Characterization of food chain-derived *Listeria monocytogenes* and the role of *Listeria* genomic island (LGI1) in virulence, survival, and tolerance to food-related stress

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2014

Abstract

The presence of *Listeria* spp. and *L. monocytogenes* (*Lm*) was investigated in provincially inspected food processing and retail facilities in British Columbia. *Lm* (n=56) was recovered in food processing environment (FPE) of dairy, meat and fish facilities, and in ready-to-eat fish products. The majority of *Lm* belonged to listeriosis causing serotypes 1/2a and 4b. Isolate fingerprinting revealed 14 sequence types, and 38 pulsotypes, with 66% of *Lm* possessing the full-length *inlA*, a causally linked virulence determinant. Unexpectedly, 4b serotypes more readily acquired point mutations leading to rifampicin resistance compared to other serotypes ($p<0.05$). *Lm* that adapted more quickly to cold (4°C) also more often encoded a full-length *inlA*. No resistance to antibiotics used in listeriosis treatment was observed; however, a large proportion of isolates possessed resistance or reduced susceptibility (RSC) to ciprofloxacin (CIP, 75%) and clindamycin (CLI, 98%). When eight isolates were experimentally adapted to high concentrations of CIP, minimum inhibitory concentrations (MICs) of benzalkonium chloride (BAC) increased (n=5), gentamicin MICs remained the same (n=6) or increased 2-fold (n=2), and led to RSC to linezolid (n=1) and resistance to CLI (n=8). Tolerance to quaternary ammonium (QAC) sanitizers, BAC and E-San, was also investigated in a clinical *Lm* strain (08-5578) that possessed a previously uncharacterized island, LGI1. High tolerance to acid, cold, high salt conditions, and QACs was seen. Deletion of LGI1 genes *lmo1851*, *emrE*, and *sel1*, with putative regulatory, efflux, and adhesion functions, respectively, did not affect the acid, cold and salt tolerance, or the adhesion and invasion of TC-7 and HeLa cells. The Δ*emrE* mutant had impaired growth at sub-lethal concentrations of QACs, and up to three times lower MICs. No change in MICs to aminoglycosides and other antibiotics, acriflavine, and triclosan, was observed for the Δ*emrE* mutant,

suggesting the primary role of EmrE in *Lm* is to increase its tolerance of QACs. Overall, findings from this research provide evidence that differences in stress survival and virulence potential exist among food chain-derived *Lm*. However, better understanding of the pressures occurring in FPEs that may contribute to strain persistence, and co-selection of antibiotic and sanitizer resistance mechanisms is needed.

Preface

The research performed in this thesis was approved by the University of British Columbia Biosafety Committee, Certificate Number B10-0010.

A version of Chapter 2 has been published in a government report [Kovacevic, J. and Environmental Health Services Division, 2010. Occurrence and distribution of *Listeria* species in facilities producing ready-to-eat foods under provincial inspection authority in British Columbia. B.C. Centre for Disease Control. Available at: http://www.bccdc.ca /NR/rdonlyres/659E872B-A803-4F99-8C6A1902A143CCC7/0/ListeriaReportFINAL withAppendicesJan122011corrected.pdf. Accessed 20 July, 2014.], and a peer-reviewed manuscript [Kovačević, J., Mesak, L.R., and Allen, K.J., 2012. Occurrence and characterization of *Listeria* spp. in ready-to-eat retail foods from Vancouver, British Columbia. Food Microbiol. 30, 372-378]. It is based on the work I conducted in UBC's Food Microbiology Laboratory and British Columbia Centre for Disease Control (BCCDC). I was responsible for conducting the research, analysis of data, as well as writing the manuscripts. Allen K.J. and Kosatsky T. were project supervisory authors, while Mesak L.R., was involved in manuscript review and edits.

A version of Chapter 3 has been published in the Applied and Environmental Microbiology journal [Kovacevic, J., Arguedas-Villa, C., Wozniak, A., Tasara, T., and Allen, K.J., 2013. Examination of food chain-derived *Listeria monocytogenes* of different serotypes reveals considerable diversity in *inlA* genotypes, mutability, and adaptation to cold temperature. Appl. Environ. Microbiol. 79, 1915-1922]. I was responsible for the majority of the work and manuscript preparation. Arguedas-Villa C. and Wozniak A.

helped with generation of cold growth adaptation and mutability data, respectively. Allen K.J. drafted the introduction and helped with critical review and editing of the manuscript. The majority of multilocus sequence typing, and serotyping data was obtained from the Canadian National Microbiology Laboratory, where I was also trained and completed parts of the work under the guidance of Dr. Matthew Gilmour. Pulsed-field gel electrophoresis isolate fingerprinting was performed at the BCCDC and Canadian Listeriosis Reference Service Laboratory.

Chapter 4 was published in Food Microbiology [Kovacevic, J., Sagert, J., Wozniak, A., Gilmour, M.W., and Allen, K.J., 2013. Antimicrobial resistance and co-selection phenomenon in *Listeria* spp. recovered from food and food production environments. Food Microbiol. 34, 319-327]. I was responsible for the majority of the work and manuscript preparation. Wozniak A. helped with co-selection experiments, and Sagert J. performed plasmid screening and prepared the gel shown in Figure 4-2 (page 130). Allen K.J. and Gilmour M.W. were supervisory authors. Allen K.J. drafted the introduction, and helped with critical review and editing of the manuscript.

I performed the research described in Chapter 5. Allen K.J. was involved in the concept formation, and Mesak L.R. and Wałecka-Zacharska E. helped with the generation of mutants.

Table of contents

List of tables

List of figures

List of abbreviations

a.a.	Amino acid
ActA	Actin-assembly inducing protein
ADI	Arginine deiminase
Ami	Amidase, autolysin
AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAC	Benzalkonium chloride
B.C.	British Columbia
BCCDC	British Columbia Centre for Disease Control
BHI	Brain heart infusion broth or agar
bp	Base pair
Bsh	Bile salt hydrolase
BUG5	Tn*1545*-induced *inlA* mutant of a EGD-SmR laboratory reference *L. monocytogenes* strain
Caco-2	Human colorectal adenocarcinoma epithelial cells
CC	Clonal complex
cDNA	Complimentary deoxyribonucleic acid
CFC	Close-to-food contact surface
CFIA	Canadian Food Inspection Agency
CFU	Colony forming units
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLI	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
Ct	Cycle threshold
DE	Dairy environment sample
DF	Dairy food sample
DMEM	Dulbecco's modified Eagle medium

DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EGD	Laboratory reference strain of *L. monocytogenes* recovered by E. G. D. Murray
ERY	Erythromycin
FBS	Fetal bovine serum
FC	Food contact surface
FE	Fish environment
FF	Fish food
FOX	Cefoxitin
FPE	Food processing environment
FQ	Fluoroquinolones
GABA	γ-aminobutyrate
GAD	Glutamate decarboxylase
GEN	Gentamicin
HeLa	Human cervical cancer cells
InlA	Internalin A protein
InlB	Internalin B protein
IPM	Imipenem
KAN	Kanamycin
LGI1	*Listeria* genomic island 1
LB	Luria-Bertani broth
LIPI-1	*Listeria* pathogenicity island 1
LLO	Listeriolysin O
Lm	*Listeria monocytogenes*
LPD	Lag phase duration in hours
LZD	Linezolid
MGR	Maximum growth rate
MIC	Minimum inhibitory concentration
ME	Meat environment
MF	Meat food
MFS	Major facilitator superfamily
MHA (-B)	Mueller-Hinton agar (with 5% sheep blood)

MLST	Multilocus sequence typing
MST	Minimum spanning tree
NAL	Nalidixic acid
NAPS	Nucleic Acid Protein Service Unit at the University of British Columbia
ncRNA	Non-coding ribonucleic acid
NFC	Non-food contact surface
OD	Optical density
OE	Meat (other) environment
OF	Meat (other) food
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PF	Food processing facilities
PFGE	Pulsed-field gel electrophoresis
PLC	Phospholipase C
PMSC	Premature stop codon
Ppm	Parts per million
PrfA	Positive regulatory factor A; virulence regulator
QACs	Quaternary ammonium compounds
qPCR	Quantitative real-time polymerase chain reaction
QRDR	Quinolone resistance-determining regions
RIF	Rifampicin
RF	Retail facilities
RNA	Ribonucleic acid
RSC	Reduced susceptibility
RTE	Ready-to-eat
RUF	Raw, unprocessed food
SD	Standard deviation
SMR	Small multidrug resistance family
SNP	Single nucleotide polymorphism
SOE-PCR	Splicing by overlap extension polymerase chain reaction
SSI-1	Stress survival islet
ST	Sequence type, based on the multilocus sequence typing

STR	Streptomycin
SXT	Cotrimoxazole
TC-7	A subclone of Caco-2 cells
TET	Tetracycline
Tm	Melting temperature
TMP	Trimethoprim
TSA	Tryptic soy agar
TSA-YE	Tryptic soy agar with yeast extract
TSB	Tryptic soy broth
US	The United States of America
VAN	Vancomycin
WT	Wild type

Acknowledgements

I would like to express my sincere gratitude to Dr. Kevin J. Allen, Dr. David Kitts and Dr. B. Brett Finlay for their support and guidance throughout my graduate research program. I would especially like to thank Dr. David Kitts and Dr. Eunice Li-Chan for providing help and encouragement during my last year of graduate studies.

I thank Dr. Matthew Gilmour and Dr. Brent Skura, my supervisory committee members, for providing valuable discussions and direction in my research.

Special thanks are also owed to Lorraine F. McIntyre, Dr. Kristie Keeney, and Dr. Lili R. Mesak for their expertise, and their patience and guidance during my training and development as a scientist. I am extremely grateful to have had such accomplished and well-rounded scientists to teach and inspire me throughout my studies.

I appreciate the friendship of Dr. Lynn M. McMullen, who instilled the love of food microbiology in me, and greatly influenced my professional and personal growth. She has been a true mentor who inspired me to go beyond what I thought was possible.

I would also like to extend my thanks to the faculty, staff, and my fellow students at the Allen and Finlay laboratories for their assistance, help, and support. Special thanks to Ana Cancarevic, who shared the joys of late night working, endless talks on gene expressions, and conference adventures with me. I also appreciate all the help from Drs. Ewa Wałecka-Zacharska and Anna Woźniak. Sincere thanks to Barbara Wakal for providing encouragement and making me laugh on the days when everything seemed endless and impossible.

I am also very grateful for technical expertise provided by the scientists at the British Columbia Centre for Disease Control, and their generous donation of the *Listeria* isolates

collection. I would like to extend my thanks to Drs. Monique Rousset (Centre de Recherche des Cordeliers, Paris, France), Pascale Cossart (Instituit Pasteur), Catherine Jumarie (Université du Québec à Montréal, Canada), and Zhaoming Xu (UBC) for providing Caco-2 cells and bacterial controls I used in my experiments. I appreciate the financial support obtained through the Four Year Doctoral Fellowship from the University of British Columbia, the Dr. Karl C. Ivarson Agricultural Scholarship from the Agricultural Institute of Canada Foundation, and the support for the research provided by the National Sciences and Engineering Research Council of Canada.

Finally, I would like to thank my family and friends for their endless support and encouragement throughout this endeavor. In particular, I would like to express my extreme gratitude and love to Jared, for providing unconditional love and taking care of me when I was too busy to eat, and sleep. Your understanding and encouragement provided great inspiration in all my academic achievements.

Dedication

To my family, who provided unconditional love, support, and inspiration during this journey of discovery and personal growth.

Chapter 1: Introduction and literature review

1.1 Introduction

Listeria monocytogenes is a ubiquitous, psychrotrophic microorganism, naturally present in soil and decaying vegetation. However, in the last two decades *L. monocytogenes* has received particular interest for concerns related to its presence and persistence in food processing facilities, and subsequent contamination of ready-to-eat (RTE) foods (Gianfranceschi et al., 2003; Little et al., 2009; Low and Donachie, 1997; Van Coillie et al., 2004; Vàzquez-Boland et al., 2001b). Challenges in controlling this widespread bacterium are associated with its unique characteristics that promote its survival in food production environments. These include the ability to form biofilms, grow at refrigeration temperature, and tolerate various extrinsic and intrinsic parameters that are used to control foodborne pathogens (Donnelly, 2001; Rørvik et al., 1995; Tasara and Stephan, 2006; Vàzquez-Boland et al., 2001b). Even with the increased attention given to cleaning and sanitation, and implementation of food safety plans (e.g., Hazard Analysis Critical Control Points), *L. monocytogenes* continues to contaminate food products and result in foodborne disease. In fact, in most developed countries, including Canada, documented cases of listeriosis are on the rise, for reasons yet unexplained (Allerberger and Wagner, 2010; Clark et al., 2010; Gillespie et al., 2009; Koch and Stark, 2006).

Once *L. monocytogenes* is ingested, primarily through the consumption of contaminated foods, it elicits a strong immune response in the host. In healthy individuals this typically results in complete eradication of the pathogen or a mild gastrointestinal disturbance (Painter and Slutsker, 2007; Seavey et al., 2008). However, in individuals where

immune systems are underdeveloped, suppressed or impaired, such as neonates and young children, pregnant women, people with organ transplants, and those undergoing chemotherapy, the bacteria are able to infect and disseminate rapidly. As a result, a variety of serious conditions result, including septicemia, meningitis, encephalitis, spontaneous abortions, and stillbirths, with mortality rates ranging from 20 to 40% (Bortolussi, 2008; Dussurget et al., 2004; Painter and Slutsker, 2007). In addition to host immune status having a role in the development of severe listeriosis, it has been suggested that some *L. monocytogenes* strains are more virulent, which can lead to more severe manifestations of the disease (Jacquet et al., 2004; Orsi et al., 2011; Painter and Slutsker, 2007; Wiedmann et al., 1997). While a number of common virulence factors that allow *L. monocytogenes* to invade and take advantage of host cell processes have been described, more subtle genetic variations that result in increased virulence and/or persistence of some strains are presently not well understood.

More than 95% of listeriosis infections are caused by 1/2a, 1/2b, and 4b serotypes (Graves et al., 2007; Jacquet et al., 2004; McLauchlin et al., 2004). Interestingly, 1/2a serotype strains comprise the majority of Canadian clinical isolates. In particular, a predominant clonal complex (CC8)/epidemic clone has been responsible for sporadic listeriosis in Canada since 1988 (Knabel et al., 2012). Within this complex, the majority of 1/2a strains were found to possess a 50 kb genomic island (LGI1), first identified in a strain associated with the Maple Leaf outbreak and linked to 23 deaths (Gilmour et al., 2010). This island encodes a combination of putative antimicrobial resistance (AMR), stress response, and virulence genes, possibly enhancing *L. monocytogenes*' ability to survive in the food chain (Gilmour et al., 2010). However, data on the prevalence of the island, and genetic

determinants associated with persistence and/or virulence amongst *L. monocytogenes* isolates recovered from the Canadian food chain are lacking. Considering that conditions encountered in food processing affect many virulence and stress response factors of *L. monocytogenes*, it would be prudent to understand the effect they may have on the expression of genes in recently described genomic island LGI1.

The purpose of this thesis, therefore, was to improve our understanding of physiological and genotypic properties of *Listeria* spp., and in particular *L. monocytogenes*, originating from the food chain, and to further characterize the role and contribution of LGI1 to *L. monocytogenes* survival in the food chain.

1.2 Literature review

1.2.1 The *Listeria* genus

The *Listeria* genus is comprised of at least 15 species, six of which have been studied in detail. These include *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri*, and the nine recently characterized, *L. aquatica*, *L. cornellensis*, *L. fleischmannii*, *L. floridensis, L. grandensis*, *L. marthii*, *L. riparia*, *L. rocourtiae*, and *L. weihenstephanensis* with the *species nova* designation (Bertsch et al., 2013; den Bakker et al., 2014; Graves et al., 2010; Lang Halter et al., 2013; Leclercq et al., 2010; Orsi et al., 2011). The majority of the species within the genus are avirulent and well adapted to saprophytic life. *Listeria monocytogenes* and *L. ivanovii* are the only recognized pathogenic species, with *L. monocytogenes* being a significant pathogen in humans and animals, while *L. ivanovii* causes illness almost exclusively in animals (Liu and Busse, 2009; Vàzquez-Boland et al., 2001b). On rare occasions, illnesses caused by atypical *L. innocua* and

L. seeligeri have been reported (Perrin et al., 2003; Rocourt et al., 1986). Taxonomically, the *Listeria* genus is closely related to the genera *Brochothrix, Bacillus*, *Lactobacillus*, and more distantly related to *Streptococcus*, *Lactococcus*, *Enterococcus* and *Staphylococcus* (Farber and Peterkin, 1991). Furthermore, they are Gram positive, facultatively anaerobic and non-sporulating rods, which can grow at a wide range of temperatures (-0.4 to 50°C), pH conditions (4.4 and greater) and salt concentrations (10%), as well as in biofilm consortia (Farber and Peterkin, 1991; Liu et al., 2005). All *Listeria* spp. exhibit motility at room temperature (e.g., 20 to 25°C) using peritrichous flagella, while this phenomenon is markedly suppressed at 37°C (Rocourt and Buchrieser, 2007).

Species of *Listeria* can be differentiated based on their somatic (O) and flagellar (H) antigens. Currently, there are 13 different serotypes of *L. monocytogenes*, one *L. grayi*, at least three amongst *L. innocua,* one *L. ivanovii*, four amongst *L. seeligeri*, and two *L. welshimeri* serotypes (Allerberger, 2003; Liu, 2006). Noticeable heterogeneity in virulence and origins has been reported amongst the 13 *L. monocytogenes* serotypes. In fact, three different categories have been suggested: serotypes 1/2b, 3b, 4b, 4d and 4e, which are responsible for more than 90% of all the listeriosis and include outbreak causing strains, belong to lineage I; serotypes 1/2a, 1/2c, 3a and 3c are known as lineage II strains, which have been isolated from humans, animals, foods, food processing environments, and sporadic cases of listeriosis but less commonly related to outbreaks; and lineage III strains, including serotypes 4a and 4c, which are exclusively linked to animals (Wiedmann et al., 1997). Although serotyping offers little information regarding virulence properties and relatedness of strains in epidemiological and outbreak settings, it is still used as a “gold standard” in

clinical investigations in many reference laboratories, as linkage of particular serotypes to listeriosis has been established (Chen and Knabel, 2008; Graves et al., 2007).

However, assigning strains to specific lineages has been challenging. As different molecular techniques are now available to assess strain relatedness variability in virulence, survival fitness, and host preferences has been observed within *L. monocytogenes* isolates belonging to the same serotype. Using molecular biology techniques that focus on the genetic content of 4b serotypes has shown that this group of *L. monocytogenes,* historically linked to a number of large human listeriosis outbreaks, in fact harbors strains of animal origin rarely associated with human listeriosis (Liu, 2006; Ward et al., 2004). Studies employing multilocus sequence typing (MLST), which is based on DNA sequencing to characterize alleles present in seven housekeeping genes, have provided improved knowledge regarding the evolution of *L. monocytogenes* strains originating from different regions, sources, and time periods (Ragon et al., 2008; Salcedo et al., 2003). Combining MLST with DNA sequencing of virulence-associated genes can contribute to a greater understanding of global distribution and evolution of virulence determinants, as well as improved discriminatory power for epidemiology studies (Zhang et al., 2004). Nucleotide sequence polymorphisms in the *inlA* gene, which encodes for a protein involved in *L. monocytogenes* host colonization, have been shown to effectively predict the virulence potential of a strain, as well as suggest the existence of still unknown ecological factors driving the adaptation and selection for virulence-attenuated strains (Knabel et al., 2012; Nightingale et al., 2008; Orsi et al., 2007; Ragon et al., 2008; Tsai et al., 2006).

1.2.2 *Listeria* genomics

Listeria has a circular chromosome ranging from 2.7 to 3 Mb in length (Cabanes et al., 2011; Glaser et al., 2001; Kuenne et al., 2013). Their G+C content (i.e. the proportion of guanine and cytosine nucleotides within a genome) is about 39%, placing them into the low G+C content group of bacteria (Kuenne et al., 2013). The first complete *L. monocytogenes* EGD-e genome became publicly available in 2001 (Glaser et al., 2001). Since then, a large number of *L. monocytogenes* strains have been sequenced and this is likely to continue to increase in the near future. Presently, there are 39 complete *L. monocytogenes* genomes available at NCBI's GenBank (Bécavin et al., 2014), and 187 entries in the genome online database (Pagani et al., 2012) from either completed or on-going projects (Genomes OnLine Database, 2012).

Comparative genomics of *Listeria* spp. reveal an overall conservation in genome organization (Cabanes et al., 2011; Kuenne et al., 2013; Nelson et al., 2004). Approximately 65% of gene functions have been identified for *Listeria* spp. (Cabanes et al., 2011; Gilmour et al., 2010). A large number of these genes encode putative transport systems, transcriptional regulators, and surface and secreted proteins. These functions are in accordance with the capacity of *Listeria* to adapt to a variety of environmental conditions and inhabit different niches (Cabanes et al., 2011). Studies have shown that a number of genes present in 4b serotypes (e.g., *L. monocytogenes* CLIP80459 and *L. monocytogenes* F2365) are missing from 1/2a serotypes, particularly *L. monocytogenes* EGD-e (Doumith et al., 2004; Milillo et al., 2009; Nelson et al., 2004). Most of the differing genes encode for surface proteins, and fitness factors such as genes involved in sugar metabolism and virulence (Cabanes et al., 2011; Nelson et al., 2004).

Recently, the three most extensively used *L. monocytogenes* strains to study virulence, persistence, and stress adaptation, EGD, EGD-e, and 10403S, have been compared for genomic differences (Bécavin et al., 2014). EGD is a reference laboratory *L. monocytogenes* strain belonging to 1/2a serotype. It was isolated from guinea pigs, and described by E. G. D. Murray in 1926 (Bécavin et al., 2014; Glaser et al., 2001; Murray et al., 1926). Its derivative, EGD-e, was the first isolate to have its whole genome sequenced by the European consortium (Bécavin et al., 2014; Glaser et al., 2001). EGD-e was obtained in 1986 by Trinad Chakraborty, and has been passaged through mice over the years to maintain virulence (Bécavin et al., 2014). *Listeria monocytogenes* 10403S, where S stands for streptomycin resistance, is a derivative of a 1/2a serotype clinical strain (i.e. 10403) recovered from human skin lesions in Bozeman, Montana, and a reference strain used in many studies in the United States (US) (Bécavin et al., 2014; Edman et al., 1968).

EGD and 10403S strains were found to be genetically similar, with only 317 single nucleotide polymorphisms (SNPs) observed, and both strains belong to clonal complex (CC) 7. In contrast, EGD-e was found genetically highly distinct from both EGD and 10403S. This strain belongs to CC9, and its closest relatives are a 1/2c serotype clinical strain recovered in 1935 and a 3c strain of unknown origins from 1966. As a result, the authors suggest a possibility that EGD-e was originally mislabeled and exchanged for a different strain (Bécavin et al., 2014). The implications of this finding could be significant, as EGD and EGD-e have been used interchangeably in studies that looked at the basis of *L. monocytogenes* virulence.

