Microbiol.* 37: 1771-1776.
- Chae, M.S., and Schraft, H. 2001. Cell viability of *Listeria monocytogenes* biofilms. *Food Microbiol.* 18: 103-112.
- Chasseignauz, E., Toquin, M.-T., Ragimbeau, C., Salvat, G., Colin, P., and Ermel, G. 2001. Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry- and pork-processing plants. *J. Appl. Microbiol.* 91: 888-899.
- Cheng, Y., Siletzky, R.M. and Kathariou, S. 2008. Genomic divisions/lineages, epidemic clones, and population structure. Pages 337-354 *In* D. Liu (ed), *Handbook of Listeria monocytogenes*. CRC Press, New York, N.Y.

- Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*. 280: 295-298.
- Deibel, V. 2003. Biofilms. *Int. J. Food Safety*. 1: 6-7.
- Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., and Ianieri, A. 2007. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. *J. Appl. Microbiol.* 104: 1552-1561.
- Djordjevic, D., Weidmann, M., and McLandsborough, L.A. 2002. Microtitre plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* 68: 2950-2958.
- Donlan, R.M. 2002. Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* 8: 881-890.
- Frank, J.F., and Koffi, R.A. 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to sanitizers and heat. *J. Food Prot.* 53: 550-554.
- Garcia-Almendarez, B.E, Cann, I.K.O., Martin, S.E., Guerra-Legarreta, I., and Regalado, C. 2007. Effect of *Lactococcus lactis* UQ2 and its bacteriocin on *Listeria monocytogenes* biofilms. *Food Cont.* 19: 670-680.

- Gauthier, M., and Blais, B.W. 2004. Cloth-based hybridization array system for the detection of multiple antibiotic resistance genes in *Salmonella enterica* subsp. *enterica* serotype Typhimurium DT104. Lett. Appl. Microbiol. 38: 265-270.
- Gilot, P., Genicot, A., and Andre, P. 1996. Serotyping and esterase typing for analysis of *Listeria monocytogenes* populations recovered from foodstuffs and from human patients with listeriosis in Belgium. J. Clin. Microbiol. 34: 1007-1010.
- Gorski, L. 2008. Phenotypic identification. Pages 156, 159 In D. Liu (ed.), Handbook of *Listeria monocytogenes*. CRC Press, New York, NY.
- Graham, T.A., Golsteyn-Thomas, E.J., Thomas, J.E., and Gannon, V.P.J. 1997. Inter- and intraspecies comparison of the 16S-23S rRNA operon intergenic spacer regions of six *Listeria* spp. Int. J. Syst. Bacteriol. 47: 863-869.
- Graham, T., Golsteyn-Thomas, E.J., Gannon, V.P.J., and Thomas, J.E. 1996. Genus- and species-specific detection of *Listeria monocytogenes* using polymerase chain reaction assays targeting the 16S/23S intergenic spacer region of the rRNA operon. Can. J. Microbiol. 42: 1155-1162.
- Graves, L.M, Swaminathan, B., and Hunter, S.B. 2007. Subtyping *Listeria monocytogenes*, pp 284 . In E.T. Ryser and E.H. Marth (eds.), *Listeria, Listeriosis and Food Safety* 3rd ed. CRC Press. New York, NY.
- Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prevost, H., and Dousset, X. 2005. Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR. Appl. Environ. Microbiol. 71: 2190-2194.