Virulence factors of *L. monocytogenes* are clustered in genomic islands along the chromosome. *Listeria* pathogenicity island 1 (LIPI-1) is a 9-kb cluster of six virulence

determinants that have a major role in *L. monocytogenes* virulence (Vàzquez-Boland et al., 2001a). Additionally, internalin islets comprising two or more *inl* genes that occur in different loci on the chromosome aid the bacterium's invasion of different cell types in the host (Dussurget et al., 2004; Vàzquez-Boland et al., 2001a). Some of the other notable chromosomal loci associated with virulence include Clp (caseinolytic proteases) stress tolerance mediators, Ami amidase protein, and Hpt hexose phosphate transporter, while a number of genes contributing to bacterial invasion of different host cells are also scattered throughout the genome (Schmid et al., 2005; Vàzquez-Boland et al., 2001a).

Recently, a 50 kb operon-like structured region termed LGI1 (Figure 1-1) was discovered in *L. monocytogenes* isolates originating from Canada (Gilmour et al., 2010). Subsequent testing of the archived clinical isolates revealed the presence of LGI1 in a number of *L. monocytogenes* strains, some of them dating back to 1988 (Knabel et al., 2012). These findings suggest LGI1 has been in the Canadian *L. monocytogenes* population for more than two decades. Interestingly, this island has not been reported outside of Canada to date (Knabel et al., 2012). Presently, it is not known whether this island is exclusive to Canadian isolates, or simply has not been observed globally due to limited number of *L. monocytogenes* genomes currently available. With the next-generation sequencing technologies becoming increasingly available this will likely change in the near future. Our knowledge of the origins and global distribution of LGI1 will likely improve as the genomes of *L. monocytogenes* isolates originating from different geographical regions become available.

1.2.2.1 *Listeria* genomic island 1 (LGI1)

LGI1 has been described in isolates of 1/2a serotype, linked to deli meats and a severe listeriosis outbreak in Canada (Gilmour et al., 2010). Further investigation revealed that serotype 1/2a isolates belonging to CC8, and possessing similar pulsed-field gel electrophoresis (PFGE) patterns (e.g., *Asc*I/*Apa*I profile LMACI.0001/LMAAI.0001), caused a substantial proportion of the sporadic cases, clusters, and outbreaks in Canada since 1988 (Knabel et al., 2012). Examination of more than 1,000 *L. monocytogenes* isolates collected from 1995 to 2010 revealed the presence of this clone in 22.3% of isolates, with the nationwide distribution believed to have occurred by the mid-1990s. Among 71 *L. monocytogenes* isolates examined by Knabel et al. (2012), 49 belonged to CC8, and all but six possessed LGI1.

This island (Figure 1-1) was most likely horizontally acquired due to the presence of putative serine recombinases (e.g., loci 1855-58) and 16 bp imperfect inverted repeats at the borders of the intergenic regions (e.g., loci 1849/50 and 1903/04) (Gilmour et al., 2010). Regions flanking the island are homologous to *L. monocytogenes* EGD-e *lmo1702* and *lmo1703* (Gilmour et al., 2010), while coding sequences within LGI1 possess partial homology to sequences found in a number of environmental firmicutes, such as *Desulfitobacterium dehalogenans, D. hafniense*, *Clostridium kluyver*i, and *C. ljungdahli* (Ziegler, 2012).

Ziegler (2012) observed an increase in minimum inhibitory concentrations (MIC) of benzalkonium chloride (BAC) and benzethonium chloride in three Canadian isolates possessing LGI1, suggesting it may play a role in *L. monocytogenes* resistance to sanitizers.

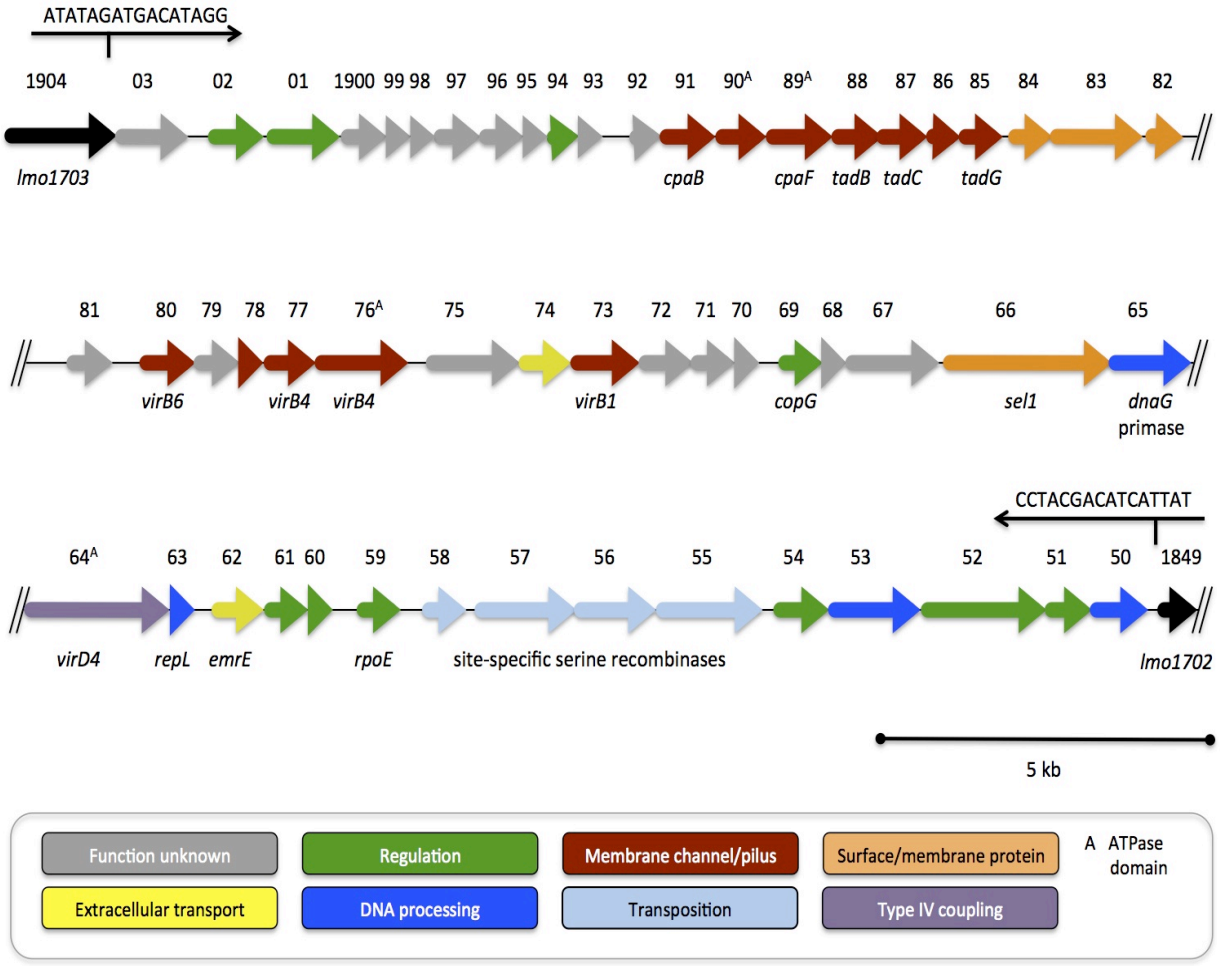


Figure 1-1. Genetic organization and putative functions of the *Listeria* genomic island 1 (LGI1). Adapted from Gilmour et al. (2010). Numbers above coding sequences map represent locus tags in *L. monocytogenes* 08-5578, and putative gene names in italics are denoted below. Different colors denote putative gene functions.

Further, the presence of genes typically involved in stress response, such as a two-component signal transduction system, with a response regulator (locus 1851) and a sensor histidine kinase (locus 1852), and a putative small RNA polymerase sigma-24 subunit (locus 1859), indicates strains possessing LGI1 may be better equipped to battle environmental and/or food processing stresses (Gilmour et al., 2010; Ziegler, 2012). It is also tempting to speculate that, to some extent, the island is contributing to virulence, considering it was found in a number

of clinical isolates, spanning more than two decades (Knabel et al., 2012). The presence of genes homologous to type IV secretion-like systems (e.g., *virB4*, *virD4*, *cpa* and *tad*), as well as a putative adhesin (i.e. *sel1*) further supports the idea (Gilmour et al., 2010; Ziegler, 2012); though evidence of increased virulence due to LGI1 is currently lacking. In fact, the actual function of genes located on LGI1, and their contribution to fitness and/or virulence of *L. monocytogenes* have not yet been confirmed.

1.2.3 *Listeria monocytogenes* pathogenesis and listeriosis

As an intracellular pathogen, *L. monocytogenes* has very successfully evolved to exploit a number of host cell processes. It facilitates its own uptake into cells and further spreads from cell to cell, generally causing little toxicity to the host cell (Cossart and Bierne, 2001; Portnoy et al., 2002). The gastrointestinal tract is the primary entrance route for *L. monocytogenes*, *via* contaminated foods (Farber and Peterkin, 1991). Once ingested, *L. monocytogenes* can either be internalized into professional phagocytes or induce its endocytosis into nonprofessional phagocytic cells, such as epithelial, endothelial and hepatocytic cells, by the use of highly sophisticated mechanisms (Vàzquez-Boland et al., 2001b).

1.2.3.1 Invasion and spread in human host cells

There has been some controversy pertaining to the entry point of the bacterium, depending on the infection models used. Earlier studies with mice and rats showed that *L. monocytogenes* preferentially colonize Peyer's patches through the use of M-cell epithelium (Marco et al., 1997; Marco et al., 1992). A study by Lecuit et al. (1999)

demonstrated that mouse and rat E-cadherin, a receptor for internalin A (InlA) protein that promotes invasion, differs from human E-cadherin. A difference in one amino acid residue (i.e. substitution of proline residue at position 16 seen in human E-cadherin with glutamate in mice and rats) renders cells resistant to InlA-mediated invasion, suggesting that mice and rat models are not representative of the *L. monocytogenes* invasion of human cells. In contrast, studies with guinea pigs showed that *L. monocytogenes* penetrates the host cell through Inl-A mediated invasion of intestinal villous epithelium (Lecuit et al., 2001; Rácz et al., 1972). More recently, studies using transgenic mice expressing human E-cadherin, and Madin-Darby canine kidney cells demonstrated that *L. monocytogenes* invasion and translocation of the small intestine occurs at apical tips of the intestinal villi (Pentecost et al., 2006), villus epithelial folds, and junctions between mucus-secreting goblet cells (Nikitas et al., 2011). While the small intestine is a preferential invasion site for *L. monocytogenes*, it has been established that the bacterium can also invade the caecum and the colon of the large intestine in gerbils and transgenic mice expressing human E-cadherin (Figure 1-2) (Disson et al., 2008).

Internalization is followed by lysis of the phagocytic vacuole, and bacterial release into the cytosol where it can undergo cell replication and spread into adjacent cells (Cossart and Bierne, 2001). Utilizing the actin-polymerization phenomenon *L. monocytogenes* is propelled from cell to cell, resulting in rapid dissemination from the small intestine to the liver, spleen, brain, and placenta tissues (Figure 1-2) (Cossart and Bierne, 2001; Dramsi and Cossart, 2003; Vàzquez-Boland et al., 2001b). Most of the bacteria accumulate in the liver, where they are killed by resident macrophages known as Kupffer cells. For any *L. monocytogenes* that survive, the principal site of multiplication is the hepatocytes

(Vàzquez-Boland et al., 2001b). Dissemination is often through macrophages (i.e. white blood cells that take up foreign materials) and dendritic cells (i.e. antigen-presenting cells, responsible for stimulating activation of T cells and immune response), allowing *L. monocytogenes* to avoid direct contact with the complement system (e.g., plasma proteins that opsonize pathogens and induce a series of inflammatory responses that facilitate pathogen removal) (Geginat and Grauling-Halama, 2008). Successful elimination of *L. monocytogenes* is dependent on effective innate immunity, also known as a first line of defense in listeriosis infection (e.g., natural killer cells, dendritic cell-primed CD4 T helper cells, and cytokines such as gamma interferon), followed by adaptive immunity (e.g., activation of $CD4^+$ T cells, and expansion of cytotoxic $CD8^+$ T cells) (Mitsuyama, 2008).

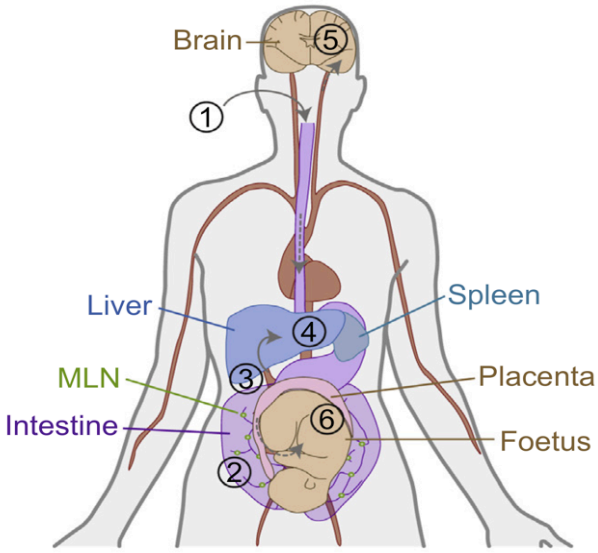


Figure 1-2. *Listeria monocytogenes* infectious process in the human body, following the ingestion of contaminated food (1), colonization of the digestive tract and crossing of the intestinal barrier (2), systemic circulation (3), infection of the liver and spleen (4), crossing of the blood-brain barrier (5), and the placental barrier (6). Re-printed from Cossart and Lebreton (2014) with permission from the authors and Elsevier.

1.2.3.2 Listeriosis manifestation

Immunocompetent individuals that develop listeriosis typically exhibit subclinical symptoms, such as those seen in mild gastroenteritis. These symptoms generally resolve within four to five days following infection. In contrast, severe infections often occur in individuals whose immune systems are suppressed (Farber and Peterkin, 1991; Vàzquez-Boland et al., 2001b). Mortality rates associated with listeriosis infections in vulnerable populations and perinatal cases range from 20 to 40%, placing *L. monocytogenes* amongst the deadliest foodborne pathogens (Clark et al., 2010).

When manifested as a feto-maternal listeriosis, the infection is generally asymptomatic in the mother or it may be present as a mild flu-like illness. However, in most cases this infection leads to spontaneous abortions and stillbirths (Farber and Peterkin, 1991). If the infection of the fetus occurs inside the uterus and the illness symptoms result following the birth or shortly after, this is typically referred to as early-onset listeriosis. A late-onset listeriosis can also present, typically several weeks after the birth. Sepsis and meningitis are often seen in early-onset cases, while meningitis and neonatal growth retardation are usually linked to the late-onset listeriosis (Painter and Slutsker, 2007; Vàzquez-Boland et al., 2001b). Numerous microabscesses and placental villitis have been reported to occur during listeriosis infections in pregnant women, as bacteria in maternal blood infect the trophoblast layer and translocate across the endothelial barrier (Lecuit et al., 2004; Vàzquez-Boland et al., 2001b). More specifically, InlA is believed to mediate attachment to E-cadherin receptors present in villous cytotrophoblasts (i.e. inner layer of trophoblasts) and at the basal and apical plasma membranes of the placental syncytiotrophoblasts (i.e. outer layer of trophoblasts) (Disson et al., 2008; Lecuit, 2005; Lecuit et al., 2004). While earlier studies (Lecuit et al., 2004)

suggested that InlA is solely responsible for mediating placental invasion, Disson et al. (2008) demonstrated that functional pathways including both InlA and InlB proteins are necessary for *in vivo* invasion of the human placenta.

In non-pregnant adults, sepsis, meningitis or meningoencephalitis can be the result of listeriosis, especially in immunocompromised individuals for whom listeriosis is often fatal (Painter and Slutsker, 2007). Infection of the central nervous system (CNS) is typically the result of bacterial dissemination *via* the bloodstream (Drevets et al., 2004). These infections can occur when *L. monocytogenes* invade the CNS vasculature using both InlA and InlB mediated internalization, or exploit phagocytes to facilitate invasion of the CNS (Grundler et al., 2013; Vàzquez-Boland et al., 2001b). Infected leukocytes can adhere to endothelial cells of the CNS, which allows *L. monocytogenes* to be spread from cell to cell, using actin-mediated movement (Vàzquez-Boland et al., 2001b). Additionally, if there are any breaches in oral cavities during the consumption of contaminated food, it is possible that the macrophages and blood phagocytes sent to abrasion sites by the immune system function as *L. monocytogenes* vehicles thereby aiding spread into cranial nerve neurites (Drevets et al., 2004). Eventually, bacteria are able to reach the CNS, where they result in meningitis, as well as systemic diseases in immunocompromised individuals (Vàzquez-Boland et al., 2001b).

1.2.4 *Listeria monocytogenes* virulence factors and their regulation

Depending on the point of entry, different *L. monocytogenes* surface proteins are employed. For instance, InlA protein plays a role in the invasion of human enterocyte-like Caco-2 cells, while internalin B (InlB) is involved in invasion of Vero, HeLa, Hep-2 and CHO (i.e.

Chinese hamster ovary) cells (Braun et al., 1998; Ireton et al., 1996; Lingnau et al., 1995; Yamada et al., 2006). Besides internalins, other molecular determinants, such as: amidase (Ami), a cell wall hydrolase (p60), fibronectin binding surface protein (FbpA), surface actin-polymerizing protein (ActA), a pore forming toxin, listeriolysin O (LLO), and an unconventional myosin and its ligand vezatin, aid in *L. monocytogenes* adhesion to and invasion of eukaryotic cells (Dussurget et al., 2004; Sousa et al., 2004).

Once *L. monocytogenes* adheres to and successfully enters the host cell, intracellular survival is dictated by its ability to avoid the immune system response. Virulence traits important for this stage of the *L. monocytogenes* life cycle include: phospholipases C (PLC) in concert with LLO, actin filaments, and Hpt (Dussurget et al., 2004; Vàzquez-Boland et al., 2001b).

Generally, the expression of virulence genes is highly dependent on the stage of life cycle, as well as the location and conditions surrounding *L. monocytogenes* (Chaturongakul et al., 2011; Mueller and Freitag, 2005). Genes encoding the major virulence factors are located in the central virulence gene cluster, LIPI-1, under control of a positive regulatory factor A (PrfA) (Scortti et al., 2007; Sheehan et al., 1995; Vàzquez-Boland et al., 2001a; Vàzquez-Boland et al., 2001b). High temperature (Leimeister-Wachter et al., 1992; Scortti et al., 2007), stress conditions (Sokolovic et al., 1990; Sokolovic et al., 1993), sequestration of extracellular growth medium components (Scortti et al., 2007), and host cell contact (Renzoni et al., 1999) have been shown to trigger the expression of various virulence genes.

1.2.4.1 Motility, adherence and surface proteins

Flagellar motility plays a role in virulence in a number of pathogens, including *Campylobacter jejuni* (Grant et al., 1993), *Legionella pneumophila*, *Clostridium difficile*, *Helicobacter pylori*, *Salmonella enterica* serovar Typhi, and *Vibrio cholerae* (Salyers and Whitt, 2002). Similarly, peritrichous flagella in *L. monocytogenes* allow bacterial movement when located outside the host cells. There are about 41 genes responsible for the flagellar complex of *Listeria*, and the expression is controlled by at least five different regulators: FlaR, PrfA, DegU, MogR, GmaR (Desvaux and Hébraud, 2008).

Flagellar filaments are produced and assembled at growth temperatures between 20 and 25°C. However, the production is significantly decreased at 37°C, the temperature of the human body (Dons et al., 2004). *Listeria monocytogenes* mutants lacking certain flagellar genes (e.g., *lmo0713* and *lmo0716*) are unable to successfully adhere to and enter nonphagocytic epithelial cells (Bigot et al., 2005). The products encoded by these genes are similar to FliI, flagellar basal body component, and FliF, an ATPase enzyme involved in energizing of the flagellum component export (Bigot et al., 2005). Bigot et al. (2005) observed the presence of flagella for some *L. monocytogenes* strains at 37°C that led to the decreased adhesion to epithelial cells; however, there was no impact on the survival of *L. monocytogenes* inside the cytosol. These findings indicate that flagella play an important role in the initial steps of listerial infection, especially if bacteria enter through the gastrointestinal tract. However, once *L. monocytogenes* are internalized this virulence trait is replaced with other forms of motility more appropriate for the surrounding environment, such as actin-based motility (Vàzquez-Boland et al., 2001b).

Actin filaments are used for intracellular movement by *L. monocytogenes* and *L. ivanovii*, as well as *Shigella flexneri* and *Rikettsia conori* (Cossart, 2000). Some strains of *Salmonella* and pathogenic *Escherichia coli* also use this mechanism to alter the cell cytoskeleton and promote infection (Cantarelli et al., 2006; Guiney and Lesnick, 2005). Following the release of *L. monocytogenes* from the phagocytic vacuole, actin polymerization is rather quickly induced allowing bacteria to move inside the cytosol, and spread from cell to cell (Cossart, 2000; Dussurget et al., 2004; Pfeuffer et al., 2000). ActA, a surface protein responsible for polymerization of actin in *L. monocytogenes*, mimics cellular eukaryotic protein WASP [Wiscott-Aldrich syndrome protein; (Higgs et al., 1999)]. Similar to WASP, ActA directly activates the Arp2/3 complex, which in turn is responsible for actin filament nucleation and organization (Cossart, 2000; Higgs et al., 1999).

A number of additional actin-binding proteins and ligands that play a role in motility have been identified. These include: α-actinin (Dold et al., 1994); profilin (Theriot et al., 1994); a vasodilator stimulated phosphoprotein (VASP) important in the speed of bacterial movement (Chakraborty et al., 1995); CapZ; and LaXp180, a protein involved in signal transduction associated with microtubule networks (Cossart and Bierne, 2001). However, the exact roles for many of these proteins in motility have not been fully elucidated.

While motility plays an important role in bacterial movement, evasion of adverse conditions and dissemination, adherence is just as important for successful uptake and colonization of the host cells. Several adhesins and autolysins have been found to be important in the initial stage of colonization. They typically play a role in the digestion of cell wall peptidoglycan, regulation of cell growth, cell division, peptide-glycan maturation, protein secretion, as well as pathogenicity (Seveau et al., 2007). It has been suggested that

adhesins aid the adherence of bacteria to host surfaces by recognizing molecules analogous to their natural receptors, teichoic and lipoteichoic acids (Dussurget et al., 2004; Milohanic et al., 2001). Autolysins Ami and Auto, found in *L. monocytogenes*, have been shown to play a role in virulence (Seveau et al., 2007). Mutants possessing deletions in *ami* exhibited reduced attachment to different cell lines (e.g., human melanoma cell line SK-MEL 28, human enterocyte-like colon carcinoma cell line Caco-2, hepatocellular carcinoma cell line Hep-G2), and attenuated virulence in the liver of mice inoculated intravenously (Milohanic et al., 2001); moreover, isolates with inactivated *aut* displayed reduced entry into various host cells (e.g., African green monkey kidney cell line Vero, Caco-2, guinea-pig epithelial cell line GPC16, human laryngeal epithelial cell line Hep-2, murine fibroblast cell line L2), and attenuated virulence in guinea pigs (Cabanes et al., 2004).

Similarly, p60 (also known as CwhA), a cell wall hydrolase encoded by the *iap* gene, is important for the invasion of mouse fibroblasts (Kuhn and Goebel, 1989; Pilgrim et al., 2003). *In vitro*, p60 is important in cell division, as it breaks the cell chains into single cells (Wuenscher et al., 1993). *Listeria monocytogenes* mutants lacking this protein have a rough-colony morphology, and decreased ability to spread from cell to cell due to defective actin-tail filaments (Pilgrim et al., 2003). The exact mechanism behind the action of p60 is not well understood, since the invasiveness of p60 mutants seems only slightly diminished in some cells while complete attenuation is observed in others (Kuhn and Goebel, 1989; Pilgrim et al., 2003).

FbpA is another example of a surface protein of *L. monocytogenes* important in the listerial adherence to host cells. It is highly similar to fibronectin-binding proteins PavA of *Streptococcus pneumoniae*, Fbp54 of *S. pyogenes*, and FbpA of *S. gordonii* (Dussurget et al.,

2004). Dramsi et al. (2004) reported FbpA binding to immobilized human fibronectin, while in the presence of exogenous fibronectin it increases adherence of wild-type (WT) *L. monocytogenes* to Hep-2 cells. In addition, the role of FbpA as a chaperone for other important virulence factors, such as LLO and InlB, has been suggested (Dramsi et al., 2004). It prevents degradation of LLO and InlB (Dussurget et al., 2004). However, a similar effect was not observed for InlA or ActA, suggesting that it may be specifically involved in aiding adherence and invasion of liver and nonepithelial cells (Dramsi et al., 2004).

Recently, *Listeria* adhesion protein (LAP) has been identified as important for the adherence of *L. monocytogenes* to intestinal epithelial cells (Pandiripally et al., 1999). LAP is a 104-kD protein, encoded by the *aad* or *lap* gene (Kim et al., 2006; Pandiripally et al., 1999). It is expressed in five of the six well characterized *Listeria* spp., including *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*, but not *L. grayi* (Jagadeesan et al., 2010). It has been suggested that this protein is unable to re-associate on the surface of non-pathogenic strains, thereby rendering them unable to adhere to mammalian cells (Jagadeesan et al., 2010). Strains of *L. monocytogenes* not expressing LAP but possessing hemolytic and phospholipase activity exhibited attenuated virulence in Caco-2 cells (Pandiripally et al., 1999). The inability of mutant strains to adhere properly to the target cells indicates that LAP plays a role in adherence and virulence of *L. monocytogenes*; however, the specific fashion by which LAP adds to virulence has not been fully elucidated. Further, evidence suggests that environmental factors such as nutrient-limiting conditions induce the expression of LAP, while high glucose levels repress the production of LAP (Jaradat and Bhunia, 2002). Since these conditions are present in food processing

environments, it has been proposed they may play a role in the subsequent ability of *L. monocytogenes* to invade human host cells (Jaradat and Bhunia, 2002).

1.2.4.2 Invasion and intracellular survival

A number of invasion mechanisms are deployed by *L. monocytogenes* during the infection process, depending on the location of the bacterium inside the host (Vàzquez-Boland et al., 2001b). Typically, pathogens can recognize a variety of host cell receptors (e.g., transmembrane glycoprotein E-cadherin, C1q complement fraction receptor, the Met receptor for hepatocyte growth) and extracellular matrix components from which they are able to sense their environment and produce virulence factors required for survival in a particular milieu (Vàzquez-Boland et al., 2001b).

Proteins known as internalins play an important role in the uptake of *L. monocytogenes* that has adhered to, or come into close proximity to the target cell. The most studied internalins in *L. monocytogenes* are InlA and InlB, which are directly responsible for internalization of *L. monocytogenes* into various mammalian cells (Figure 1-3). The 800-amino acid long InlA surface protein recognizes and binds to human receptor E-cadherin expressed by a number of cells (e.g., epithelial cells in the intestine, intracerebral microvascular endothelial cells, placenta, hepatocytes and dendritic cells) (Schubert et al., 2001) and has a key role in *L. monocytogenes* invasion of human epithelial cells (Lecuit et al., 1997). InlA and E-cadherin binding is species specific, and requires recognition of a proline at position 16 in the E-cadherin molecule (Lecuit et al., 1999).

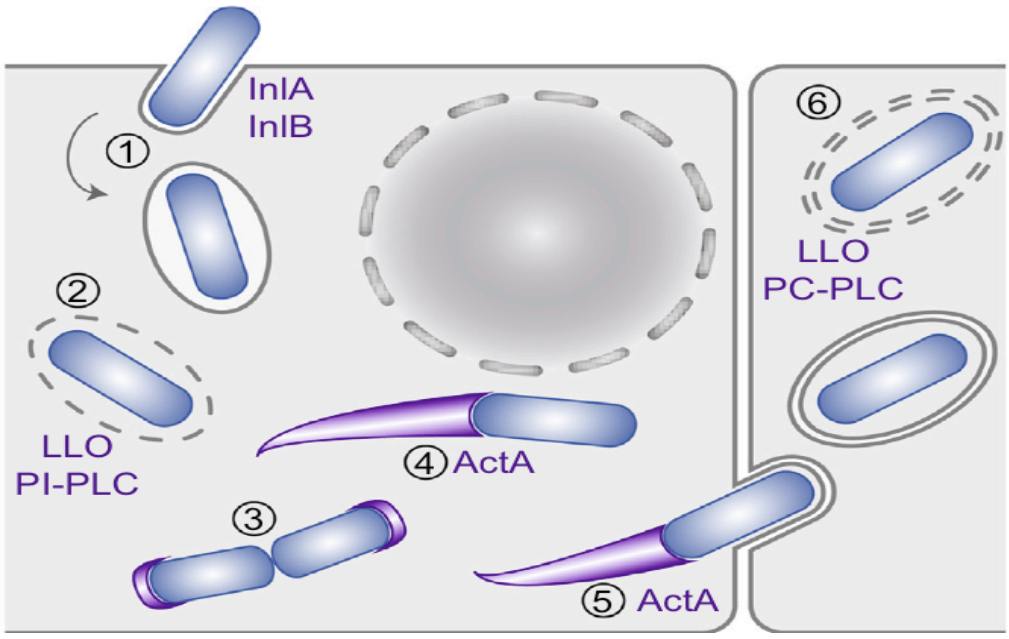


Figure 1-3. Intracellular life cycle of *L. monocytogenes* represented by the bacterial entrance into host cells using internalins InlA and InlB (1), release from the endocytic vacuole using listeriolysin O (LLO) and phosphatidylinositol phospholipase C (PI-PLC) (2), replication in the cytosol (3), polymerization of cellular actin via the recruitment of the Arp2/3 complex using ActA (4), cell to cell spread using actin comet tails (5), and breakdown of the two-membrane vacuole using LLO and phosphatidylcholine-specific phospholipase C (PC-PLC). Re-printed from Cossart and Lebreton (2014) with permission from the authors and Elsevier.

Furthermore, together with the 630-amino acid long InlB protein that interacts with human receptors C1q (i.e. complement fraction receptor) and Met (i.e. receptor for hepatocyte growth), InlA facilitates listerial invasion of the human placenta (Disson et al., 2008) and crossing of the brain barrier (Grundler et al., 2013).

Research has shown that differences linked to *inlA* affect virulence of *L. monocytogenes* strains. In particular, a significant proportion (35-45%) of strains recovered from RTE foods have been reported to possess mutations in *inlA* resulting in a premature stop codon (PMSC) (Felicio et al., 2007; Jacquet et al., 2004; Nightingale et al., 2008), and the production of either a truncated or non-secreted InlA protein. Assays conducted *in vitro* (Nightingale et al., 2005) and in mammalian models (Nightingale et al.,

2008; Roldgaard et al., 2009; Van Stelten et al., 2011) have both shown that strains possessing PMSCs exhibit virulence-attenuated phenotypes. Interestingly, PMSCs are more commonly observed in 1/2a and other serotypes that belong to lineage II strains, and are not associated with 4b strains (Orsi et al., 2011). It has been suggested that lineage II 1/2a strains are better able to survive conditions associated with the food chain, while 4b serotype strains appear more recalcitrant to genetic flux. As such, 4b strains are less likely to acquire or possess plasmids and to experience homologous recombination events that may afford rapid adaptation to niche-specific stresses (Orsi et al., 2011).

Other internalins such as InlC2, InlD, InlE, InlG, InlH, and InlJ have been suggested to contribute to infection, but presently their roles are not well defined (Tsai et al., 2006). Deletion of *inlJ* (*lmo2821* in EGD-e strain), which is present in the genomes of *L. monocytogenes* but absent from *L. innocua* (Cabanes et al., 2002; Doumith et al., 2004), resulted in attenuated virulence in intravenously or orally infected mice expressing human E-cadherin (Sabet et al., 2005). Sabet et al. (2008), further demonstrated that this protein is an adhesin specifically expressed during infection *in vivo.* It is produced and localized on the bacterial surface when *L. monocytogenes* is in the liver and blood, but not *in vitro* in brain heart infusion medium or when replicating in the cytosol of tissue-culture cells (e.g., JEG-3 placental cells, HT29 intestinal cells, J774 murine macrophages) (Sabet et al., 2008). While the extent to which InlJ and other internalins contribute to *L. monocytogenes* virulence remains elusive, together with InlA and InlB they are believed to work in synergy to achieve optimal invasiveness (Dramsi et al., 1995).

In addition to internalins, listeriolysin O (LLO) and phospholipase C (PLC) are extremely important in the intracellular survival of *L. monocytogenes*. LLO is a member of

the pore-forming, cholesterol-dependent cytolysin family, with similarities to some other cytolysins produced by Gram positive bacteria such as *Streptococcus*, *Bacillus*, and *Clostridium* spp. (Salyers and Whitt, 2002). LLO lyses the primary phagosomes engulfing the bacteria upon cell internalization and disrupts the secondary phagosomal membrane during intercellular movement (Figure 1-3) (Gedde et al., 2000). LLO is crucial for listerial survival inside the host, since bacteria that are internalized but cannot breach the vacuolar membrane and exit into the cytosol have very remote chance of survival due to immune response action. Even if the bacteria are able to survive inside the vacuole, the inability of *L. monocytogenes* to multiply decreases their spread, which consequently results in lower virulence capacity (Cossart et al., 1989; Vàzquez-Boland et al., 2001b).

Similar to LLO, PLCs enzymes aid the escape of *L. monocytogenes* from the vacuoles into the cytosol and contribute to successful cell to cell spread (Figure 1-3) (Camilli et al., 1991; Grundling et al., 2003). PI-PLC is the enzyme responsible for propagation of *L. monocytogenes* in the host tissue, due to its activity on phosphatidylinositol (PI) and glycosyl-PI-anchored proteins, while phosphatidylcholine-specific PLC (PC-PLC) is a zinc dependent, broad range enzyme capable of recognizing a range of different substrates and responsible for cleaving most phospholipids (Camilli et al., 1991). The PC-PLC enzyme is excreted in an inactive form, which then needs to be matured and activated with the help of a metalloprotease (Mpl) (Vàzquez-Boland et al., 2001b).

1.2.4.3 Regulation of virulence

The expression of major virulence genes is mediated by PrfA, a positive regulatory factor responsible for activation and repression of genes located on LIPI-1 and a number of other

genes on the chromosome (e.g., *inlAB*, *bsh*) (Cabanes et al., 2011; Ward et al., 2004). PrfA regulation is subject to the changes in transcriptional activity of the protein, with the amount of PrfA produced being controlled at the transcriptional and translational levels (Johansson et al., 2002; Scortti et al., 2007). It is also thermally regulated, with the expression of PrfA suppressed at temperatures below 30°C and up-regulated as the temperature increases, for example in a warm-blooded host (Kuhn et al., 2008; Scortti et al., 2007). In particular, at low temperatures *prfA* 5' untranslated region (UTR) adopts a stable hairpin structure that masks the Shine-Delgarno site and prevents its interaction with the 30S ribosomal subunit, thereby inhibiting translation. In contrast, at high temperatures (e.g., 37°C) this hairpin structure denatures, allowing PrfA translation (Johansson et al., 2002; Loh et al., 2006; Mellin and Cossart, 2012).

A PrfA mutation in EGD, where glycine is replaced with serine at amino acid position 145, results in constitutive expression of the core PrfA regulon [e.g., genes located on LIPI-1, the *inlA*-*inlB* operon, *inlC* (*lmo1786*), and *hpt* (*lmo0838*)] (Bécavin et al., 2014). This leads to higher *in vitro* (e.g., HeLa, JEG3 cells, and mouse macrophages Raw264) invasion efficiencies compared to EGD-e and 10403S *L. monocytogenes* strains. Interestingly, the same effect is not observed when the invasion of EGD is tested in mice (Bécavin et al., 2014). Further, when 39 *L. monocytogenes* genomes were compared, this mutation was observed only in EGD and M7 (i.e. a non-pathogenic serovar 4a strain isolated from cow's milk) strains. The authors suggest this mutation is likely not advantageous and may be a consequence of repeated passage through mice (Bécavin et al., 2014). *prfA* mutations resulting in attenuated virulence have also been described (Herler et al., 2001; Miner et al., 2008; Velge et al., 2007). A naturally occurring substitution of lysine with

threonine at amino acid position 220 (K220T), and a seven nucleotide insertion in the *prfA* gene that leads to a PMSC and a truncated protein (PrfAΔ174-237) have been found in low-virulence *L. monocytogenes* isolates from food products and production environments in France (Roche et al., 2005; Roche et al., 2012). Interestingly, the K220T mutation has been associated with multilocus sequence types (ST) 13, and PrfAΔ174-237 has been seen in isolates belonging to ST31 (Roche et al., 2012).

In addition to PrfA, σ^B, an alternative sigma factor that plays a role in the general stress response in *L. monocytogenes*, contributes to regulation of genes encoding internalins (e.g., *inlA* and *inlB*) (Kuhn et al., 2008; McGann et al., 2007). One of the PrfA promoters (*prfAp2*) is partially σ^B regulated (Kazmierczak et al., 2006; Nadon et al., 2002), however, its contribution to PrfA transcription has been debated. Kazmierczak et al. (2006) demonstrated that σ^B plays a limited or non-existent role in the activation of PrfA or in the transcription of PrfA-dependent genes during invasion. Instead, they suggest that *L. monocytogenes* switches from σ^B-mediated expression of stress response and selected virulence genes to PrfA-mediated expression of virulence genes required for intracellular survival and growth once it senses stressful environment inside the gastrointestinal tract (Kazmierczak et al., 2006).

In the last decade, several other virulence regulators have been identified in *L. monocytogenes*; however, the regulatory extent and exact functions for most remain elusive (Roche et al., 2008). To date, 16 two-component systems have been described in *L. monocytogenes* (Mandin et al., 2005). Most of these systems are related to stress sensing and response (e.g., CesR/CesK, involved in beta-lactam resistance; CheY/CheA, involved in chemotaxis; LisR/LisK, implicated in acid stress), with the recently described response regulator VirR playing a role in virulence. A study by Mandin et al. (2005) demonstrated a

severe virulence defect in mice and Caco-2 cell lines in the *virR* deletion mutant. Further, they found that VirR regulates 12 genes that are involved in bacterial surface components modifications. Other regulators, such as DegU, regulate the expression of flagella-specific genes and play a role in *in vivo* virulence in mice (Knudsen et al., 2004), while a serine-threonine phosphatase (Stp) regulates translation elongation factor (EF)-Tu, and controls bacterial survival inside the host (Archambaud et al., 2005).

Recent RNA studies have identified a number of small non-coding RNAs (ncRNAs) in *L. monocytogenes* that are absent from *L. innocua*, and are likely candidates in virulence gene regulation (Christiansen et al., 2006; Mandin et al., 2007; Toledo-Arana et al., 2009). These are divided into cis-acting RNAs that serve as riboswitches and typically affect either transcription or translation; cis-encoded antisense RNAs (asRNAs) that may interfere with RNA polymerase affecting mRNA translation and stability; and trans-encoded RNAs (sRNAs) that modulate translation by base-pairing with target mRNA or direct binding with proteins (Mellin and Cossart, 2012; Toledo-Arana et al., 2009). Among some of the well-studied and described *L. monocytogenes* ncRNAs are the translational attenuator and RNA thermosensor regulating the translation of PrfA (Johansson et al., 2002; Loh et al., 2006), and an asRNA regulator of the flagellar biosynthetic genes and their repressor, MogR (Toledo-Arana et al., 2009). A number of other ncRNAs, with putative regulatory roles, have recently been identified in *L. monocytogenes*; however, the exact targets and mechanisms of action for most are presently unknown (Mandin et al., 2007; Oliver et al., 2009; Toledo-Arana et al., 2009). As this type of research is still relatively new, the increased availability of next-generation sequencing technologies will likely expand and improve knowledge of non-coding regulatory RNAs in the coming years.

1.2.5 Survival mechanisms of *Listeria monocytogenes*

Listeria monocytogenes encounter a variety of stresses as they travel from natural environments such as soil and decaying vegetation to food processing environments and, ultimately, mammalian hosts. These often include fluctuations in temperature, pH and osmolarity, competition with other microorganisms and nutrient limitation (Stack et al., 2008; Wesche et al., 2009). The ability of *L. monocytogenes* to persist in various environmental niches, and to successfully colonize animal and human hosts is due to a network of sophisticated survival mechanisms. These include a number of virulence factors, approximately 331 transport, 133 surface and 86 secreted proteins, and overall 7% of its coding capacity dedicated to transcriptional regulators (Dussurget et al., 2004).

1.2.5.1 Sigma B (σ^B) and other alternative sigma factors

The crucial component and the main regulator in *L. monocytogenes* energy-related and environmental stress response network is σ^B (Oliver et al., 2010). It is one of the four alternative sigma factors found in *L. monocytogenes* (e.g., σ^B, σ^C, σ^H, σ^L) (Chaturongakul et al., 2008; Glaser et al., 2001). Sigma factors associate with a core RNA polymerase under appropriate environmental conditions. Through the recognition of specific promoter regions they help initiate and regulate the transcription of specific genes (Chaturongakul et al., 2008; Glaser et al., 2001). Earlier studies demonstrated that σ^B positively regulates at least 168 genes in *L. monocytogenes*, while 128 genes are negatively regulated (Kazmierczak et al., 2003; Raengpradub et al., 2008). More recently, Oliver et al. (2009; 2010) showed that the number of genes under σ^B regulation is strain-dependent, when four strains belonging to lineages I, II, III, and IV were characterized. As many as 400 σ^B-dependent genes were

observed to be positively regulated in at least one of the four strains tested, representing the σ^B pan-regulon, while 63 genes were identified within the σ^B core regulon (Oliver et al., 2010). Under normal growth conditions σ^B is typically maintained inactive through the association with an anti-sigma factor, RsbW (O'Byrne and Karatzas, 2008). When the cells experience stress phosphatase RsbU unphosphorylates an anti-anti-sigma factor, RsbV, which in turn reacts with the anti-sigma factor RsbW (O'Byrne and Karatzas, 2008; Shin et al., 2010). This process leads to dissociation of the σ^B-RsbW complex, thereby releasing σ^B and allowing it to induce the expression of appropriate stress response genes (Chaturongakul et al., 2008).

Less is known about the specific targets and mechanisms of action for the other three alternative sigma factors found in *L. monocytogenes*. The σ^C and σ^H-dependent genes have not been characterized; however, studies have shown that σ^C, belonging to the family of extracytoplasmic function factors, is seen exclusively in lineage II strains, and likely contributes to heat resistance (Zhang et al., 2005; Zhang et al., 2003), while the σ^H deletion mutants exhibited reduced growth in minimal medium, and alkaline conditions (Rea et al., 2004). Based on the growth of σ^C and σ^H-deletion mutants at 4°C, Chan et al. (2008) suggested these sigma factors may be involved in cold adaptation of *L. monocytogenes* 10403S strain; however, their contribution to listerial growth at 4°C in rich medium could not be demonstrated. A more diverse role has been proposed for σ^L. A study by Arous et al. (2004) described 77 σ^L-dependent genes, most of which are involved in carbohydrate and amino acid metabolism. Additional studies used σ^L deletion mutants to show this sigma factor plays a role in osmotolerance (Okada et al., 2006; Raimann et al., 2009), and growth in the presence of organic acids and cold temperatures (Raimann et al., 2009). Recently,

sequencing of the *sigL* gene in a diverse population of *L. monocytogenes* strains revealed that strains capable of fast adaptation to refrigeration possessed identical amino acid substitutions, suggesting potential structural and functional changes in the protein that may promote differences in cold growth behavior (Arguedas-Villa et al., 2014).

1.2.5.2 Acid stress response

Listeria monocytogenes produces a number of acid shock proteins that aid the bacterium in maintaining its pH homeostasis (e.g., pH 6 to 7) when exposed to acidic environments. Glutamate decarboxylase (GAD), arginine deiminase (ADI), and F_0F_1ATPase systems (Figure 1-4) play major roles in *L. monocytogenes* survival in low pH foods, the gastrointestinal environment, or upon excretion from the host cell (Stack et al., 2008).

In particular, the GAD system reduces the proton concentration within the cell and contributes to alkalization of the environment by irreversibly decarboxylating free glutamate present in the environment, and by producing γ-aminobutyrate (GABA). During this reaction, intracellular protons are consumed, GABA is transported outside and glutamate uptake occurs *via* an antiporter. As a result, the cytosol becomes less acidic and a slight increase in extracellular pH occurs, contributing to pH stability (Hill et al., 2002; Stack et al., 2008). Since glutamate is commonly present in foods, its presence greatly enhances *L. monocytogenes* survival in acidic conditions (Rundlett and Armstrong, 1994).

The expression of genes involved in the GAD system is acid-induced and to some extent under control of σ^B (Hill et al., 2002; Stack et al., 2008). Recently, a five-gene operon (e.g., *lmo0444* to *lmo0448* in EGD-e), termed the stress survival islet (SSI-1), was shown to encode for a glutamate decarboxylase homolog (*gadD1*), and an antiporter (*gadT1*) involved

in *L. monocytogenes* adaptation to mildly acidic environments (Ryan et al., 2010). Interestingly, this islet is absent from the majority of strains belonging to serotype 4b (Arguedas-Villa et al., 2014; Stack et al., 2008), suggesting its presence is not required for the survival of *L. monocytogenes* in the gastrointestinal environment.