- Hall-Stoodley, L., Nistico, L., Sambanthamoorthy, K., Dice, B., Nguyen, D., Mershon, W., Johnson, C., Hu, F.Z., Stoodley, P., Ehrlich, G.D., and Post, J.C. 2008. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in *Streptococcus pneumonia* clinical isolates. BMC Microbiol. 8: 173-189.
- Harvey, J., Keenan, K.P., and Gilmour, A. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiol. 24: 380-392.
- Health Canada. 2010. Policy on *Listeria monocytogenes* in ready-to-eat foods. Modified final version (April 1, 2011). Available at: http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php
- HCMC (Health Canada Microbiology Methods Committee). 2010. Health Canada compendium of analytical methods, MFLP 41: Environmental sampling for the detection of microorganisms. Available at: http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/res-rech/mflp41-eng.pdf
- Hood, S.K., and Zottola, E.A. 1997. Growth media and surface conditioning influence the adherence of *Pseudomonas fragi*, *Salmonella typhimurium*, and *Listeria monocytogenes* cells to stainless steel. J. Food Prot. 60: 1034-1037.
- Huigens, R.W., Ma, L., Gambino, C., Moeller, P.D.R., Basso, A., Cavanagh, J., Wozniak, D.J., and Melander, C. 2008. Control of bacterial biofilms with marine alkaloid derivatives. Mol. BioSyst. 4: 614-621.

- Ingham, S. C., Borneman, D. L., Ané, C., and Ingham, B. H. 2010. Predicting growth-no growth of *Listeria monocytogenes* on vacuum-packaged ready-to-eat meats. J. Food Prot. 73: 708-714.
- Janseens, J.C.A., Steenackers, H., Robijns, S., Gellens, E., Levin, J., Zhao, H., Hermans, K., De Coster, D., Verhoeven, T.L., Marchal, K., Vanderleyden, J., De Vos, D.E., and De Keersmaecker, C.J. 2008. Bromated furanones inhibit biofilm formation by *Salmonella* enteric serovar *Typhimurium*. Appl. Environ. Microbiol. 74: 6639-6648.
- Janzten, M.M., Navas, J., Corujo, A., Moreno, R., López, V., and Martínez-Suárez, J.V. 2006. Review: specific detection of *Listeria monocytogenes* in foods using commercial methods: from chromogenic media to real-time PCR. Spanish J. Agric. Res. 4: 235-247.
- Jay, J.M., Loessner, M.J., and Golden, D.A. 2005. Modern Food Microbiology, 7th ed. Springer, New York, NY.
- Jeffers, G. T., Bruce J.L., McDonough, P.L., Scarlett, J., Boor, K.J., and Wiedmann, M. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiology 147:1095-1104.
- Johnson, J., Jinneman, K., Stelma, G., Smith, B.G., Lye, D., Messer, J., Ulaszek, J., Evsen, L., Gendel, S., Bennett, R.W., Swaminathan, B., Pruckler, J., Steigerwalt, A., Kathariou, S., Yildirim, S., Volokhov, D., Rasooly, A., Chizhikov, V., Wiedmann, M., Fortes, E., Duvall, R.E., and Hitchins, A.D. 2004. Natural atypical

Listeria innocua strains with *Listeria monocytogenes* pathogenicity island 1 genes. Appl. Environ. Microbiol. 70: 4256-4266.

Jordon, S.J., Perni, S., Glenn, S., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R.P., Chambel, L., Barata, B., Zilhao, I., Aldsworth, T.G., Adria, A., Faleiro, M.L., Shama, G., and Andrew, P.W. 2008. *Listeria monocytogenes* biofilm-associated protein (BapL) may contribute to surface attachment of *L. monocytogenes* but is absent from many field isolates. Appl. Environ. Microbiol. 74: 5451-5456.

Kalmokoff, M.L., Austin, J.W., Wan, X.D., Sanders, G., Banerjee, S., and Farber, J.M. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. J. Appl. Microbiol. 91: 725-734.

Kang, D., Eifert, J., Williams, R., and Pao, S. 2007. Evaluation of quantitative recovery methods for *Listeria monocytogenes* applied to stainless steel. J. AOAC Int. 90: 810- 816.

Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H., and Ramasubbu, N. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. Antimicrob. Agents Chemother. 48: 2633-2636.

Kathariou, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65: 1811–1829.

Kerrigan, J.E., Ragunath, C., Kandra, L., Gyémánt, G., Lipták, A., Jánossy, L., Kaplan, J.B., and Ramasubbu, N. 2008. Modeling and biochemical analysis of the activity of antibiofilm agent Dispersin B. Acta. Biol. Hung. 59: 439-451.

- Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. 2010. D-amino acids trigger biofilm disassembly. *Science* 328: 627-629.
- Leggate, J., and Blais, B.W. 2006. An internal amplification control system based on primer-dimer formation for PCR product detection by DNA hybridization. *J. Food Prot.* 69: 2280-2284.
- Lemon, K.P., Freitag, N.E., and Kolter, R. 2010. The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. *J. Bacteriol.* 192: 3969-3976.
- Lequette, Y., Boels, G., Clarisse, M., and Faille., C. 2010. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. *Biofouling*. 26: 421-431.
- Leriche, V., Sibille, P., and Carpentier, B. 2000. Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. *Appl. Environ. Microbiol.* 66: 1851-1856.
- Leriche, V., and Carpentier, B. 2000. Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. *J. Appl. Microbiol.* 88: 594-605.
- Lianou, A., and Sofos, J.N. 2007. A Review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *J. Food Prot.* 70: 2172-2198.
- Libras, C.M., and Rose, B.E. 1989. Antibacterial properties of retail sponges. *J. Food Prot.* 52: 49-50.

- Liu, D., Ainsworth, A.J., Austin, F., and Lawrence, M. 2003. Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. J. Med. Microbiol. 52: 1065-1070.
- Liu, D., Lawrence, M., Austin, F., and Ainsworth, A.J. 2007. A multiplex PCR for species-and virulence-specific determination of *Listeria monocytogenes*. J. Micro. Meth. 71: 133-140.
- Lourenço, A., Machado, H., and Brito, L. 2011. Biofilms of *Listeria monocytogenes* produced at 12°C either in pure culture or in co-culture with *Pseudomonas aeruginosa* showed reduced susceptibility to sanitizers. J Food Sci. 76: M143-M148.
- McClure, P.J., Kelly, T.M., and Roberts, T.A. 1991. The effects of temperature, pH, sodium chloride and sodium nitrate on the growth of *Listeria monocytogenes*. Int. J. Food Microbiol. 14: 77-91.
- McLauchin, J. 1997. The pathogenicity of *Listeria monocytogenes*: a public health perspective. Rev. Med. Microbiol. 8: 1-14.
- McLauchlin, J., Hall, S.M., Velani, S.K., and Gilbert, R.J. 1991. Human listeriosis and pâté: a possible association. Br. Med. J. 303: 773-775.
- Mead, P., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P., and Tauxe, R.V. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5: 607-625.