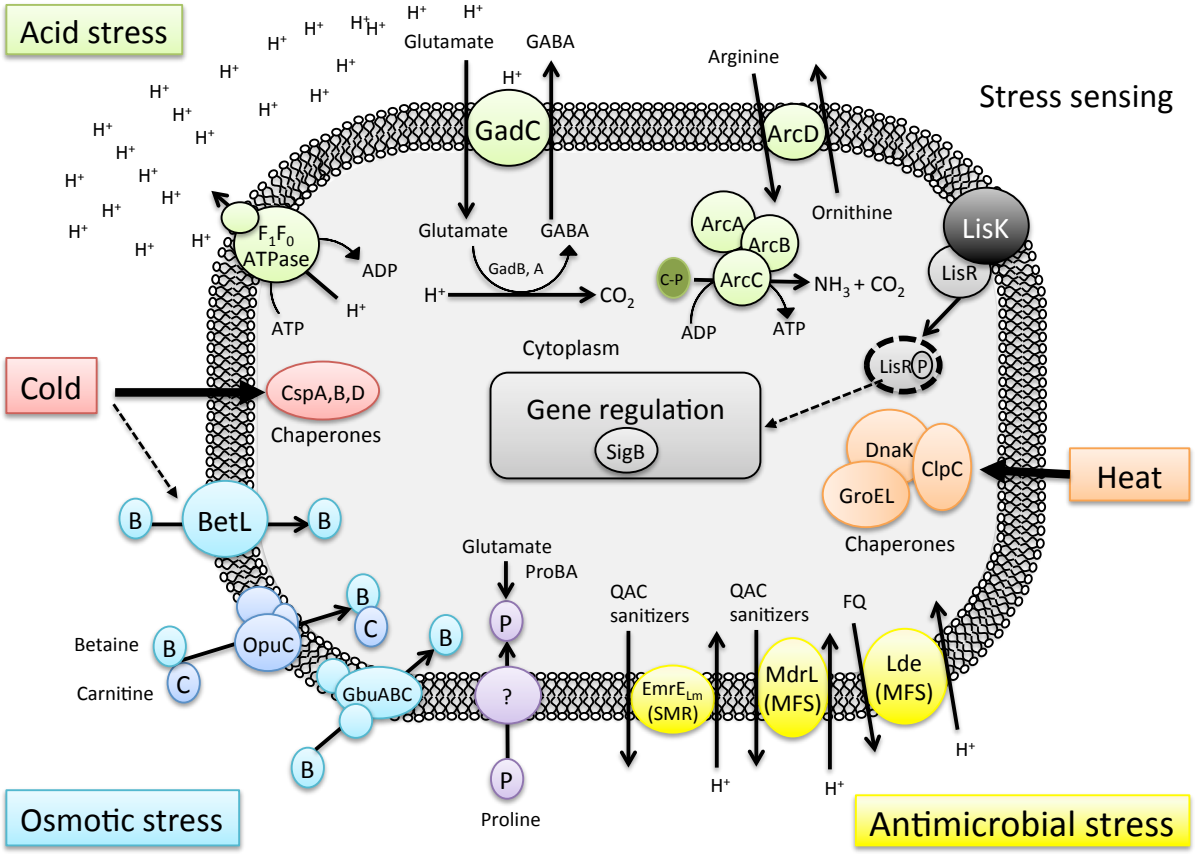


Figure 1-4. A schematic representation of simplified *Listeria monocytogenes* stress mechanisms discussed in the text and their proposed roles in stress protection/adaptation. Adapted from Hill et al. (2002). B, betaine; C, carnitine; C-P, Carbamoyl-phosphate; Ceph, cephalosporins; FQ, fluoroquinolones; GABA, gamma-aminobutyrate; LM, lincosamides; P, proline; circled P, phosphate; QAC, quaternary ammonium compounds; SMR, small multidrug resistance; MFS, major facilitator superfamily; ML, macrolides. Intermittent arrows represent putative functions.

In addition to the GAD system, *Listeria* spp. utilize the ADI system when faced with acidic environments. The ADI system is comprised of three enzymes: arginine deiminase, catabolic ornithine carbamoyltransferase, and carbamate kinase (Ryan, 2006). In this pathway, arginine is converted to ornithine, ammonia and carbon dioxide; ornithine is transported outside the cell *via* an antiporter, while ammonia reacts with intracellular protons resulting in ammonium ions (NH_4^+) and increased intracellular pH. In the ADI system, both extracellular and intracellularly synthesized arginine are used, with the latter being more important for the reactions (Stack et al., 2008). Regulation of the ADI system is rather complex and under the influence of at least four regulators: ArgR, Lmo0041, PrfA and σ^B (Ryan, 2006; Stack et al., 2008).

The third system *L. monocytogenes* employs under acidic conditions is an F_0F_1ATPase multisubunit enzyme complex (Cotter et al., 2000). It is based on adenosine triphosphate (ATP) synthesis and/or hydrolysis, and proton translocation. The F_0 portion of the enzyme is membrane bound and responsible for proton translocation across the membrane, while F_1 is the ATPase component in the cytoplasm, involved in the ATP synthesis and hydrolysis (Stack et al., 2008). Under aerobic conditions this system is typically used to synthesize ATP by utilizing proton influx into the cell, while under anaerobic conditions a proton motive force is generated and protons are excreted. It has been suggested that *L. monocytogenes* lacks a respiratory chain, but rather has an alternative method for ATP synthesis by the decarboxylation of amino acids (Cotter et al., 2000; Stack et al., 2008). In bacteria that lack a respiratory chain the role of F_0F_1ATPase system is to create a proton gradient driven by ATP hydrolysis, and expel hydrogen ions, which in turn helps establish pH homeostasis (Cotter et al., 2000; Stack et al., 2008).

1.2.5.3 Osmotic stress response

Survival of *L. monocytogenes* in food processing environments and the gastrointestinal tract requires bacteria to appropriately and quickly respond to wide osmolarity oscillations caused by desiccation and high amounts of salt and sugars present in the intestinal chyme. Bacteria typically accumulate compatible solutes, such as glycine betaine, proline, and carnitine to fight osmotic stress (Bayles and Wilkinson, 2000; Patchett et al., 1992; Sleator et al., 2003a; Sleator et al., 2003b; Stack et al., 2008). Glycine betaine is commonly found in plant tissues, fish and baked goods, while carnitine is predominantly present in mammalian tissues (Beumer et al., 1994; McNeil et al., 1999). Three important ATP-dependent transporters have been identified in *L. monocytogenes*, BetL and Gbu for transport of glycine betaine, and OpuC responsible for carnitine transport (Sleator et al., 2003a). In addition, various other proteins are up-regulated or down-regulated in order for *L. monocytogenes* to successfully combat osmotic stress, such as RelA, Ctc, HtrA, KdpE, LisRK, ProBA and BtlA (Stack et al., 2008). Regulation of compatible solutes is rather complex and is believed to occur at transcriptional, translational and post-translational levels (Sleator et al., 2003b). Putative σ^B promoter binding sites have been found upstream of *betL*, *gbu*, and *opuC*, suggesting σ^B may play a partial role in their expression. In addition, σ^A dependent promoters have been described for *betL* (Hoffmann et al., 2013) and *gbu* (Sleator et al., 2003b; Spiegelhalter and Bremer, 1998).

1.2.5.4 Temperature induced stress response

Heat shock response mechanisms in *L. monocytogenes* include a variety of molecular chaperones and ATP-dependent proteases (Figure 1-4). Their roles involve maintaining the

integrity of cellular proteins during adverse conditions (e.g., proteins GroES, GroEL, DnaK, DnaJ), and preventing the accumulation of altered or misfolded proteins through degradation (e.g., Clp, HtrA) (Liu et al., 2002; Stack et al., 2008). The expression of these genes is tightly regulated, with repressors HrcA and CtsR, and a positive regulator LisRK believed to play important roles (Nair et al., 2000; Stack et al., 2008). In addition, most of the proteins identified in the heat shock response either directly or indirectly contribute to *L. monocytogenes* virulence (Stack et al., 2008; Stack et al., 2005).

Listeria monocytogenes also elicits a strong stress response comprised of a network of adaptation mechanisms when exposed to cold temperatures. These include changes in the cell membrane [e.g., increase in $C_{15:0}$ and short chain fatty acids (Jones et al., 1997; Puttmann et al., 1993)], uptake of cryoprotective osmolytes and peptides (Bayles and Wilkinson, 2000; Hoffmann et al., 2013), and the production of cold shock (Csps) and acclimation proteins (Caps), as well as general stress response proteins (Bayles et al., 1996; Liu et al., 2002; Tasara and Stephan, 2006).

Three main Csp family proteins of low-molecular weight (e.g., CspA, CspB, and CspD) have been characterized, and found within the sequenced *L. monocytogenes* genomes (Bayles et al., 1996; Glaser et al., 2001; Nelson et al., 2004; Phan-Thanh and Gormon, 1995; Tasara and Stephan, 2006). They typically act as chaperones that bind RNA and DNA, promoting transcription and translation function during cold stress (Horn et al., 2007). Recent studies have also demonstrated the role of these proteins in osmotic stress tolerance (Schmid et al., 2009), and the regulation of LLO production (Scharer et al., 2013), suggesting they may play a role in virulence.

However, the extent of different mechanisms contributing to strain differences when it comes to cold adaptation and growth of *L. monocytogenes* are not well understood. Studies have reported differences in the lag phase duration in *L. monocytogenes* strains of human, food, and food production environment origins (Arguedas-Villa et al., 2014; Arguedas-Villa et al., 2010; Kovacevic et al., 2013a). While three cold adapting groups were observed (i.e. fast, < 70 h lag; intermediate 70 to 200 h lag; and slow, > 200 h lag), these variations could not be attributed to differences in the expression of nine cold adaptation genes (e.g., *lmo0501*, *cspA*, *cspD*, *gbuA*, *lmo0688*, *pgpH*, *sigB*, *sigH*, *sigL*) (Arguedas-Villa et al., 2014), or the presence of SSI-1 (*lmo0444-lmo0448*) previously linked to improved survival of *L. monocytogenes* in mildly acidic and osmotic environments (Ryan et al., 2010). Interestingly, fast cold adaptors exhibited five identical substitutions (e.g., Met90Leu, Ser203Ala/Ser203Thr, Ser304Asn, Ser315Asn, and Ile383Thr) in their SigL proteins, suggesting these may play a role in cold stress adaptation. Similarly, an experimentally induced single point mutation in the *betL* σ^A-dependent promoter, where one of a string of seven thymines within the spacer region between the -10 and -35 binding sites was deleted, resulted in higher transcript levels under cold stress conditions, and simultaneous increase in *L. monocytogenes* growth under refrigeration (Hoffmann et al., 2013). However, the extent of this mutation in WT *L. monocytogenes* strains and possible correlation with cold adapting groups has not been reported.

1.2.6 *Listeria monocytogenes* in the food industry

Although the widespread distribution of *L. monocytogenes* in rivers, soils, and industrial and farming effluents has been recognized as inevitable due to the ubiquitous nature and the

propensity of *Listeria* spp. to adapt to arduous conditions, their presence and persistence in food processing environments is of concern. The ability of *L. monocytogenes* to survive multiple hurdles imposed in food processing, through stress-adaptation mechanisms and formation of biofilms, has highlighted the need for improved food safety plans in food processing facilities (Møretrø and Langsrud, 2004; Wong, 1998). Since it was recognized as an important foodborne pathogen in the 1980s, many efforts have been made to eliminate *L. monocytogenes* from foods and food processing environments; however, three decades later *Listeria* are very much present in the food supply worldwide (Bērziņš et al., 2009; Bohaychuk et al., 2006; Garrido et al., 2009; Inoue et al., 2000; Sauders et al., 2009).

1.2.6.1 Incidence in foods and food processing environments

The prevalence of *Listeria* spp., and particularly *L. monocytogenes*, in foods has been studied extensively, with evidence of listerial growth in almost all food categories (Farber et al., 2007; Ryser, 2007a; Ryser, 2007b; Ryser, 2007c; Uyttendaele et al., 2009). Different levels of contamination have been seen across dairy, meat, fish and produce facilities and their products in different geographical regions; however, in most cases strains or serotypes of *L. monocytogenes* are not facility category- or food-specific (Kovačević et al., 2012a; Kovačević et al., 2012b; Liu, 2008). Moreover, listerial contamination is frequently linked but not limited to the level of hygiene practiced in a facility, including inadequacies in processing, cleaning and sanitation (Kabuki et al., 2004; Møretrø and Langsrud, 2004; Wong, 1998). Failures in food processing and/or sanitary handling, and inadequate cleaning and sanitation may lead to bacterial build-up and continuous contamination of processing machines and food products with *L. monocytogenes* (Holah et al., 2004; Lin et al., 2006;

Lundén et al., 2002). In fact, a number of large listeriosis outbreaks have occurred in the last decade as a result of *L. monocytogenes* contaminating food products (Table 1-1) (CDC, 2011; Gaulin et al., 2012; Health Canada, 2011; Weatherill, 2009).

There have been eight listeriosis outbreaks reported in Canada, linked to a variety of RTE foods (Table 1-1) (Clark et al., 2010; Health Canada, 2011). The most notable, however, was the 2008 nationwide outbreak associated with contaminated deli meats that originated from a single food processing facility and resulted in 57 invasive listeriosis cases and 23 deaths (Weatherill, 2009). The originating source of contamination was suspected to be a large commercial slicer (Weatherill, 2009). Environmental sampling records showed the intermittent presence of *L. monocytogenes* on two processing lines within the facility for almost a year prior to the outbreak. Similar scenarios have been reported in other listeriosis outbreaks where *L. monocytogenes* in the processing environment led to contamination of RTE products (CDC, 2002; Mead et al., 2006; Olsen et al., 2005). It is well established that food product contamination is associated with food processing environments harboring *L. monocytogenes* and subsequent post- processing transfer to finished products (Lappi et al., 2004; Lundén et al., 2002; Olsen et al., 2005; Tompkin, 2002). Strains of *L. monocytogenes* capable of persisting in food-processing environments for up to 12 years and intermittently contaminating products have been reported (Holah et al., 2004; Lundén et al., 2002; Olsen et al., 2005; Senczek et al., 2000).

1.2.6.2 Control and monitoring

In effort to minimize the survival of *L. monocytogenes* in foods, food additives, such as *Carnobacterium maltaromaticum* CB1, potassium lactate, sodium acetate, sodium diacetate

Table 1-1. Major foodborne listeriosis outbreaks reported worldwide. Adapted from Health Canada's policy on *Listeria monocytogenes* in ready-to-eat foods (Health Canada, 2011) and government reports on listeriosis outbreaks worldwide (CDC, 2011; CDC, 2012; CDC, 2013; CDC, 2014; Fretz et al., 2010; NSW Ministry of Health, 2013).

Food type / Year	Location	Invasive/ Non-invasive	No. cases (deaths)	Foods
Meat and poultry				
1987-89	UK and Ireland	Invasive	355 (94)	Pâté
1990	Australia	Invasive	11(6)	Pâté
1992	France	Invasive	279 (85)	Jellied pork tongue
1993	France	Invasive	39 (12)	Pork rillettes (pâté-like RTE meat)
1998-99	U.S.A.	Invasive	108 (14)	Meat frankfurters
1999	U.S.A.	Invasive	11	Pâté
1999-2000	France	Invasive	10 (3)	Rillettes (pâté-like RTE meat)
1999-2000	France	Invasive	32 (10)	Jellied pork tongue
2000	U.S.A.	Invasive	30 (7)	Deli turkey meat
2000	Australia	Non-invasive	31	RTE corned beef and ham
2001	U.S.A.	Non-invasive	16	Precooked sliced turkey
2002	U.S.A.	Invasive	54 (8)	Sliceable turkey deli meats
2008	Canada	Invasive	57 (23)	RTE deli meats
Dairy products				
1983	U.S.A.	Invasive	49 (14)	Pasteurized milk
1983-87	Switzerland	Invasive	122 (31)	Soft cheese
1985	U.S.A.	Invasive	142 (48)	Mexican-style fresh cheese
1989-90	Denmark	Invasive	26 (6)	Blue mould cheese or hard cheese
1994	U.S.A.	Invasive	45	Chocolate milk
1995	France	Invasive	37 (11)	Raw milk soft cheese
1997	France	Invasive	14	Soft cheeses
1998-99	Finland	Invasive	25 (6)	Butter made from pasteurized milk
2000	Canada (Manitoba)	Invasive	7	Flat whipping cream
2000-01	U.S.A.	Invasive	13	Mexican-style fresh cheese
2001	Sweden	Non-invasive	> 120	Fresh cheese made from raw milk in a summer farm
2001	Japan	Non-invasive	38	Washed-type cheese
2001	Belgium	Invasive	2	Frozen ice cream cake
2002	Canada (BC)	Invasive	47	Cheese
2002	Canada (Quebec)	Invasive	17	Soft and semi-hard raw milk cheese
2002	Canada (British Columbia)	Non-invasive	86	Cheese made from pasteurized milk
2003	U.S.A.	Invasive	13 (2)	Mexican-style fresh cheese

Table 1-1. Continued.

Food type / Year	Location	Invasive/ Non-invasive	No. cases (deaths)	Foods
Dairy products				
2005	Switzerland	Invasive	10 (3)	Soft cheese
2007	U.S.A.	Invasive	5 (3)	Pasteurized milk
2008	Canada (Quebec)	Invasive	38 (2)	Cheeses
2009-10	Australia, Germany, Czech Republic	Invasive	34 (8)	Acid curd cheese "Quargel"
2012	U.S.A.	Invasive	22 (2)	Ricotta salata cheese imported from Italy
2013	U.S.A.	Invasive	6 (2)	Farmstead Cheeses
2013	Australia	Invasive	18 (3)	Variety of soft cheeses
2014	U.S.A.	Invasive	8 (1)	Variety of cheese products
Fish and seafood products				
1989	U.S.A.	Non-invasive	9 (1)	Shrimp
1991	Australia (Tasmania)	Non-invasive	4	New Zealand produced smoked mussels
1992	New Zealand	Invasive	4 (2)	Smoked mussels
1994-95	Sweden	Invasive	6 (1)	"Gravad" rainbow trout and cold- smoked rainbow trout
1996	Canada	Invasive	2	Imitation crab meat
Unknown	Finland	Non-invasive	5	Cold-smoked rainbow trout
Fruits and vegetables				
1981	Canada	Invasive	41 (17)	Coleslaw mix
1997	Italy	Non-invasive	1566	Corn and tuna salad
1998-99	Australia	Invasive	6 (5)	Commercially prepared fruit salad
2011-12	U.S.A.	Invasive	147 (34)	Cantaloupe
Other foods				
1993	Italy	Non-invasive	23	Rice salad
2003	UK	Invasive	5	Pre-packed sandwiches
2009	Australia	Unknown	8	Chicken wrap

and sodium lactate, have been approved as growth repressors or inhibitors in Canada (Health Canada, 2011). Health Canada (2011) also encourages processors to use post-processing treatments, such as surface pasteurization of products with hot water, steam, infrared processes and radiant oven heating, or high-pressure processing, in conjunction with antimicrobial treatments. More recently, research on naturally occurring bacteriocins and bacteriophages has shown promise in the control *L. monocytogenes* in foods and processing environments, respectively (Carlton et al., 2005; Guenther et al., 2009; Ming et al., 1997; Muriana, 1996). However, even with these applications, it has been acknowledged that the complete elimination of *L. monocytogenes* from the food chain is unrealistic. Instead, efforts should be placed on minimizing the contamination of food products and implementing effective controls in the food processing environments (FAO and WHO, 2004; Health Canada, 2011).

The use of environmental sampling to detect the contamination and spread of *Listeria* spp. in food processing has been recognized as a valuable tool (Health Canada, 2011). However, in Canada, these practices are required only in federally licensed facilities that export foods out of the province of origin, or country. In a large number of food processing facilities that are licensed by provincial authorities and do not export foods out of province or country, environmental sampling or food testing for *Listeria* spp. is not required or practiced. As such, there is a concern that consumers are potentially exposed to pathogenic *L. monocytogenes*, leading to unfavorable health outcomes.

1.2.7 Susceptibility of *Listeria monocytogenes* to antimicrobials

1.2.7.1 Antibiotic resistance

If diagnosed promptly, listeriosis can be treated successfully with antibiotics. Ampicillin, in combination with aminoglycosides (e.g., gentamicin) is the preferred line of treatment, while, cotrimoxazole (SXT; sulfamethoxazole with trimethoprim) is often administered to patients with allergies to β-lactams (Hof et al., 1997).

Although resistance to antimicrobials used in listeriosis treatment (i.e. aminopenicillins, aminoglycosides, and SXT) remains rare (Granier et al., 2011; Lyon et al., 2008; Morvan et al., 2010), an increasing trend in resistance or reduced susceptibility to a number of clinically relevant drugs historically effective against *Listeria* spp. has been reported worldwide (Aureli et al., 2003; Charpentier and Courvalin, 1999; Kovacevic et al., 2013b; Lungu et al., 2011; Morvan et al., 2010; Safdar and Armstrong, 2003; Troxler et al., 2000). In particular, there have been a number of reports describing reduced susceptibility and resistance to ciprofloxacin, clindamycin, and tetracycline in clinical, food and food processing environment (FPE) isolates in North America (Chen et al., 2010; Kovacevic et al., 2013b; Lyon et al., 2008) and Europe (Aureli et al., 2003; Morvan et al., 2010). Within these reports, *L. monocytogenes* has been shown to harbor genetic elements conferring resistance/reduced susceptibility to ciprofloxacin (*lde*), chloramphenicol (*cat*), erythromycin *(erm*), streptomycin (*aad6*), and trimethoprim (*dfrD*) (Chen et al., 2010; Granier et al., 2011; Morvan et al., 2010).

Examination of clinical *L. monocytogenes* isolates collected in the US from 1955 to 1997 found no resistance to the fluoroquninolone ciprofloxacin and high susceptibility (96.9%) to tetracycline (Safdar and Armstrong, 2003). Similarly, 202 *L. monocytogenes*

isolates recovered from food sources in France from 1996 to 2006 did not exhibit any fluoroquinolone resistance and only two isolates were resistant to tetracycline (Granier et al., 2011); however, when a large collection of clinical strains (n=4,668) collected from 1989 to 2007 in France were examined, 20 (0.4%) and 34 (0.7%) isolates were resistant to ciprofloxacin and tetracycline, respectively, indicating a slow emergence of these phenotypes that had previously been absent or rare in *L. monocytogenes* (Morvan et al., 2010). Similar concerns have been raised in a recent Canadian study where 7% of food-derived *L. monocytogenes* were resistant and 67% possessed reduced susceptibility to ciprofloxacin (Kovacevic et al., 2013b). The same study showed that 6% of *L. monocytogenes* strains from fish possessing plants possessed resistance to tetracycline, while 8% of isolates were resistant in a study by Zhang et al. (2007). Fluoroquinolone resistance has been attributed to active efflux due to over-expression of *lde* in *L. monocytogenes* (Godreuil et al., 2003; Jiang et al., 2012; Lismond et al., 2008). This is in contrast to ciprofloxacin resistance observed in *E. coli* (Vila et al., 1996) and *Streptococcus pneumoniae* (Piddock et al., 2002), where mutations in the quinolone resistance-determining regions (QRDR) of DNA gyrase and topoisomerase IV confer resistance. In fact, QRDR-based mutations are absent in resistant isolates in *L. monocytogenes* or do not significantly impact tolerance to ciprofloxacin (Jiang et al., 2012; Lampidis et al., 2002).

Clindamycin resistance seems to vary by geographical region and origin. Studies by Safdar and Armstrong (2003) and Chen et al. (2010) reported 96% of clinical and 69% of food-derived *L. monocytogenes* isolates from the US were resistant to clindamycin, respectively. In a separate US study by Davis and Jackson (2009), resistance to clindamycin was seen in 21% of *L. monocytogenes* isolates from animals, food, FPE, and humans, while

33% and 65% of food-derived Canadian isolates were resistant or possessed reduced susceptibility, respectively (Kovacevic et al., 2013b). Interestingly, no resistance to clindamycin was observed in clinical isolates from France (Morvan et al., 2010), or from French food and environmental isolates collected between 1996 and 2006 (Granier et al., 2011). The mechanism(s) behind *L. monocytogenes* resistance to clindamycin is not fully understood, but modifications of 23S ribosomal RNA or the presence of an enzyme capable of modifying clindamycin are believed to contribute to this phenomenon (Chen et al., 2010; Depardieu et al., 2007). Similar mechanisms may be associated with reduced efficacy of chloramphenicol and erythromycin, which raises concerns about the potential for cross-resistance to differing drug classes through a common resistance mechanism and selective pressure (Depardieu et al., 2007).

1.2.7.2 Resistance to sanitizers

A number of different hypotheses have been proposed to explain the ability of *L. monocytogenes* to persist for prolonged periods of time on various surfaces in food processing facilities (Giovannacci et al., 2000; Lundén et al., 2002). Some of these pertain to the conditions practiced and controlled at the facility level. For instance, inadequate cleaning and sanitation practices can promote bacterial persistence through the formation of biofilms (Barbalho et al., 2005; Jeyasekaran et al., 2000). Moreover, particular food environments and surfaces (e.g., rubber and plastics) are more prone to harboring pathogenic microorganisms (Lundén et al., 2002; Wilks et al., 2006; Wong, 1998). Additional hypotheses have been put forward that suggest strain-associated differences in stress adaptation or the capacity to form biofilms influence persistence in the food processing environment (Chae and Schraft, 2000;

Lundén et al., 2003). Studies have also suggested that strains exhibit a range of tolerance and resistance to sanitizers commonly used in food processing environments (Jeyasekaran et al., 2000; Lemaître et al., 1998).

Although there have been no reports of *L. monocytogenes* resistance to concentrations of sanitizer equal to or higher than those commonly recommended by manufacturers when bacteria are grown in a liquid suspension, inter-strain variation in the tolerance of sub-lethal concentrations of different sanitizers has been observed (Aase et al., 2000; Earnshaw and Lawrence, 1998; Lundén et al., 2003). This enhanced tolerance has been attributed to cell membrane modifications that reduce permeability, acquisition of genes conferring resistance, as well as the function of efflux pumps (Casey et al., 2014; Dutta et al., 2013; Elhanafi et al., 2010; Romanova et al., 2006; To et al., 2002).

Casey et al. (2014) reported that approximately 600 genes were up-regulated when a persistent *L. monocytogenes* serotype 1/2a strain recovered from a cheese processing environment was exposed to sub-lethal concentration (4 ppm) of benzethonium chloride. Sanitizers such as benzethonium chloride and BAC are cationic agents, belonging to the QAC family. They are frequently used as sanitizers in food processing facilities due to their non-corrosive properties (Holah et al., 2002). Studies have shown that in Gram positive bacteria such as *Staphylococcus aureus* these compounds display ionic and hydrophobic interactions with bacterial cytoplasmic membranes, with the cationic head group positioned outwards and the hydrophobic tails tucked into the lipid bilayer (Casey et al., 2014; Ioannou et al., 2007). This interaction results in the rearrangement and solubilization of hydrophobic cell membrane components (e.g., phospholipids and lipoteichoic acids) leading to cell leakage (Gilbert and Moore, 2005). To battle this stress *L. monocytogenes* up-regulate genes

associated with stress sensing (e.g., *cheA*/*cheY*), chemotaxis (e.g., methyl-accepting chemotaxis protein, MCP), and motility (e.g., *flaA*, *fliG*, *fliM*, and *fliN*) to favor a quick and effective response (Casey et al., 2014). Fatty acid and peptidoglycan biosynthesis and metabolism are simultaneously increased to maintain proper membrane fluidity and reinforce structural integrity and stability of the cell wall, respectively (Casey et al., 2014). A shift in the synthesis of longer saturated fatty acids upon exposure to sub-lethal concentrations of BAC has been observed to decrease membrane fluidity (To et al., 2002). Some of the genes up-regulated during this time include the *acpP* gene, which is responsible for the expression of the acyl carrier protein; *fabHA, fabF*, and *fabR,* which are implicated in the initiation and catalyzation of fatty acid elongation and regulation of membrane phospholipid biosynthesis, respectively; the *mur* family of peptidoglycan synthesis genes; and *mnaA* involved in the synthesis of teichoic acids and polysaccharides (Casey et al., 2014). Interestingly, genes involved in cold stress response, such as *cspC* and *cspD*, are also up-regulated during QAC exposure, along with many other general stress response genes (e.g., *rsbRA*, *ftsHi*), and putative stress proteins (e.g., YloU, involved in alkaline-shock) (Casey et al., 2014). This suggests that many of the stress proteins in *L. monocytogenes* offer cross-protection during adaptation and survival under harsh environmental conditions.

While these physiological changes occur to some extent in all *L. monocytogenes,* additional sanitizer-induced stress mechanisms have been noted and may contribute to variation in sanitizer tolerance. For instance, the presence of multi-drug transporters and efflux pumps has been described in isolates of different origins (Casey et al., 2014; Dutta et al., 2013). Romanova et al. (2006) demonstrated increased expression of the *mdrL* gene responsible for the production of the MdrL chromosomal efflux-pump system when

previously sensitive *L. monocytogenes* isolates were experimentally adapted to BAC. However, this pump did not seem to have a significant effect on the resistance of strains naturally resistant to BAC (Romanova et al, 2006). Recently, several other mechanisms have been described in WT strains possessing high levels of resistance to QACs. Elhanafi et al. (2010) characterized a plasmid-based (pLM80) BAC resistance gene cassette (*bcrABC*) in a strain that caused a multistate hot-dog outbreak in 1998-99. This plasmid was found to possess three transposable units, with one carrying genes that confer resistance to cadmium and BAC and others carrying either cadmium or BAC resistance (Elhanafi et al., 2010). Located on the cassette are a putative transcriptional regulator of the TetR family (*bcrA*), followed by two putative small multidrug resistance (SMR) genes, *bcrB* and *bcrC*. A subsequent study that examined the distribution of the *bcrABC* cassette revealed that all but one of 71 BAC resistant *L. monocytogenes* strains, originating from clinical, food and food processing environments, possessed the cassette (Dutta et al., 2013). Interestingly, it was carried by a plasmid in some isolates, while in others it was chromosomally integrated (Dutta et al., 2013).

More recently, Müller et al. (2013) discovered a transposon (Tn*6188*)-based BAC resistance determinant in *L. monocytogenes* strains, predominantly of the 1/2a serotype recovered from foods and food processing environments in the US. It is chromosomally incorporated within the *radC* gene, and is comprised of three transposase genes (*tnpABC*), a putative transcriptional regulator of the TetR family, and a protein similar to SMR proteins described in *E. coli* (e.g., EmrE) and *Staphylococcus* spp. (e.g., QacC/Smr and QacJ in *S. aureus*, and QacH in *S. saprophyticus*). This SMR efflux pump has been termed QacH in *L. monocytogenes* (Müller et al., 2013).

SMR efflux-pump systems have been shown to confer resistance to select antibiotics, such as aminoglycosides in *Pseudomonas aeruginosa* (Li et al., 2003), tetracycline, erythromycin, and sulfadiazine resistance in *E. coli* (Yerushalmi et al., 1995), and *Mycobacterium smegmatis* resistance to quinolones (Li et al., 2004). Resistance due to the activity of SMR pumps has not yet been described in *L. monocytogenes*. However, other transposons described in some *L. monocytogenes* isolates confer tolerance to cadmium (e.g., Tn*5422*), antibiotics (e.g., Tn*6198*), and arsenic (e.g., Tn*554*-like) (Müller et al., 2013). Similarly, the MFS efflux pump Lde has been implicated in the resistance of *L. monocytogenes* to quinolones (e.g., Lde) and dyes (Godreuil et al., 2003). Recently, two additional MFS efflux pumps, MdrM and MdrT, have been described in *L. monocytogenes*, though these pumps are strongly induced by host bile cholic acid, and are believed to transport endogenous rather than exogenous materials (Quillin et al., 2011).

While there has been no definitive evidence to suggest that proper use of sanitizers in food processing will lead to development of resistant microorganisms, the increased presence of multiple potential resistance mechanisms in *L. monocytogenes* is alarming. These mechanisms could confer competitive advantages to some *L. monocytogenes* strains in food processing environments, particularly when they are embedded in equipment niches sheltered from cleaners and sanitizers.

1.2.7.3 Antimicrobial resistance and co-selection

While increased tolerance to some sanitizers has been observed in different *L. monocytogenes* strains, the mechanisms underlying sanitizer resistance are not well understood. Moreover, little is presently known about co-selection phenomenon between

commonly used sanitizers and antibiotics. Different mechanisms of BAC resistance have been proposed to explain differences between inherently resistant *L. monocytogenes* strains and experimentally adapted ones (To et al., 2002). The increase in activity of an efflux pump, encoded by *mdrL*, confers resistance to BAC when sensitive strains of *L. monocytogenes* are experimentally adapted to high concentrations of BAC (Romanova et al., 2006). However, the efflux pump MdrL has a minor role in strains inherently resistant to BAC, and other mechanisms such as changes in cell surface antigens and fatty acid profiles (To et al. 2002), and the presence of plasmid- (e.g., *bcrABC*) and transposon-borne (e.g., *qacH*) elements have been suggested to contribute to this phenomenon. The extent to which these changes contribute to antibiotic resistance has not been determined.

Additionally, when *L. monocytogenes* were repeatedly cultured in the presence of triclosan, an antibacterial agent widely used in household cleaning products, up to 16-fold increase in tolerance to gentamicin was observed (Christensen et al., 2011). Similarly, Rakic-Martinez et al. (2011) demonstrated that adaptation of clinical *L. monocytogenes* strains to ciprofloxacin (2 μg/ml) or BAC (10 μg/ml) leads to a subsequent increase in gentamicin resistance. In a separate study, an increase in gentamicin MIC was observed in only two of eight food chain *L. monocytogenes* isolates experimentally adapted to ciprofloxacin (30-240 μg/ml) (Kovacevic et al., 2013b). This contrasts with another report where the antimicrobial resistance of food chain isolates adapted to BAC (6-7 μg/ml) did not increase, suggesting the phenomenon may be associated with genetic traits not common in all *L. monocytogenes* (Aase et al., 2000).

Considering the widespread distribution of *L. monocytogenes* in nature and its recurring encounter with sanitizers in different food processing environments, a better

understanding of the co-selection phenomenon and pressures contributing to antimicrobial resistance in *L. monocytogenes* is needed.

1.3 Research objectives and hypotheses

The overall objective of this research was to improve the understanding of physiological and genotypic properties of *Listeria* spp., and in particular *L. monocytogenes*, that are encountered in B.C.'s food chain to better understand the risks posed to consumers with potential exposure to *Listeria*. The characteristics associated with virulence, enhanced stress resistance and survival along the food chain were investigated in a large proportion of *Listeria* isolates originating from food processing and retail facilities in B.C. to achieve this objective.

The research described in this thesis was carried out in two parts. The first part focused on investigations of the occurrence and distribution of *Listeria* spp. in food processing and retail facilities, followed by characterization of the genetic properties associated with population structure and virulence. Phenotypic characteristics pertaining to AMR, adaptive mutability, and adaptation to refrigeration were examined. The results of relevant experiments are described in Chapters 2, 3, and 4. The second part of the thesis research focused on elucidating the role of LGI1 in *L. monocytogenes* virulence and tolerance to food chain-relevant stresses (e.g., low pH, high salt, low temperature, and sanitizers). Hypotheses and sub-hypotheses were generated based on the putative functions of three selected genes. The results of these experiments are described in Chapter 5.

Thesis hypotheses and sub-hypotheses investigated included:

Chapter 2

1. Pathogenic *L. monocytogenes* are present in dairy, meat and fish processing environments, and ready-to-eat foods distributed in B.C. retail trade.

Chapter 3

1. Strains belonging to clonal complexes associated with clinical listeriosis cases, and virulent *L. monocytogenes* strains possessing wild type *inlA* genes are present in B.C. food chain.
    1.1. Serotype 1/2a is more prone to point mutations than other serotypes.
    1.2. Differences exist in the ability of food chain-derived *L. monocytogenes* to adapt to cold temperatures.

Chapter 4

1. *Listeria* spp. recovered from the B.C. food chain possess resistance to clinically relevant antimicrobials.
    1.1. *L. monocytogenes* adaptation to high concentrations of ciprofloxacin leads to resistance to benzalkonium chloride.

Chapter 5

1. *emrE* contributes to enhanced tolerance of *L. monocytogenes* to
    1.1. Antibiotics,
    1.2. Quaternary ammonium sanitizers and cationic dye acriflavine.
2. Regulator gene *lmo1851* contributes to enhanced survival of *L. monocytogenes* under
    2.1. Refrigeration,
    2.2. Acidic pH, and
    2.3. High salt environments.

3. *sel1* contributes to *L. monocytogenes* increased adhesion and invasiveness of Caco-2 and HeLa cells.

Findings from this research will provide a better understanding of the characteristics of *Listeria* isolates consumers may encounter through contaminated foods in B.C. In particular, the data obtained will improve our understanding of virulence and stress response factors, and the contribution of presently uncharacterized genomic island LGI1 to the virulence and stress tolerance of *L. monocytogenes*. Such knowledge is needed to develop more effective *Listeria* mitigation strategies, prevent virulent *L. monocytogenes* from contaminating foods, and to aid the development of future detection methods focusing on virulence profiling rather than species detection.

Chapter 2: Occurrence and distribution of *Listeria* spp. in food processing facilities producing ready-to-eat foods, and retail establishments in British Columbia (B.C.), Canada

2.1 Introduction

Listeria monocytogenes is commonly associated with food processing environments and ready to eat (RTE) foods. Although it is an infrequent cause of foodborne disease, *L. monocytogenes* has been linked to disproportionately high levels of morbidity and mortality (Clark et al., 2010; Weatherill, 2009). Its presence in RTE products is particularly troublesome for pregnant women and their fetuses, young children and the elderly. Mortality rates associated with vulnerable populations can range between 20 and 40% (Clark et al., 2010). Evidence suggests the risk of invasive listeriosis may be even higher if virulent strains of *L. monocytogenes* in RTE foods are encountered (Chen et al., 2006; Gilmour et al., 2010).

In Canada, eight listeriosis outbreaks linked to a variety of RTE foods have been reported over the years (Clark et al., 2010; Health Canada, 2011). The most notable, however, was the 2008 nationwide outbreak associated with contaminated deli meats that originated from a single food processing facility, and resulted in 57 invasive listeriosis cases and 23 deaths (Weatherill, 2009). The originating source of contamination was suspected to be a large commercial slicer harboring *L. monocytogenes* (Weatherill, 2009). The facility's environmental sampling records showed the intermittent presence of *L. monocytogenes* on two processing lines for almost a year prior to the outbreak. Similar scenarios have been reported in other listeriosis outbreaks where *L. monocytogenes* in the processing environment

led to contamination of RTE products (CDC, 2002; Mead et al., 2006; Olsen et al., 2005). It is well established that food product contamination is associated with food processing environments that harbor *L. monocytogenes* and thus are the source of subsequent post-processing transfer to finished products (Lappi et al., 2004; Lundén et al., 2002; Olsen et al., 2005; Tompkin, 2002). Numerous studies have focused on the prevalence of *Listeria* spp. in production environments and contamination patterns in these facilities (Barros et al., 2007; Chasseignaux et al., 2002; Chasseignaux et al., 2001; Eklund et al., 1995; Norton et al., 2001). Strains of *L. monocytogenes* capable of persisting in food processing environments for up to 12 years and intermittently contaminating products have been reported (Holah et al., 2004; Lundén et al., 2002; Olsen et al., 2005; Senczek et al., 2000). Retail facilities of RTE foods, however, have received less attention and consequently there are fewer data available that examine the prevalence of *L. monocytogenes* at retail.

Canadian data on the presence of *L. monocytogenes* in RTE foods have varied across studies (Bohaychuk et al., 2006; Dillon et al., 1994; Farber, 1991; Farber, 2000). As a result, contradicting messages have been conveyed regarding the safety of Canadian RTE foods. In 1991, Farber (1991) reported results of a limited sampling survey of wholesale and retail seafood products originating from Canada and other countries. Based on the low recovery of *L. monocytogenes* in shrimp and smoked salmon, they concluded the observed levels did not represent a serious health hazard. In 1994, however, a study examining *Listeria* spp. contamination of retail RTE fish in Newfoundland found 18.3% (11/60) of cod samples were contaminated with *L. monocytogenes* (Dillon et al., 1994). In contrast, a report on government seafood testing in 2000 revealed *L. monocytogenes* contamination in 0.3-0.88% of imported products and its absence in domestic products (Farber, 2000). Similarly, a low

prevalence of *L. monocytogenes* in raw and RTE meats from retail outlets in Alberta was reported (Bohaychuk et al., 2006).

Looking at other Canadian provinces, and particularly at B.C., there exist limited data on the occurrence of *Listeria* spp. in RTE products and the associated risks of listeriosis linked to consumption of contaminated RTE foods. In B.C., there are no provincial regulations or guidelines, which refer specifically to *Listeria* spp. and/or *L. monocytogenes* in the environment or in products from provincially licensed but not federally registered dairy, fish and meat processing facilities that produce RTE foods, or retail facilities. While food processing facilities are routinely inspected, environmental and food product testing for *Listeria* spp. or *L. monocytogenes* is not considered a requirement.

In dairy processing facilities regulated by the B.C. Milk Industry Act (Anonymous, 1981), microbial testing of RTE dairy products for indicator microorganisms and/or pathogens (including *L. monocytogenes*) is required on at least six occasions during each six month period. In contrast, in fish processing facilities regulated by the B.C. Fish Inspection Act (Chapter 148) (Anonymous, 1996) there is no specific requirement for foods or environmental samples to be tested, although an inspector may collect samples during investigations or inspections. In meat processing facilities regulated by the Meat Inspection Regulation of the B.C. Food Safety Act (Anonymous, 2004) (slaughterhouses that produce RTE foods under provincial inspection authority) and meat facilities regulated by the Food Premises Regulation of the B.C. Public Health Act (Anonymous, 1999) (e.g., deli, butcher and other processors that produce RTE meat under provincial inspection authority), there are currently no specific regulations or guidelines for control of *Listeria* spp. or

L. monocytogenes; however, according to the B.C. Food Safety Act (Chapter 28) (Anonymous, 2010), inspectors may collect and examine any samples they deem appropriate.

Regardless of the size, retail establishments that sell foods within B.C. are not required to test products or food handling areas for *Listeria* spp. However, these establishments are inspected at least once per year and their foods tested as part of the provincial Food Quality Check Sampling Program (BCCDC, 2010). This program is primarily educational, with bacteriological tests (e.g., indicator organisms) used as sanitation checks to inform inspectors and producers of the effectiveness of current food handling practices. These procedures, however, do not include testing for *Listeria* spp. or other foodborne pathogens. Foods produced within B.C. for retail outside of the province are inspected by the federally regulating Canadian Food Inspection Agency (CFIA), and are subject to more intensive microbiological monitoring programs, including testing for *Listeria* spp. (CFIA, 2011).

Generally, food testing for *Listeria* spp. that occurs at the food processing level provides little information about the microbial quality and safety of food at retail. This is a concern because *L. monocytogenes* populations can increase during shipping and prolonged storage, particularly if RTE foods are stored at temperatures above 4°C (Farber, 2000; Glass and Doyle, 1989). Additional handling of RTE foods at the retail level, with such activities as slicing, weighing, and packaging, may increase the potential for cross-contamination (Lin et al., 2006). The current Canadian policy on *Listeria* allows 100 CFU/g of *L. monocytogenes* in RTE foods, in which proliferation of the organism to levels above this before the end of the product shelf-life is not possible (Health Canada, 2011). However, extensive microbial challenge of retail products is required to determine listerial growth potential. Failure to

control the proliferation of *L. monocytogenes* using extrinsic and intrinsic mitigation strategies may result in situations where unacceptable levels of the organism exist in products, particularly towards the end of the shelf-life period.

Currently, there is a lack of data on the occurrence of *Listeria* spp. and *L. monocytogenes* in RTE foods at food processing and retail levels in B.C. Furthermore, there are inconsistent reports on consumer health risks associated with *L. monocytogenes* contamination of RTE foods in Canada. Considering that several large listeriosis outbreaks have occurred in Canada in the past, two of them linked to B.C. cheese manufacturers, investigating the presence of *L. monocytogenes* in the B.C. food chain seems prudent. Therefore, the objectives of the experiments performed in Chapter 2 were three-fold and included: (1) examination of the occurrence and distribution of *Listeria* spp. and *L. monocytogenes* in RTE foods and food processing environments of food processing facilities under provincial inspection authority in B.C.; (2) testing of RTE products from retail facilities for the presence of *Listeria* spp., and *L. monocytogenes*; and (3) investigation of the relatedness of recovered *L. monocytogenes* isolates using serotyping and genetic fingerprinting methods.

2.2 Materials and methods

2.2.1 Sample collection from food processing facilities (PF)

The selection of PF was guided by three principles: the inclusion of representative facilities and RTE products from three major producer classes, namely dairy, fish and meat; coverage of the geographical territories of B.C.'s Regional Health Authorities; and practicability within a three month sampling period. Overall, 17 dairy, 13 fish, and 23 meat (5

slaughterhouses that also produce RTE products, and 18 smaller retail and butcher deli shops) facilities were visited between August and October 2009 (Figure 2-1)[1]. Overall 262 RTE food samples, and 305 food processing environment swabs (referred to as environmental samples) were collected.

Environmental swabs included 101 samples from non-food contact (NFC) surfaces (Table 2-1), 101 from close-to-food contact (CFC) surfaces, and 103 were obtained from food contact (FC) surfaces.