- Mengaud, J., Vicente, M.F., Chenevert, J., Pereira, J.M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.C., and Cossart, P. 1988. Expression in *Escherichia coli* and sequence analysis of the Listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. 56: 766-772.
- Nishibori, T., Cooray, K., Xiong, H., Kawamura, I., Fujita, M., and Mitsuyama, M. 1995. Correlation between the presence of virulence-associated genes as determined by PCR and actual virulence to mice in various strains of *Listeria* spp. Microbiol Immunol. 39: 343-349.
- Pagotto, F., Daley, E., Farber, J., and Warburton, D. 2001. Health Canada compendium of analytical methods, MFHPB 30: Isolation of *Listeria monocytogenes* from all food and environmental samples. Available at: http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/res-rech/mfhpb30-eng.pdf
- Pan, Y., Breidt Jr., F., and Kathariou, S. 2006. Resistance of *Listeria monocytogenes* Biofilms to Sanitizing Agents in a Simulated Food Processing Environment. Appl. Environ. Microbiol. 72: 7711-7717.
- Peccio, A., Autio, T., Korkeala, H., Rosmini, R., and Trevisani, M. 2003. *Listeria monocytogenes* occurrence and characterization in meat-producing plants. Lett. Appl. Microbiol. 37:234-238.
- Pradhan, A.K., Ivanek, R., Grohn, Y.T., Geornaras, I., Sofos, J.N., and Wiedmann, M. 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: 978–989.
- Promadej, N., Fielder, F., Cossart, P., Dramsi, S., and Kathariou, S. 1999. Cell wall teichoic acid glycosylation in *Listeria monocytogenes* serotype 4b requires gtcA, a novel, serogroup-specific gene. *J. Bacteriol.* 181: 418-425.
- Ramasubbu, N., Thomas, L.M., Ragunath, C., and Kaplan, J.B. 2005. Structural analysis of Dispersin B, a biofilm-releasing glycoside hydrolase from the periodontopathogen *Actinobacillus actinomycetemcomitans*. *J. Mol. Biol.* 349: 475-486.
- Ray, B., and Bhunia, A. 2008. *Fundamental Food Microbiology*, 4th ed. CRC Press, New York, NY.
- Roche, S.M., Velge, P., and Liu, D. 2008. Virulence determination. Pages 241, 255-260 *In* D. Liu (ed.), *Handbook of Listeria monocytogenes*. CRC Press, New York, NY.
- Sabet, C., Lecuit, M., Cabanes, D., Cossart, P., and Bierne, H. 2005. LPXTG protein *InlJ*, a newly identified internalin involved in *Listeria monocytogenes* virulence. *Infect. Immun.* 73: 6912-6922.
- Soni, K.A., Nannapaneni, R. 2010. Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. *J. Food Prot.* 73: 1519-1524.
- Swaminathan, B., and Gerner-Smidt, P. 2007. The epidemiology of human listeriosis. *Microbes Infect.* 9:1236–1243.

- Swaminathan, B., Cabanes, D., Zhang, W., and Cossart, P. 2007. *Listeria monocytogenes*. Pages 464-466 In M.P. Doyle and L.R. Beuchat (eds.), Food microbiology: fundamentals and frontiers 3rd Ed. ASM Press, Washington D.C.
- Thévenot, D., Delignette-Muller, M.-T., Christieans, S., Leroy, S., Kodjo, A., and Vernozy-Rozand, C. 2006. Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products. Int. J. Food Microbiol. 112:153-161.
- Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehlan, J., and Kreft, J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14: 584-640.
- Vivas, J., Padilla, D., Real, F., Bravo, J., Grasso, V., and Acosta, F. 2007. Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains. Vet. Microbiol. 129: 150-155.
- Vu, B., Chen, M., Crawford, R.J., and Ivanova, E.P. 2009. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules. 14: 2535-2554.
- Watnick, P., and Kolter, R. 2000. Biofilm, city of microbes. J. Bacteriol. 182: 2675-2679.
- Weatherill, S. 2009. What led to the outbreak? Listeriosis Investigative Review. Government of Canada. July 23, 2009. Available at: http://www.listeriosis-listeriose.investigation-enquete.gc.ca/index_e.php?s1=rpt&page=chap05#na51

- Wimpenny, J.W.T., Kinniment, S.L. and Scourfield, M.A. The physiology and biochemistry of biofilm. Pages 53, 56 *In* Denyer, S.P., Gorman, S.P., and Sussman, M. (Eds.) Microbial biofilms: formation and control. Cambridge University Press, Cambridge, UK.
- Wong, A.C.L. 1998. Biofilms in Food Processing Environments. *J. Dairy Sci.* 81: 2765-2770.
- Xue, J.Z., Pagotto, F., and Blais, B.W. 2010. Cloth-based hybridization system for the identification of food-borne *Listeria* and confirmation of *Listeria monocytogenes*. *Int. J. Food Safety.* 12: 87-94.
- Zameer, F., Kreft, J., and Gopal, S. 2010. Interaction of *Listeria monocytogenes* and *Staphylococcus epidermidis* in dual species biofilms. *J. Food Safety.* 30: 954-968.
- Zhang, W., and Knabel, S.J. 2005. Multiplex PCR assay simplifies serotyping and sequence typing of *Listeria monocytogenes* associated with human outbreaks. *J. Food Prot.* 68: 1907-1910.