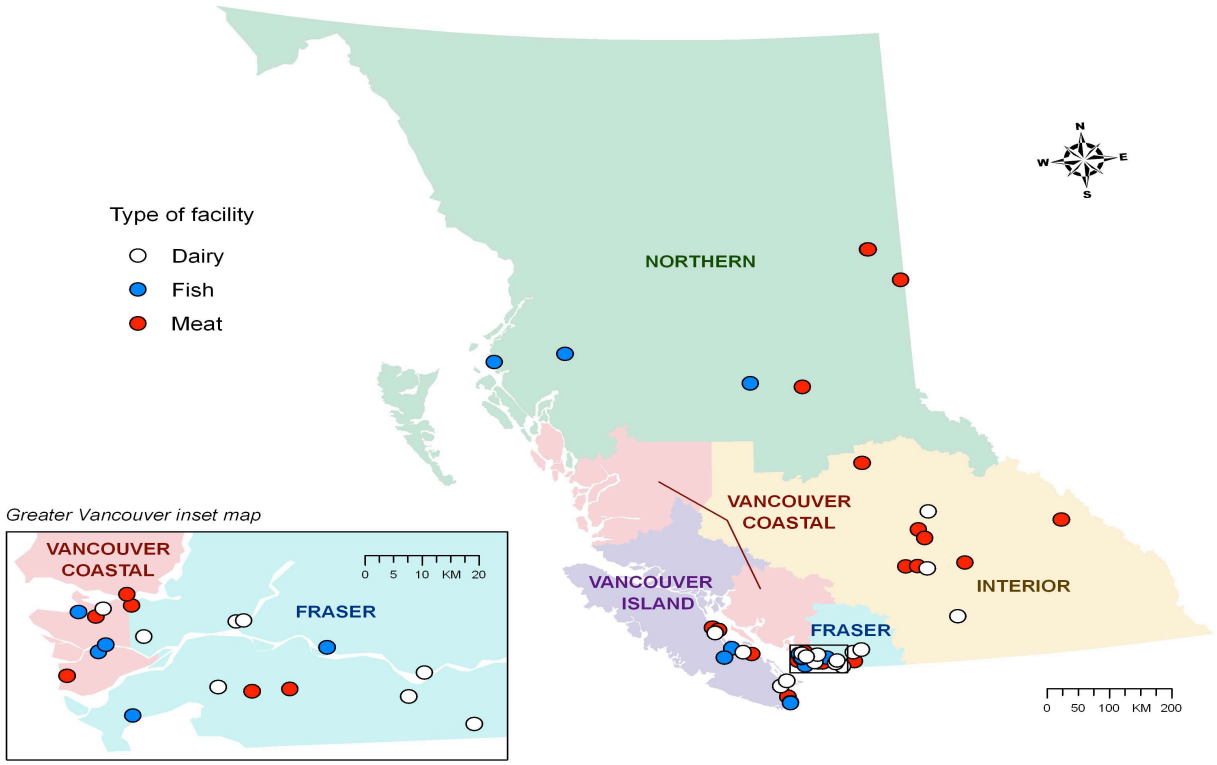


Figure 2-1. Geographic distribution of facilities producing ready-to-eat foods under provincial inspection authority (n=53) visited during the survey that assessed the prevalence of *Listeria* spp. in food facilities, by Health Authority regions in British Columbia.

[1] Re-printed with permission from the report prepared by Kovacevic, J. and Environmental Health Services Division (2010), available at: http://www.bccdc.ca/NR/rdonlyres/659E872B-A803-4F99-8C6A-

Table 2-1. List of surfaces sampled in food processing facilities.

Non-food contact	Close-to-food contact	Food contact
Drain	Walls adjacent to food handling surfaces	Work-table
Sides/Legs:	Sides/Legs:	Packaging counter
Cart	Slicer	Food racks/shelves
Conveyor	Packaging table	Slicer
Vat	Shrink wrapper	Cutting board
Table	Work-table	Food bin
Refrigerator	Vacuum packer	Food display basket/bin/insert
Doors	Counter space	Food mold
Area under wash-sink	Silent cutter	Filler bowl
Support beams	Scale	Inside of vat pipes
Trolley wheels	Cup/jug filler	Cutting utensils
Bottom shelves of packaging/ wrapping tables	Show-case/display cooler door handle and interior	
Trolley wheels		

Swabs were collected during food processing, and at least three hours after the facility began operations in order to increase the likelihood of obtaining positive results for *Listeria* spp. Sterile pre-wetted sponge applicators (Qualicum Scientific Ltd., Nepean, ON) were used to swab 30 by 30 cm areas, five times vertically and five times horizontally. Sponges were then placed in sterile bags (Qualicum) and refrigerated for no more than 48 hours. Food samples (150 g) were collected either aseptically in sterile sample bags (Fisherbrand, Thermo Fisher Scientific, Ottawa, ON) or as pre-packaged consumer-ready products. Foods sampled had been produced on the day of the visit, or, in the case of foods normally aged prior to shipment (e.g., aged cheeses and meats), were collected at the end-stage of production ready for shipment to retailers. Samples recovered from dairy processing facilities included milk and fluid dairy products, hard and soft cheeses, yogurt and ice cream. Fish and seafood products primarily included cooked, heat dried or hot smoked salmon products with various flavors (e.g., teriyaki, honey garlic, Cajun, candied), as well as cold smoked and lox salmon

products, smoked sablefish, sardines and cooked prawns. Meat samples tested included varieties of beef and pork sausages, "smokies", pepperoni, prosciuttino salami, meatloaf, hot dogs/wieners, beef and deer jerky, turkey, chicken, ham and beef deli meats, as well as buffalo and bison salami and sausages. While the objective was to examine primarily RTE samples, in a few instances a small number of raw meat samples were collected and tested (n=13).

2.2.2 Sample collection from retail facilities (RF)

Ready-to-eat meat and fish products were purchased from seven large chain retail establishments and 10 smaller retailers in the Metro Vancouver area (B.C., Canada) in September and October 2010. Overall, 80 samples were collected: 40 deli meats and 40 RTE fish products. Meat samples included: beer sausage, bologna, cervelat and genoa salami, cheese loaf, chicken and turkey breast, cooked ham, corned beef, meat macaroni loaf, mortadella, variety pack sausages, and different types of pepperoni (e.g., beef, chicken, turkey). Fish samples consisted of different flavored, candied and/ or smoked fish jerky, nuggets, and pepperoni samples, as well as lox, sockeye sticks, smoked steelhead trout, and tuna. Samples (approximately 50 g) were purchased as sliced/weighed deli products or in manufacturer-sealed packaging. Samples were transported to the laboratory in coolers on the day of purchase, and tested prior to best before/expiry date.

2.2.3 Isolation of *Listeria* spp. and confirmation

Samples were analyzed according to Health Canada's MFLP-74 enumeration (Pagotto et al., 2002) and MFHPB-30 two-step enrichment (Pagotto et al., 2001) methods. Confirmation of

Listeria spp. was based on Gram stain, catalase and oxidase reactions, and motility at room temperature. Isolates were speciated by standard biotyping (Microgen Listeria ID, Microgen Bioproducts Ltd., Cam- berley, Surrey, U.K. and API Listeria, BioMerieux, Marcy l'Etoile, France). Where possible, up to three isolates were characterized and saved from each positive sample. Isolates were maintained in 20% peptone glycerol solution, at -80°C.

2.2.4 Serotyping and genetic fingerprinting

Listeria monocytogenes isolates (n=111) were serotyped by slide agglutination and antisera prepared according to Seeliger and Höhne (1979) at the Canadian National Microbiology Laboratory. Genetic fingerprinting, based on pulsed-field gel electrophoresis (PFGE), was performed according to PulseNet standardized protocol at the BCCDC Public Health Microbiology and Reference Laboratory (PF isolates) or at the Canadian Listeriosis Reference Service Laboratory (Ottawa, ON) (RF isolates) using restriction enzymes *Asc*I and *Apa*I. For PF isolates, PFGE patterns were assigned according to the Tenover et al. (1995) criteria. Briefly, identical letter and number combinations represent indistinguishable patterns; the same letter with a different number (e.g., A1, A2) indicates that some variability was observed but isolates are closely related (e.g., changes are consistent with a single genetic event that may lead to two to three band differences); and a different letter indicates that strains are likely not related (i.e. $\geq$ 7 band differences) (Tenover et al., 1995). For RF isolates patterns were assigned after comparison to the PulseNet Canada database.

2.2.5 Statistical analyses

A two-tailed Fisher's exact test was used to assess differences in the proportions of facilities with environment swabs or food samples that were shown to be positive for *Listeria* spp. (all *Listeria* species, including *L. monocytogenes*) and *L. monocytogenes*, analyzed separately, among the dairy, fish and meat categories, at a 5% level of significance. Inclusion criteria for statistical analyses required that facilities have at least one swab collected in each of NFC, CFC, and FC area sampled (i.e. 51 facilities: 17 dairy, 21 meat, and 13 fish), and at least four RTE samples (i.e. 43 facilities: 17 dairy, 14 meat, and 12 fish).

Contingency table analysis was used to assess the probability of finding *Listeria* (*Listeria* spp. and *L. monocytogenes*, analyzed separately) in foods at a facility given that *Listeria* were found in the environment at that facility. In contingency tables, each facility was counted in one of four categories: (A) *Listeria* found in food and *Listeria* found in environment; (B) *Listeria* found in food and not found in environment; (C) *Listeria* not found in food and found in environment; and (D) *Listeria* not found in food and not found in environment. The odds of finding *Listeria* in foods when it is present in the environment were calculated as A / C, and the odds of finding *Listeria* in food when it is not present in the environment were calculated as B / D. The ratio of these odds [(A x D) / (B x C)] indicated the strength of the association between *Listeria* present in the environment and in foods. All analyses were performed using R software (version 2.10.1; R Foundation for Statistical Computing, Vienna, Austria).

2.3 Results

2.3.1 *Listeria* spp. contamination in food processing facilities

Of 51 facilities that met the criterion of at least one swab collected in each of the three sampling areas, *Listeria* spp.[2] were recovered from the processing environments of 21 (41%), while *Listeria monocytogenes* was found in 11 (22%) facilities. The highest contamination was seen amongst fish processing facilities, followed by meat, and dairy (Figure 2-2A).

NFC surfaces were contaminated with *Listeria* spp. and *L. monocytogenes* in 21 (41%) and 10 (20%) facilities, respectively. *Listeria* spp. (in all cases also *L. monocytogenes*) were recovered from CFC surfaces in 4 (8%), while 5 (10%) and 2 (4%) facilities had *Listeria* spp. and *L. monocytogenes*, respectively, recovered from FC surfaces (Figure 2-2B).

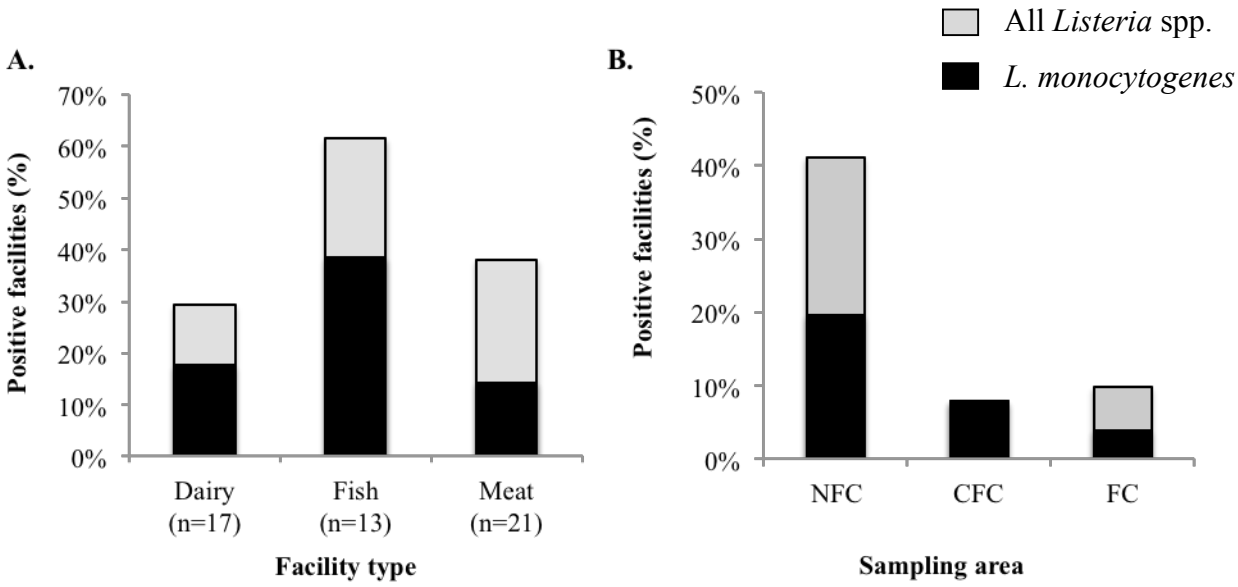


Figure 2-2. The proportion of facilities meeting the criterion of at least one swab collected in each of the three sampling areas, having environmental swab samples positive for *Listeria* spp. and *L. monocytogenes* by facility type (A) and sampling area (B). NFC, non-food contact; CFC, close-to-food; FC, food contact surface.

[2] Unless specified, *Listeria* spp. positive sample represents a sample that was identified positive prior to biotyping, and may contain any *Listeria* species, including *L. monocytogenes*.

Considering the sub-environments of processing facilities, no significant differences were found in the proportions of dairy, fish, and meat facilities having swabs of drains and other NFC or CFC surfaces positive for *Listeria* spp. The same comparison for FC surfaces indicated a higher proportion of fish facilities with one or more sample positive for *Listeria* spp. compared to dairy (5/13 *versus* 0/17, p=0.009) and meat (5/13 *versus* 0/21, p=0.005) facilities. Similarly, *L. monocytogenes* was seen on FC surfaces only in fish facilities, though differences were not statistically significant.

Considering facility contamination based on the presence of *Listeria* spp. and *L. monocytogenes* in RTE foods, 43 facilities visited met the criterion of at least four RTE food samples collected in a facility. Of these, 8 (19%) and 5 (12%) had *Listeria* spp. and *L. monocytogenes*, respectively, recovered from RTE food samples. There was a higher proportion of fish facilities compared to dairy facilities where one or more food samples were positive for *Listeria* spp. (6/12 *versus* 0/17, p=0.002) and *L. monocytogenes* (5/12 and 0/17, p=0.007). Proportionally more fish than meat facilities had a food sample positive for *Listeria* spp. (6/12 *versus* 2/14, p>0.05) and *L. monocytogenes* (5/12 *versus* 0/14, p=0.012) (Figure 2-3).

When the overall contamination was examined (i.e. environmental and RTE food), of the 43 facilities that met the criteria of at least one swab collected in each of the three sampling areas (i.e. NFC, CFC, FC) and at least four RTE food samples collected, 11 (26%) had *Listeria* spp. recovered only from the processing environment, 7 (16%) had *Listeria* spp. recovered from both environment and RTE foods, and 1 (2%) had *Listeria* spp. isolated only from foods.

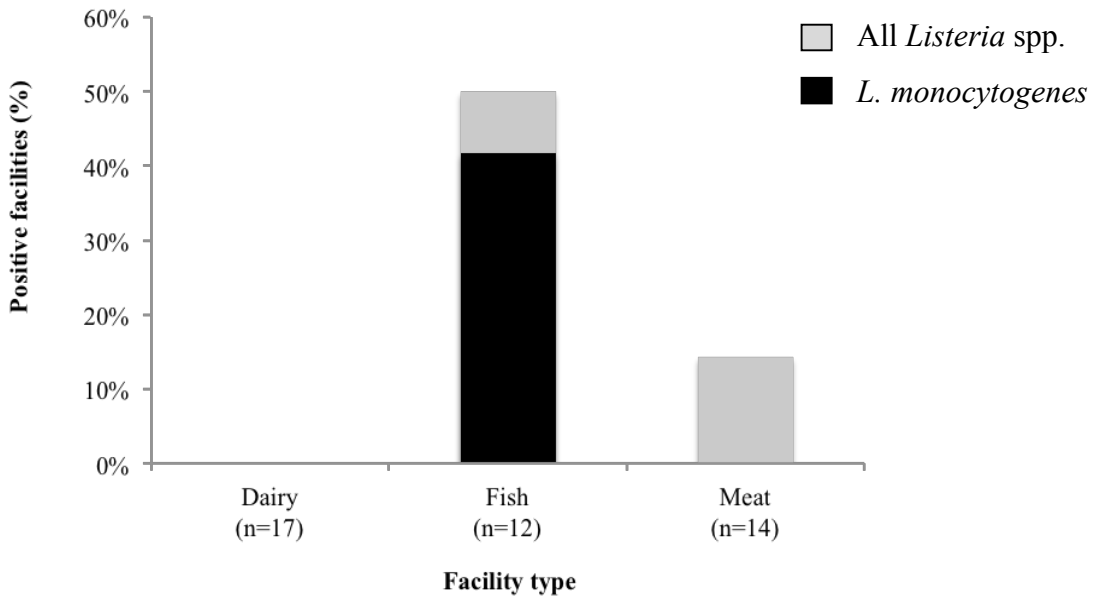


Figure 2-3. The proportion of facilities meeting the criterion of at least four RTE foods collected having samples positive for *Listeria* spp. and *L. monocytogenes*.

Contingency table analysis revealed that facilities where one or more foods were contaminated with *Listeria* spp. were 15 times (7/18 *versus* 1/25, prevalence odds ratio 15.3; p=0.005) more likely to have had *Listeria* spp. in swabs collected from the processing environment than facilities with no *Listeria* spp. positive foods.

In addition, facilities where one or more foods were contaminated with *L. monocytogenes* were highly more likely to have *Listeria* spp. found in swabs from the processing environment than facilities with no *L. monocytogenes* positive foods (5/18 versus 0/25, prevalence odds ratio infinite, p=incalculable) (Table 2-2). Interestingly, *L. monocytogenes* was never found in foods when *Listeria* spp. were not detected somewhere in the processing environment (Figure 2-4).

Table 2-2. Contingency table for *Listeria* spp. found in at least one environmental swab sample *versus L. monocytogenes* found in at least one food sample, and *Listeria* spp. found in any food sample *versus L. monocytogenes* found in any food sample, by facility. Only those facilities that met both environmental swab and food inclusion criteria are included.

		***L. monocytogenes* in food sample**		
		Yes	No	**Total**
Listeria spp. in environmental swab	Yes	5	13	**18**
	No	0	25	**25**
	Total	**5**	**38**	**43**
PPV[a] 28%				
NPV[b] 100%				
Listeria spp. in food sample	Yes	5	3	**8**
	No	0	35	**35**
	Total	**5**	**38**	**43**
PPV 63%				
NPV 100%				

[a]Positive predictive value: true positive samples divided by the sum of true positive and false positive samples, expressed as %.
[b]Negative predictive value: true negative samples divided by the sum of false negative and true negative samples, expressed as %.

Considering the joint presence of *L. monocytogenes* in foods and *Listeria* spp. in swabs of the processing environment, NFC surfaces were contaminated with *Listeria* spp. in facilities of all three food categories, but FC surfaces were contaminated only in RTE fish facilities (Figure 2-4). Interestingly, *Listeria monocytogenes* was found only in RTE fish products. In all facilities where food products were contaminated with *L. monocytogenes*, *Listeria* spp. were present in the processing environment (Figure 2-4).

2.3.2 Recovery of *Listeria* spp. from environmental samples

Listeria spp. were recovered from 13% (40/305) of all environmental samples, while 7% (21/305) harbored *L. monocytogenes*. The highest rates of contamination with *Listeria* spp., and *L. monocytogenes* were seen in swabs of NFC surfaces, at 30% (30/101) and 13% (13/101), respectively. The proportion of CFC and FC surfaces positive for *Listeria* spp. was

lower, at 5% (5/101) and 6% (6/103), respectively. *Listeria monocytogenes* was recovered from 4 and 3% of CFC and FC surfaces, respectively.

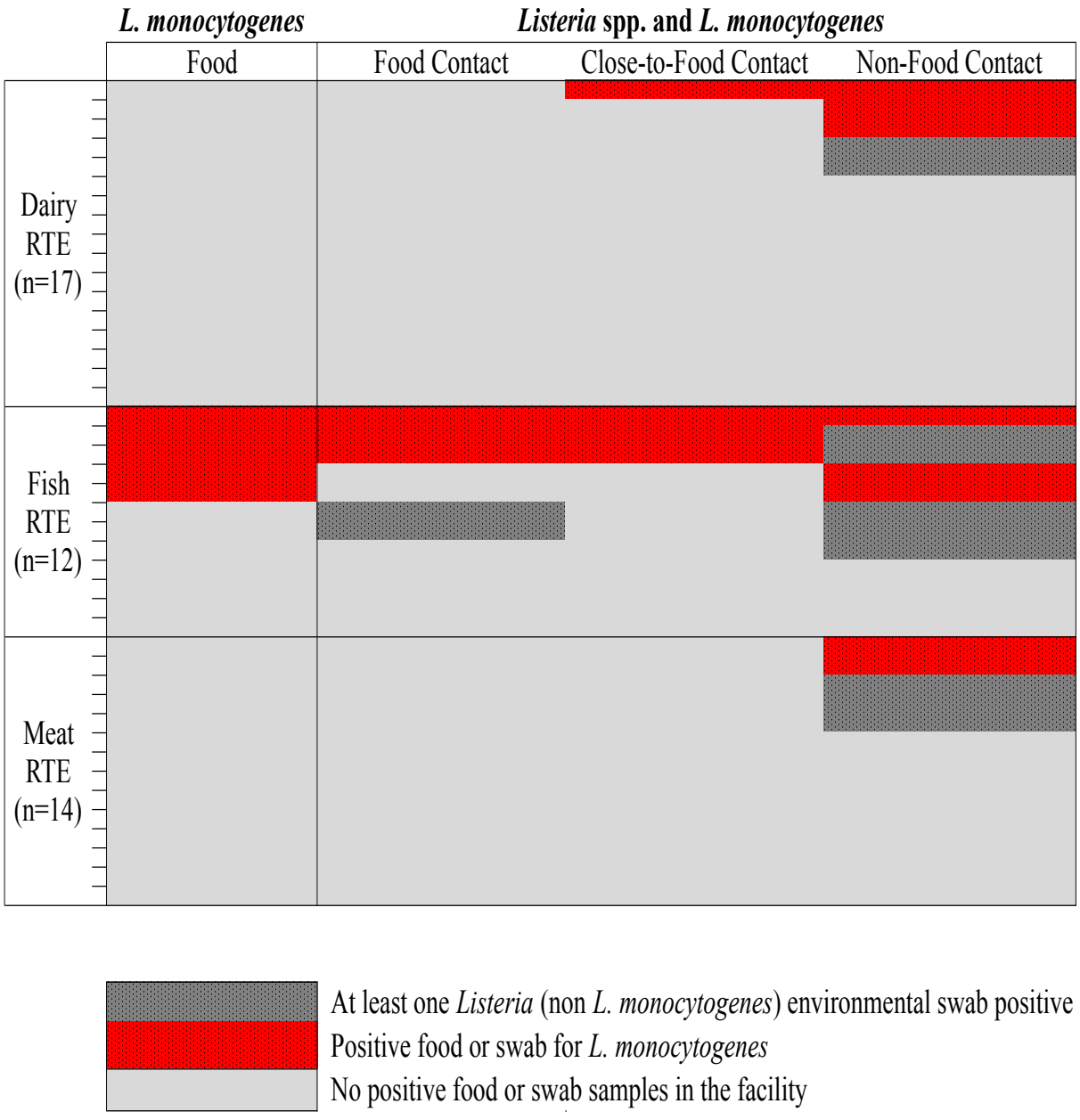


Figure 2-4. The joint presence of *L. monocytogenes* in food and other *Listeria* spp. in the processing environment, for facilities that met the criterion of at least one swab from each of the three sub-environments and no less than four RTE food products collected. Each row represents a facility.

Based on the type of facility, highest contamination of both *Listeria* spp. and *L. monocytogenes* occurred in environmental samples from fish facilities, with 23 (29%) and 13 (17%) samples positive, respectively. In dairy facilities, of the 102 environmental samples collected, 9% were positive for *Listeria* spp., while only 3% harbored *L. monocytogenes*. Similarly, 7% of 125 environmental samples collected from meat facilities tested positive for *Listeria* spp., with only 2% culturing *L. monocytogenes.*

2.3.3 Recovery of *Listeria* spp. from retail food samples

From 80 RTE food samples analyzed, eight (10%) were positive for *Listeria* spp., with all containing less than 100 CFU/g. Positive samples came from four RF; three small and one large facility. *Listeria welshimeri* was the most commonly isolated species (4/8), followed by *Listeria innocua* (2/8) and *L. monocytogenes* (2/8). Listerial contamination, seen exclusively in RTE fish samples, included smoked and candied salmon and salmon jerky.

2.3.4 *L. monocytogenes* contamination of RTE foods

In RTE foods *L. monocytogenes* was found exclusively in fish products (Table 2-3). It was recovered from 14 food samples from PF, including 13 hot smoked, heat dried or cooked fish products, and one cold smoked salmon product, and in two fish samples from RF. Further, *L. monocytogenes* was recovered from four of the contaminated products that had *Listeria* spp. counts greater than 100 CFU/g; three of which were grossly contaminated with more than 30,000 CFU/g (Table 2-3).

Table 2-3. List of foods contaminated with *L. monocytogenes*.

	Food product	*Listeria* spp. (CFU/g)	Species isolated
Food processing facilities			
Fish	Salmon nuggets	< 100	*L. monocytogenes*
	Sockeye salmon candy	< 100	*L. monocytogenes*
	Salmon leather	< 100	*L. innocua, L. welshimeri, L. monocytogenes*
	Salmon leather	< 100	*L. welshimeri, L. innocua*
	Salmon leather	< 100	*L. innocua*
	Cold smoked salmon	< 100	*L. monocytogenes*
	Salmon candy	< 100	*L. monocytogenes*
	Salmon candy	< 100	*L. monocytogenes*
	Teriyaki smoked sablefish	< 100	*L. monocytogenes*
	Spring-wood smoked salmon	< 100	*L. monocytogenes*
	Indian candy salmon	300	*L. monocytogenes, L. innocua*
	Lox whole salmon	< 100	*L. innocua*
	Lox sliced salmon Coho	400	*L. innocua*
	Prawns	< 100	*L. innocua*
	Indian candied salmon	100	*L. innocua*
	Salmon jerky	< 100	*L. monocytogenes*
	Cajun salmon	> 30,000	*L. monocytogenes*
	Shrimp meat	< 100	*L. monocytogenes*
	Teriyaki salmon	> 30,000	*L. monocytogenes*
	Honey garlic salmon	> 30,000	*L. monocytogenes*
Meat	Cheese smokie	< 100	*L. welshimeri*
	Hot pepperoni	< 100	*L. welshimeri*
	Prosciuttino salami	< 100	*L. innocua*
Retail facilities			
Fish	Smoked salmon	< 100	*L. monocytogenes*
	Wild smoked salmon fingers	< 100	*L. monocytogenes*
	Smoked salmon	< 100	*L. welshimeri*
	Wild smoked lox trim	< 100	*L. welshimeri*
	Smoked salmon nuggets	< 100	*L. innocua*
	Smoked salmon jerky	< 100	*L. welshimeri*
	Salmon candy	< 100	*L. innocua*
	Salmon strips	< 100	*L. innocua*

RF samples positive for *L. monocytogenes*, had less than 100 CFU/g, and were purchased from different RFs. They also originated from different PF; one PF was federally registered and inspected by the CFIA, while the other facility was under provincial inspection authority.

2.3.5 Species distribution among PF and RF, and food categories

Up to three isolates were saved from each positive sample, for a total of 253 *Listeria* isolates recovered from 86 samples: 32 RTE foods, 13 raw foods, and 41 swabs of PF environments. The majority of the isolates were recovered from swabs of food processing environments (n=123). Of the 119 isolates originating from foods, the majority (n=111) originated from PF, and eight were recovered from retail food samples (Table 2-4).

Table 2-4. The number of isolates[a] recovered from different types of facilities (food processing, PF, or retail facilities, RF), and different food categories (dairy, fish and meat).

Source	Dairy	Fish		Meat[b]		Total
	PF	PF	RF	PF	RF	
Food						
L. innocua	0	21	5	9[b]	0	35
L. monocytogenes	0	39	6	10[b]	0	55
L. seeligeri	0	0	0	0	0	0
L. welshimeri	0	3	8	29[b]	0	40
Total	0	63	19	48	0	130
Environment						
L. innocua	9	4	-	9	-	22
L. monocytogenes	13	34	-	9	-	56
L. seeligeri	5	28	-	0	-	33
L. welshimeri	0	3	-	9	-	12
Total	27	69	-	27	-	123

[a]The number of isolates and positive samples do not add up, as up to three isolates were recovered and archived from a positive sample.
[b]Isolates from raw meat samples: six *L. innocua*, 10 *L. monocytogenes*, and 23 *L. welshimeri*.

Listeria monocytogenes was the most often recovered species (n=111), followed by *L. innocua* (n=57), *L. welshimeri* (n=52), and *L. seeligeri* (n=33). Most of the isolates came from fish processing and retail samples (Table 2-4).

2.3.6 Serotype and PFGE pattern distribution among *L. monocytogenes* isolates

Majority of isolates belonged to listeriosis causing serotypes, including 1/2a (42%) and 4b (37%) serotypes (Table 2-5). Less commonly recovered serotypes included 1/2c (12%), 1/2b (5%), and 3a (4%) (Table 2-5).

When subjected to PFGE, 36 pulsotypes were observed among the various isolates recovered from PF (Figure 2-5; more detail given in Figure A-1 in Appendix A), and two pulsotypes among the retail isolates. Based on the PFGE data, in most instances the three isolates recovered from the same sample were clonal.

PFGE typing of isolates from PF found *L. monocytogenes* from fish facilities to be the most diverse, with 21 pulsotypes observed (Table 2-6). These isolates were genetically unrelated to isolates from dairy facilities [i.e. ≥ 7 band differences, resulting from changes consistent with three or more independent genetic events (Tenover et al., 1995)].

Table 2-5. Serotypes of *L. monocytogenes* isolates recovered in food processing environments, and raw (RUF) or ready-to-eat (RTE) foods.

Source	**No. of *L. monocytogenes* serotypes**					
	Total no. isolates	**1/2a**	**1/2b**	**1/2c**	**3a**	**4b**
Food processing environment	56	26	3	8	4	15
Food[a]						
RUF	10	8	0	2	0	0
RTE	45	13	3	3	0	26
Total No. (%)	**111**	**47 (42)**	**6 (5)**	**13 (12)**	**4 (4)**	**41 (37)**

[a]RUF, raw unprocessed food; RTE, ready-to-eat food products.

Table 2-6. Distribution of *L. monocytogenes* (n=111) sero- and pulsotypes, across different facilities (n=15).

Facility ID	Type	Number of isolates recovered	Isolate source[a]			Serotypes	Pulsotypes (PFGE)[b]
			FPE	RUF	RTE		
Food processing facilities							
d5	Dairy	9	9	-	-	1/2a	F1, G2
d7	Dairy	1	1	-	-	1/2a	N1
d11	Dairy	3	3	-	-	1/2a	G1
f19	Fish	15	12	-	3	1/2c, 3a	H1, H4, K2, K3, K4, K5
f20	Fish	21	9	-	12	1/2a, 4b	A1, L1, M1
f21	Fish	10	6	-	4	1/2a, 4b	A2, K1, K2, K4, K6, K8, K9
f28	Fish	11	6	-	5	1/2a, 4b	B1, H3
f31	Fish	16	1	-	15	1/2a, 4b	B1, C1, C2, E1, I1, I2
m38	Meat	3	-	3	-	1/2a, 1/2c	H2, J1
m44	Meat	3	-	3	-	1/2a	H6
m46	Meat	3	3	-	-	1/2a	G3, G4
m49	Meat	6	6	-	-	1/2a, 1/2b	D1, D2, F2, K10
m50	Meat	4	-	4	-	1/2a, 1/2c	H5, K7
	Total	105					
Retail facilities							
rf7	Fish	3	-	-	3	1/2a	LMACI.0001 LMAAI.0001
rf11	Fish	3	-	-	3	1/2b	LMACI.0470 LMAAI.0584
	Total	6					

[a]FPE, food processing environment; RUF, raw, unprocessed food; RTE, ready-to-eat food.
[b]Pulsotype designation is based on *Apa*I and *Asc*I enzymes and Tenover et al. (1995) composite designation in the isolate population originating from food processing facilities, where an identical letter/number indicates indistinguishable pattern; same letter and different number indicate closely related isolates and different letters represent unrelated isolates.

Isolates belonging to 1/2a serotype dominated in food processing environments, while serotype 4b was most commonly recovered from RTE foods (Table 2-5). Serotype 3a was

seen exclusively amongst isolates from food processing environments, and was present only in one fish facility (f19).

Indistinguishable patterns (K2 and K4) were seen in two fish facilities (f19 and f21) located in the Lower Mainland (Vancouver). Closely related patterns (i.e. 2-3 band differences) were seen in facilities 28 and 31, both located on Vancouver Island, facilities f20 and f21, located in Vancouver, and geographically distant facilities f19 and f28. Closely related patterns (H1 to H4) were also seen amongst fish (f19 and f28) and meat (m38, m44, and m50) facilities, geographically distant from each other. While isolates from dairy facilities were unrelated to those originating from fish facilities, patterns (G1 and G2) found in two dairy facilities (d11 and d5, respectively) were closely related to those seen in a meat facility (m46, patterns G3 and G4) (Figure 2-5).

For retail isolates, PFGE patterns were dissimilar among *L. monocytogenes* from different samples (LR39, LMACI.0001/ LMAAI.0001, and LR59, LMACI.0470/ LMAAI.0584), but were identical for all *L. monocytogenes* recovered from respective positive samples.

2.4 Discussion

The ubiquitous nature of *Listeria* has been demonstrated in numerous studies. *Listeria* species, and in particular *L. monocytogenes*, have been found in food products and retail and processing environments of fish (Eklund et al., 1995; Food Standards Agency, 2008; Johansson et al., 1999), dairy (Farber et al., 1987; Fox et al., 2009) and meat facilities (Cabedo et al., 2008; Chao et al., 2006; Farber and Daley, 1994). Considerable variability has

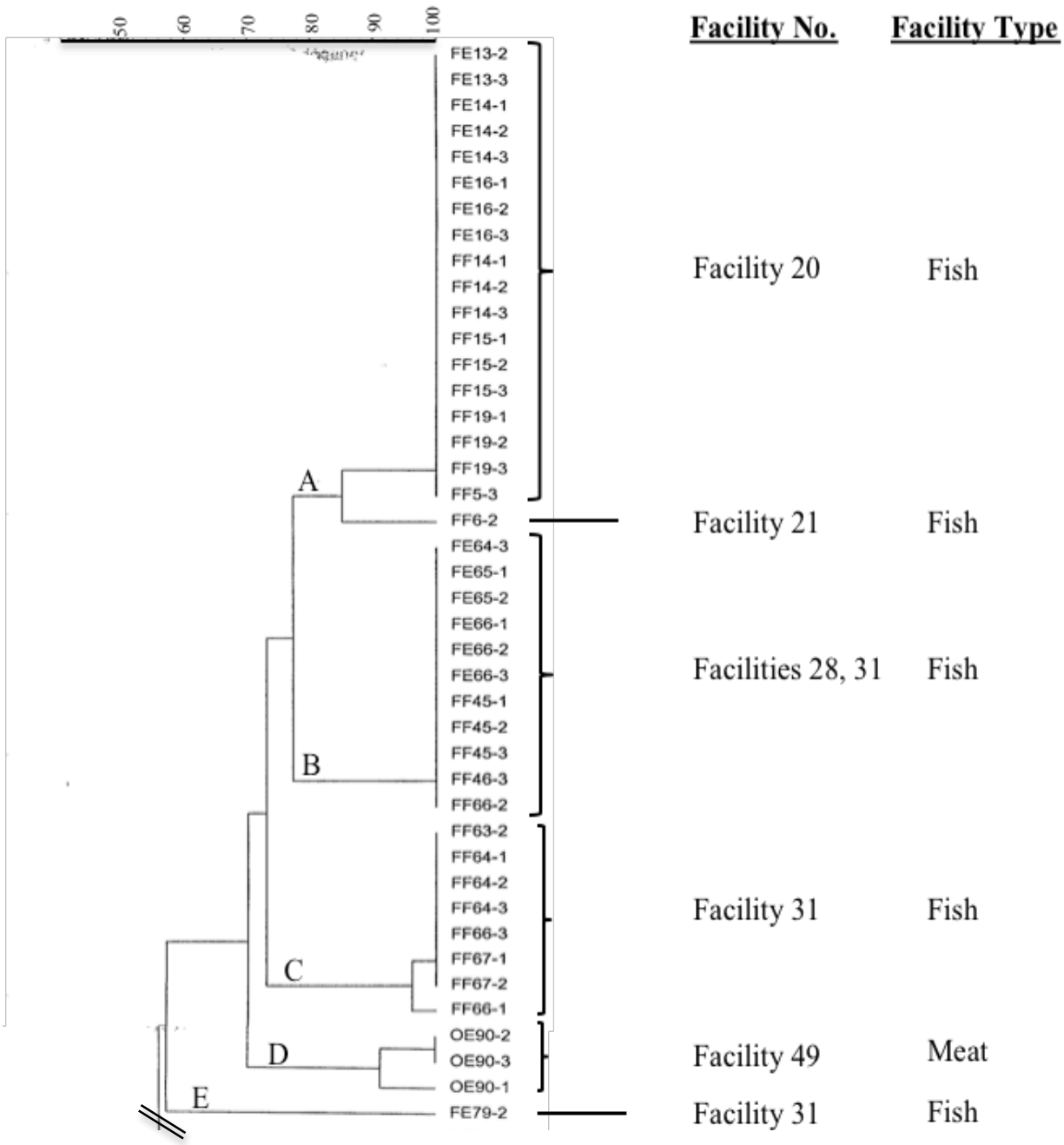


Figure 2-5. PFGE dendrogram of *L. monocytogenes* isolates recovered from food processing facilities based on *Asc*I and *Apa*I patterns; different letters represent unrelated isolates. Sample designations: DE, dairy environment; FF, fish food; FE, fish environment; MF and OF, meat food; ME and OE, meat environment. Dendrogram represents a continuous tree but is split onto two pages for visual clarity; the splitting point is indicated with \\ lines.

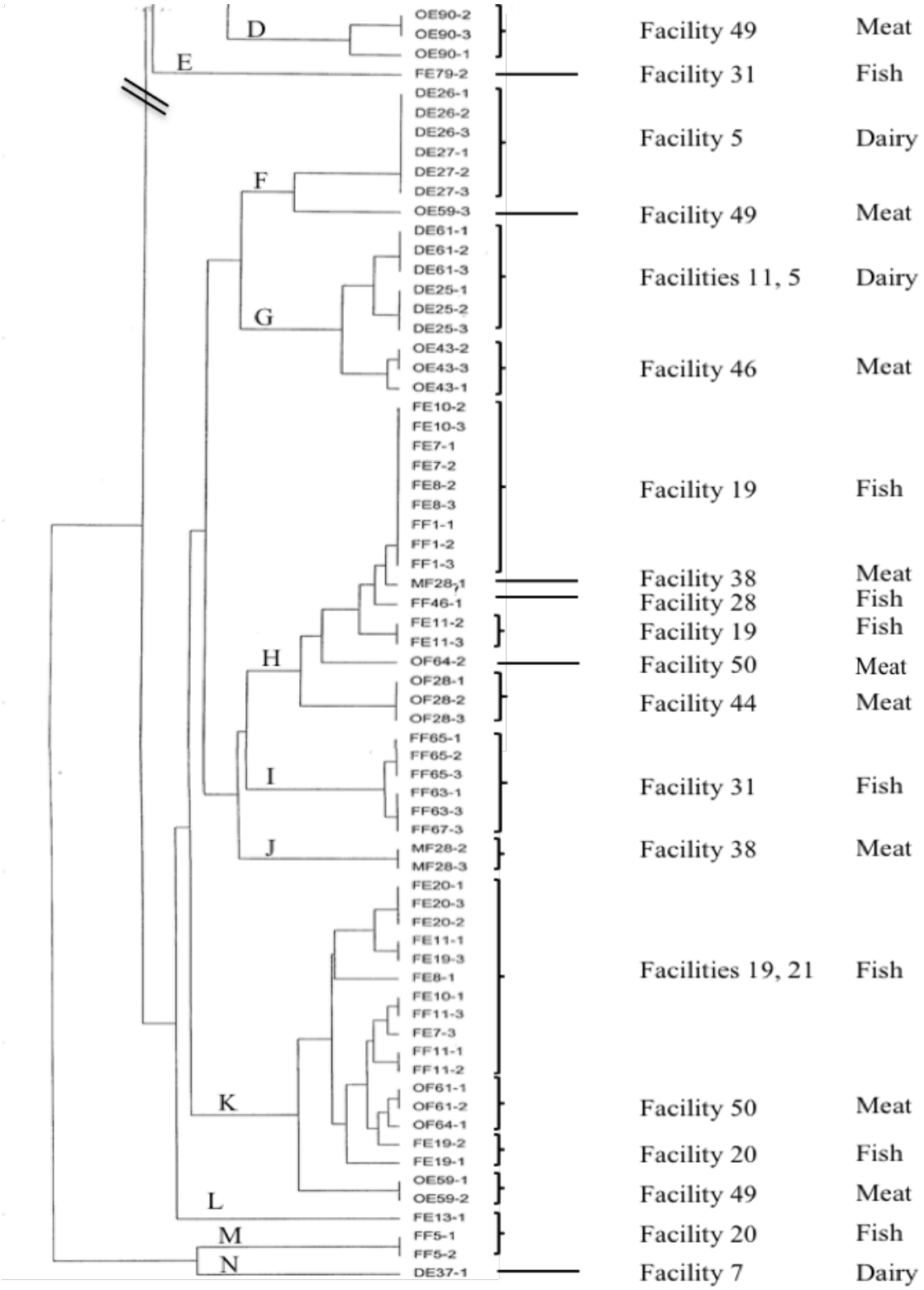


Figure 2-5. Continued.

been noted in the levels of contamination of food and food processing, and retail facilities with *Listeria* from region to region (Ryser, 2007a; Ryser, 2007b; Ryser, 2007c). The current study demonstrates such variation in the occurrence of *Listeria* spp. and *L. monocytogenes* in dairy, fish and meat RTE products from food processing and retail facilities in B.C.

Listeria spp., including *L. monocytogenes*, were recovered from RTE foods, specifically, smoked fish samples, while the pathogenic species were absent from RTE dairy and meat products. Contamination levels of fish in food processing facilities (28%) were similar to levels of *Listeria* spp. in retail fish samples (20%). While 20% of fish samples from PF harbored *L. monocytogenes*, in RF fish samples 5% of isolates were identified as *L. monocytogenes*. These data show that among different RTE food processing facilities under B.C. provincial inspection authority, the majority of fish processors harbor *Listeria* spp. *Listeria monocytogenes* was not recovered from RTE dairy or meat products, and was found at low levels and only on surfaces not in direct food contact in these facilities. In contrast, B.C. fish processing facilities were commonly contaminated with *Listeria* spp., and in two of the 13 fish processing facilities visited, *L. monocytogenes* was recovered from FC surfaces. *Listeria monocytogenes* was also recovered from food products in five fish facilities, in some cases at high levels. Surfaces not in direct contact with RTE foods, such as drains, floors, and legs of tables and carts, were where the highest prevalence of *Listeria* spp. contamination was found. However, in three of the fish facilities all three types of surfaces (FC, CFC and NFC), and RTE food products, were contaminated with *Listeria* spp. Interestingly, in two facilities where *L. monocytogenes* was found in RTE foods the bacteria were recovered only from NFC surfaces. NFC surfaces positive for *L. monocytogenes* have

been reported previously as potential contamination sources and a sensitive predictor for the presence of *L. monocytogenes* in smoked salmon (Rørvik et al., 1997; Thimothe et al., 2004).

In a study of *L. monocytogenes* contamination patterns in four smoked fish processing facilities, Thimothe et al. (2004) observed a strong positive relationship ($p<0.0001$) between *L. monocytogenes* prevalence in environmental and finished product samples. They also reported a very highly statistically positive relationship between *Listeria* spp. prevalence in the environment and *L. monocytogenes* prevalence in the environment, as well as in finished products. While investigating risk factors associated with *L. monocytogenes* contamination of smoked salmon during processing, Rørvik et al. (1997) reported that the risk of contamination in smoked salmon was positively associated with the presence of *L. monocytogenes* in drains (relative risk of 3.3). In the current study PFGE data confirmed the presence of genetically indistinguishable strains in drains and other NFC surfaces, and RTE foods (e.g., A1 in facility f20, B1 in facility f28, and H1 in facility f19) (Figure 2-5). These findings confirm that cross-contamination between processing environment and finished product is a likely occurrence in fish processing facilities.

Although *Listeria* spp. and *L. monocytogenes* contamination appears to be common in cold and hot smoked fish samples (Dominguez et al., 2001; Hartemink and Georgsson, 1991), the prevalence rate for this organism in RTE fish products from PF reported in the current study was notably higher than the rates described in previous Canadian studies (Farber, 1991; Farber, 2000), and those reported by the European Food Safety Authority (EFSA, 2010). The rates reported in B.C. samples are, however, similar to findings reported by Van Coillie et al. (2004) for Belgian samples, and those observed by Dominguez et al. (Dominguez et al., 2001) for smoked fish and fish pâté samples in Spain.

Farber (2000), reported the absence of *L. monocytogenes* in 196 and 150 Canadian RTE seafood products tested in 1997/1998 and 1998/1999, respectively. This sampling was performed as part of the Canadian Food Inspection Agency's Quality Management Program. However, limited information was provided regarding the origin of the products and characteristics of facilities from which samples were collected (Farber, 2000). In addition, only a direct plating method was used to test for *L. monocytogenes*, as opposed to both the direct plating and enrichment methods applied in the current study. The use of a direct plating method may decrease the chance of bacterial detection if microorganisms are sub-lethally injured or present in low numbers.

A Canadian study, also conducted by Farber (1991), examined 113 RTE seafood products from the wholesale level for the presence of *L. monocytogenes*. Among the 113 samples tested, only 20 salmon products originated from Canada. Overall, 13% (15/113) of the tested products contained *L. monocytogenes*, which is lower than the 20% (14/71) reported in this thesis. Of the 20 salmon products produced in Canada, 5 (25%) were positive for *L. monocytogenes* (Farber, 1991). A summary of trends and sources of foodborne outbreaks in Europe reported an overall prevalence of 9.8% for *L. monocytogenes* in 7,126 RTE fish products, derived from both retail and food processing facilities in 12 European countries (EFSA, 2010). Individual studies from coastal European countries reported varying levels of listerial contamination in retail fish samples (Cabedo et al., 2008; Garrido et al., 2009; Gianfranceschi et al., 2003; Van Coillie et al., 2004). A 2009 study conducted in Spain found *Listeria* spp. in 18.6 % of smoked salmon samples from retail, with 10 % harboring *L. monocytogenes* (Garrido et al., 2009). A Spanish study in 2008 (Cabedo et al., 2008) recovered *L. monocytogenes* from 7.9 % of smoked salmon samples, which is comparable to

levels observed at retail in the current study. In Italy (Gianfranceschi et al., 2003) and Belgium (Van Coillie et al., 2004) much higher levels of *L. monocytogenes* were reported in fish and fish products (27.9 %) and smoked halibut (33.3 %), respectively. However, it is important to note that in many instances direct comparison of results is not possible, as studies were structured differently, and testing procedures often varied from country to country. For instance, the study described here provides contamination levels obtained during a one-time sampling interval, and a small number of retail samples. In contrast, the data described in other studies involved a large number of samples (e.g., EFSA report), and in some cases a longitudinal approach, thus limiting the extent of comparison among studies.

Even though *Listeria* spp. and *L. monocytogenes* are common in fish products, listeriosis outbreaks linked to these foods are rare (Farber, 2000; Jinneman et al., 2007). It has been suggested that since cooked fish products generally contain low levels of *L. monocytogenes* and have a short shelf life, they therefore do not likely represent a serious health hazard (Farber, 2000; Rørvik, 2000). Also, while in some cases high levels of contamination of fish and fish products with *L. monocytogenes* have been reported, the population health risk is rated low when the low degree of consumption of RTE fish per capita is taken into account (Dominguez et al., 2001).

In this study, fish products were the only RTE foods positive for *L. monocytogenes*. These microorganisms were not detected in the tested RTE foods produced in dairy or meat facilities. In one particular fish processing facility, three RTE samples contained high levels of *L. monocytogenes*. Even though a low health risk from RTE fish contaminated with *L. monocytogenes* has been suggested elsewhere (Dominguez et al., 2001; Farber, 1991), the infective dose for acquiring listeriosis infection is thought to be host and dose dependent

(FAO and WHO, 2004; Iwamoto et al., 2008). While a dose of 100 organisms conveys a probability risk for infection ranging from 10^{-9} to 10^{-13}, a dose of 1,000,000 organisms increases the risk of infection to 10^{-6} to 10^{-9} (Iwamoto et al., 2008). Furthermore, persons in vulnerable groups, such as cancer and transplant patients, are 1,000 times more susceptible to the invasive listeriosis compared to healthy persons (FAO and WHO, 2004). Similarly, pregnant women and their newborns have been estimated to be 14 times more likely to acquire invasive listerial infections compared to a normal healthy population (FAO and WHO, 2004). The contaminated products in B.C. were destined for sale to a wide population, potentially including pregnant women and immunocompromised individuals. In addition, findings that the majority of the recovered isolates belonged to listeriosis causing serotypes, 1/2a and 4b, and that some samples were grossly contaminated, are of concern. Hence, a closer look into the production of RTE fish products in B.C. is warranted.

While the complete eradication of *L. monocytogenes* from food processing facilities is regarded as unrealistic, a correlation between the level of hygiene practiced in a facility and the prevalence of *L. monocytogenes* has been demonstrated in many studies (Fox et al., 2009; Kabuki et al., 2004; Klausner and Donnelly, 1991; Kozak et al., 1996). To achieve the reduction in *L. monocytogenes* levels, both stringent and continuous control strategies are required (Tompkin, 2002). Sampling of food processing environments has been suggested as a good tool to assess the level of *Listeria* control within a facility (Tompkin, 2002). In the 2008 Canada-wide deli meat listeriosis outbreak, longitudinal testing of environmental swabs revealed ongoing contamination of meat processing lines with *Listeria* spp. prior to the onset of the outbreak. A post-mortem of the outbreak highlighted the importance of following trends in microbial analyses of environmental samples as an early indicator of the potential

for contamination of RTE products (Weatherill, 2009). In Canada, federally registered food processing facilities are subject to environmental and end-product testing for *Listeria* spp. and/or *L. monocytogenes*; however, this level of inspection is not required nor practiced in most non-federally registered food processing facilities. Findings from the current study suggest that a combination of monitoring and validation of food safety practices, whether through periodic environmental sampling, end-product testing, more rigorous inspection, or a combination of these, is warranted in RTE food processing facilities in B.C. This is especially true for RTE fish processing facilities in B.C., where health inspectors noted that the presence of *L. monocytogenes* in RTE food samples was often coupled with inadequate sanitation and/or the lack of rigorous food hygiene practices in a facility. However, the absence of *Listeria* spp. in the great majority of B.C.'s dairy and meat facilities, some of which have previously been implicated in listeriosis outbreaks, suggests these bacteria can be kept at low levels in RTE facilities.

2.5 Conclusions

In summary, the results obtained from this study suggest that while control of *L. monocytogenes* in B.C.-inspected dairy and meat facilities is effective in limiting food contamination, there is a need for processors and inspectors to initiate improved monitoring and management of contamination by *L. monocytogenes* in RTE fish processing and retail sectors. Furthermore, considering that the majority of recovered isolates were found to belong to listeriosis causing serotypes, characterization of the genetic and phenotypic properties of the isolates is required in order to assess the risk posed to consumers from the consumption of the foods contaminated with *L. monocytogenes*.

Chapter 3: Assessment of the population structure, virulence potential, mutability and cold adaptation of food chain-derived *Listeria monocytogenes* isolates

3.1 Introduction

Listeria monocytogenes is an environmentally ubiquitous organism that frequently contaminates food processing environments. It is estimated that 99% of listeriosis cases are transmitted through the consumption of contaminated food (Mead et al., 1999; Scallan et al., 2011; Swaminathan and Gerner-Smidt, 2007). In healthy individuals, *L. monocytogenes* infections are rare, restricted to the gastrointestinal environment, self-limiting, and manifest as gastroenteritis and/or mild flu-like symptoms. In contrast, in susceptible populations (e.g., neonates, the elderly, and immunocompromised), infections become invasive, leading to encephalitis, meningitis, septicemia, and/or spontaneous abortions during the last trimester of pregnancy (McLauchlin et al., 2004). Mortality rates for invasive listeriosis typically range between 20 to 40% (Farber and Peterkin, 1991; Hof et al., 1997; Weatherill, 2009).

Although there are 13 *L. monocytogenes* serotypes in total, the majority of human disease is caused by 1/2a, 1/2b, and 4b serotypes (Chenal-Francisque et al., 2011; Graves et al., 2007). Historically, lineage I 4b strains have been over-represented in clinical listeriosis cases and are less frequently recovered from foods (Chenal-Francisque et al., 2011; Gray et al., 2004; Jeffers et al., 2001). In contrast, lineage II 1/2a strains have been over-represented in food and environmental samples (Chen et al., 2009; Gray et al., 2004; Swaminathan and Gerner-Smidt, 2007). It has been suggested that positive selection resulted in the adaptation of lineage II strains to a broad range of environmental and stress conditions, whereas lineage

I strains that originated from an already pathogenic ancestor likely further adapted to a narrow range of conditions specific to host colonization (Orsi et al., 2011). Interestingly, over the past decade, lineage II 1/2a strains have been increasingly linked to human disease, causing notable listeriosis outbreaks in Switzerland (Bille et al., 2006), the United Kingdom, and two separate 2008 outbreaks in Canada (Gaulin et al., 2012; Weatherill, 2009). With regards to the latter, 1/2a strains comprise the majority of Canadian clinical isolates, followed by 4b (Clark et al., 2010). Reasons for the prevalence of 1/2a strains in human disease in Canada may be linked to a recently identified clonal complex/epidemic 1/2a clone that was identified as a recurring cause of sporadic listeriosis since 1988 (Knabel et al., 2012). Within this complex, the majority of 1/2a strains were found to possess the LGI1 genomic island, which was first identified in an outbreak strain linked to 23 deaths (Gilmour et al., 2010).

Over the past decade, sequence analysis of *inlA*, which encodes a membrane-bound protein facilitating invasion into non-professional phagocytes, revealed that a significant proportion (45%) of strains recovered from RTE food possess mutations resulting in premature stop codons (PMSCs) in *inlA* (Nightingale et al., 2008; Van Stelten et al., 2010; Ward et al., 2010). Strains with *inlA* PMSCs produce either a truncated or secreted InlA (i.e. absence of cell wall anchor), resulting in virulence attenuated phenotypes, as measured both by *in vitro* cell assays (Felicio et al., 2007; Handa-Miya et al., 2007; Jonquieres et al., 1998; Nightingale et al., 2005; Rousseaux et al., 2004) and *in vivo* mammalian models (Nightingale et al., 2008; Roldgaard et al., 2009; Van Stelten et al., 2011). As a result, it has been speculated that inactivation of InlA in some way increases strain fitness in environments outside of mammalian hosts, with the exception of 4b serotype (Orsi et al., 2011). While the absence of *inlA* PMSCs in 4b strains may reflect their over-representation in clinical

listeriosis, the contrary is postulated for 1/2a strains. Being frequently recovered in food and food production environments, positive selection for strains with *inlA* PMSCs may serve as a phase switch that is important for environmental survival of the organism (Orsi et al., 2011). In-line with this, it has been suggested that lineage II 1/2a strains are better able to survive conditions associated with the food chain. Notably, 1/2a and 1/2c (lineage II) strains more frequently possess *inlA* PMSCs than 1/2b or 3b serotypes, and presently, no *inlA* PMSC mutations have been reported for 4b strains (Orsi et al., 2011). In general, serotype 4b appears more recalcitrant to genetic flux, being less likely to acquire, or possess plasmids, and to experience homologous recombination events that may afford rapid adaptation to niche-specific stresses (Orsi et al., 2011).

Considering that the majority of strains recovered from the B.C. food chain belonged specifically to serotypes 1/2a and 4b, it seemed prudent to examine genetic properties and the population structure of the isolates. Since *inlA* mutations have been linked to food chain isolates, and InlA protein is a causally linked virulence determinant, the nature and prevalence of *inlA* genotypes in *L. monocytogenes* serotypes recovered from food and food production environments in B.C. were also investigated. Furthermore, since these strains are of food origin it was of interest to examine how *L. monocytogenes* strains with differing *inlA* genotypes respond to food chain-relevant conditions. In particular, the reliance on refrigeration to maintain the quality of fresh and RTE foods makes cold temperature a suitable and relevant parameter to examine for characterizing *L. monocytogenes* response to those conditions. Lastly, whether 1/2a serotypes are more prone to mutations compared to 4b and other serotypes the capacity of these strains to acquire adaptive mutations was also measured.

3.2 Materials and methods

3.2.1 Bacterial isolates

Listeria monocytogenes used in the experiments were recovered from food processing environment (FPE) swabs (n=29), raw unprocessed food (RUF; n=6), and ready-to-eat (RTE) foods ($n_{processing}$=19, and n_{retail}=2) that were collected from three dairy, five fish, and five meat processing facilities (PF), and two retail facilities (RF) located in B.C. (described in detail in Chapter 2). Using origin of isolation (see Table B-1, Appendix B), serotyping (Figure 2-4, Chapter 2) and PFGE data (Figure 2-5; Chapter 2), a total of 56 different isolates were selected for inclusion in this study. Isolate origins and serotype data for the 56 isolates used in experiments are described in Table 3-1. *Listeria monocytogenes* cultures were maintained in peptone with 20% glycerol at -80°C. Prior to conducting the experiments, isolates were grown overnight on tryptic soy agar (TSA; Difco, Becton Dickinson Diagnostics, Mississauga, ON, Canada) at 37°C.

3.2.2 Internalin A sequencing

Conventional polymerase chain reactions (PCR) were used to amplify the 2.4 kb *inlA* gene. Briefly, 5 U of AmpliTaq Gold 360 DNA polymerase (Invitrogen, Burlington, ON) was used with one set of custom primers (*inlA*-JK-F 5'-TAC AAC GAA ACC TGA TAT TG-3' and *inlA*-JK-R 5'-GCT AGA TAT AGT CCG AAA AC-3'), each at 0.5 mM, 200 mM dNTPs (Invitrogen), and 50-100 ng DNA template (50-100 ng) obtained using a DNeasy Blood and Tissue kit (Qiagen, Toronto, ON). Thermocycling was performed as follows: initial denaturation was set at 94°C for 2 min; 20 cycles of 94°C for 1 min, 60-50°C for 1 min with touchdown decrease of 0.5°C per cycle, and 72°C for 2.5 min; 20 cycles of 94°C for 1 min,

50°C for 1 min, and 72°C for 2.5 min; and a final extension step at 72°C for 7 min (Van Stelten et al., 2010). PCR product was purified using a QIAquick PCR Purification kit (Qiagen) and sequenced at Canada's Michael Smith Genome Science Centre using the *inlA*-JK primer set and previously published primers (Van Stelten et al., 2010). Nucleotide sequences were assembled and analyzed with Geneious 5.4 software (Biomatters Ltd., Auckland, New Zealand). The presence of PMSCs was determined by comparing *inlA* sequence data to *L. monocytogenes* EGD-e (Glaser et al., 2001).

3.2.3 Multilocus sequence typing

Serotype and PFGE data were used to select a subset of isolates (n=56), representing each positive food or environmental sample for MLST analysis (Table B-1). For example, when more than one isolate originated from the same sample, and serotype and PFGE patterns were identical, only one isolate was used in the MLST analysis. MLST was performed using seven housekeeping genes as previously described by Ragon et al. (2008). Briefly, conventional polymerase chain reaction (PCR) was used to amplify the genes. DNA was isolated from overnight cultures grown on TSA (Difco). A single colony was resuspended in 100 µl of 1 x Tris-EDTA buffer, heated at 90°C for 10 min, cooled on ice for 2 min, and spun at 16,000 x *g* for 5 min. PCR reactions (50 µl) using 1 U of Platinum® *Taq* DNA polymerase (Invitrogen, Burlington, ON), 0.2 µM of each primer, 2 mM dNTPs (Roche, Mississauga, ON), and template DNA (1 µl) were cycled as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 2 min; followed by 68°C for 7 min (BioRad C1000 Thermal Cycler). All amplicons were purified (Amicon Ultra-0.5 ml Centrifugal Filter Devices) and sequenced at the Canadian National Microbiology Laboratory on both strands with an

ABI3730 machine (Applied Biosystems), and sequencing primers -20M13 (5'-GTAAAACGACGGCCAGT-3') and -29M13-Rev (5'-CAGGAAACAGCTA TGACC-3'). Sequences were assembled using BioNumerics (v.6.5, Applied Maths, St. Martens-Latem, Belgium) and subsequently uploaded to the *L. monocytogenes* MLST database (http://www.pasteur.fr/mlst) maintained by the Institut Pasteur. MLST profile or sequence type (ST) was assigned to each isolate after comparison to the online database. For any novel allele/ST, data were forwarded to the Institut Pasteur for designation of allele and ST numbers. Grouping of STs into clonal complexes (CC) was based on the scheme set by the Institut Pasteur, where STs belonging to the same CC do not have more than one allelic mismatch (Ragon et al., 2008).

Allelic profile-based comparison was performed using a minimum spanning tree (MST) analysis (BioNumerics). This analysis links ST profiles so that the sum of distances (i.e. number of distinct alleles between two STs) is minimized (Ragon et al., 2008). In the MST, each circle represents an ST with a unique number, thus indicating distinct allele combinations of the seven housekeeping genes, while different circle sizes are proportional to the number of tested isolates within an ST profile (Ragon et al., 2008).

3.2.4 Invasion of Caco-2 cells

The invasion efficiency of seven representative *L. monocytogenes* isolates from different serotypes, food and environmental samples, and *inlA* genotypes were assessed in 24-well tissue culture plates according to Gaillard et al. (1996), with minor modifications. Briefly, Caco-2 cells (~2 x10^5 cells per well; passages 5 to 20) were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; HyClone®, Thermo Scientific, Toronto, ON),

supplemented with 10% inactivated fetal calf serum (GIBCO, Life Technologies, Burlington, ON), 1% non-essential amino acids (GIBCO), and 1% GlutaMAX (GIBCO) for two days (5% CO_2, at 37°C) to reach confluency. *Listeria monocytogenes* cultures grown statically overnight in brain heart infusion (BHI; Oxoid, Ottawa, ON) broth at 30°C were pelleted by centrifugation (5,939 x *g* at 22°C; Eppendorf 5415 R), washed once with 1x Dulbecco's phosphate buffered saline (DPBS; HyClone®) with magnesium and calcium, re-hydrated in DPBS, and adjusted to OD_{600nm} = 0.5 (Genesys 10UV, Thermo Spectronic, Rochester, NY). Prior to infection, *L. monocytogenes* cultures were diluted in DMEM to approximately 4 x 10^7 colony forming units (CFU) per ml, as assessed by growth on TSA. Bacterial suspensions (0.5 ml) were added to Caco-2 cells and incubated at 37°C for 1 h to allow bacterial entry. Cells were washed three times, overlaid with fresh DMEM containing gentamicin (10 mg/l), and incubated at 37°C for 2 h. Following incubation, the cell monolayer was washed three times with DPBS and treated with 1% Triton X-100 for 10 min at 37°C. The number of viable bacteria released was quantified by spread plate method on TSA. *Listeria monocytogenes* EGD-SmR and BUG5 (Tn*1545*-induced *inlA* mutant from EGD-SmR) (Gaillard et al., 1991), and 08-5578 (Gilmour et al., 2010), kindly provided by Dr. Pascale Cossart (Institut Pasteur) and Dr. Matthew Gilmour (Public Health Agency of Canada), respectively, were used as controls. The gentamicin concentration used (10 mg/l) was confirmed to kill all extracellular bacteria by spreading post-wash medium onto TSA. Invasion assays for each isolate were carried out in triplicate and repeated twice.

3.2.5 Mutation frequency

Since differences in the occurrence of PMSCs in *inlA* exist amongst different serotypes, frequency of isolates (n=56) acquiring mutations following exposure to rifampicin (RIF) was measured. Previously published methodology described for Enterobacteriaceae, with some modifications was applied (Allen and Poppe, 2002; LeClerc et al., 1996). Briefly, isolates were grown overnight at 35°C in BHI broth and adjusted to an OD_{600nm} = 1.0. A 100 µl aliquot was spread onto BHI agar with 100 µg/ml RIF. Following incubation for 48 h at 35°C, the number of CFU was counted. The assay for each isolate was carried out in triplicate and repeated two times. The mean number of colonies for all strains was determined, and comparisons made between strains with and without PMSCs in *inlA* and across serotypes.

3.2.6 Cold growth evaluation

A subset of isolates (n=33) representing *L. monocytogenes* with full-length *inlA*, 3-codon deletion (a.a. 738-740), and each type of PMSC observed in our collection was assessed for cold growth adaptation, as described by Arguedas-Villa et al. (2010). In short, a single colony was inoculated into 10 ml BHI and grown overnight at 37°C with shaking (220 rpm) (~10^9 CFU/ml). Fresh BHI (10 ml) was inoculated with approximately 10^3 CFU/ml and incubated at 4°C until bacteria reached stationary phase. Growth was monitored by spreading 10-fold serial dilutions prepared in peptone buffered saline onto plate count agar (Oxoid), incubating at 37°C for 24 h, and CFUs were counted. The lag phase duration (LPD) and maximum growth rate (MGR) of each strain were calculated from log converted growth

(CFU/ml) data using Dmfit version 2.0 and Microfit version 1.0 programs, based on the models of Baranyi and Roberts (1994).

3.2.7 Statistical analysis

Data analysis was performed using GraphPad Prism 6.0 software. The statistical significance of differences in *inlA* genotypes based on isolate source (FPE, RUF and RTE foods) was assessed using chi-square and Fisher's exact test (RUF and RTE foods). The Student's *t*-test was used to compare invasive *inlA* genotypes to control strains (08-5578, EGD-SmR or BUG5), and to examine whether differences exist in LPD and MGR between food and environmental strains. Mutability, as indicated by the number of RIF^R colonies, among serotypes (1/2a, 1/2c, 3a and 4b) was compared using Kruskal-Wallis test for nonparametric data, followed by Dunn's multiple comparisons test. Differences between *inlA* genotypes (no PMSCs *vs.* PMSCs) were assessed by the Mann-Whitney test. A Fisher's exact test was performed to assess whether differences existed between cold adapting groups (fast, intermediate) and *inlA* genotypes (no PMSC *vs.* PMSCs). For all analyses, differences were considered significant if p was < 0.05.

3.2.8 Nucleotide sequence accession numbers

The nucleotide sequences from the isolates in this study have been deposited in GenBank under numbers KC433332 to KC433385.

3.3 Results

3.3.1 Distribution of different sero-, pulso- and sequence types

The majority of isolates examined in this chapter belonged to listeriosis causing serotypes, including 37.5% of serotype 1/2a and 4b. Other serotypes, including 1/2b (4%), 1/2c (14%), and 3a (7%), were isolated at lower rates (Table 3-1).

When subjected to PFGE, 36 pulsotypes were observed among the isolates recovered from PF, and two pulsotypes were recovered among the retail isolates (Table 3-2). Strains discriminated by PFGE and serotyping possessed different sequence types (STs). MLST grouped isolates into 14 STs, with one ST found to be novel (ST662). Distinct STs were observed among lineage I and II isolates (Figure 3-1). Two ST120 isolates, belonging to CC8, were recovered from raw meat, and a RTE fish sample. Seven STs (ST1, ST5, ST7, ST9, ST11, ST120, ST321) were common among the isolates described here and isolates associated with clinical cases of listeriosis in Canada reported by Knabel et al. (2012).

The majority of lineage I isolates (serotype 4b and 1/2b) were recovered from food and environmental samples from fish facilities, while lineage II isolates were widespread among all food categories (Figure 3-2). Interestingly, ST2, ST91, and ST296 were seen exclusively in RTE fish, while ST5 and ST662 were found in environmental and food meat samples, respectively.

ST321, which was the most common ST, included isolates recovered from food and environmental samples from meat and fish facilities, but not dairy. ST7 and ST11 were seen in dairy and meat facility environments, but not in fish processing facilities. Only one ST (ST155) was common between dairy and fish facilities (Figure 3-2).

Table 3-1. Serotypes of *L. monocytogenes* (n=56) isolates, characterized in this study, recovered from food processing environments, raw unprocessed foods (RUF) or ready-to-eat (RTE) foods.

Source	No. of *L. monocytogenes* isolates with each serotype					
	Total No. Isolates	**1/2a**	**1/2b**	**1/2c**	**3a**	**4b**
Food processing environment	29	11	1	5	4	8
Food[a]						
RUF	6	4	0	2	0	0
RTE	21	6	1	1	0	13
Total No. (%)	**56**	**21 (37.5)**	**2 (4)**	**8 (14)**	**4 (7)**	**21 (37.5)**

[a]RUF, raw unprocessed food; RTE, ready-to-eat food products.

3.3.2 *inlA* genotypes and mutability among *L. monocytogenes* strains

DNA sequencing of *inlA* in 54 *L. monocytogenes* strains originating from food and food processing environment samples recovered from dairy, fish, and meat processing facilities revealed *inlA* PMSCs in 35% of isolates, while no *inlA* PMSCs were found in the two isolates from retail samples. Isolates possessing truncated InlA due to PMSC mutations are hereafter referred to as PMSC isolates. Type 3 mutations (amino acid [a.a.] position 700) (Figure 3-3) were the most common PMSC mutation in this collection (10/19), followed by type 4 (6/19) (a.a. position 9), type 11 (2/19) (a.a. position 685), and only a single isolate (1/19) possessed a type 1 mutation (a.a. position 606) (Table 3-3) (Van Stelten et al., 2010). Overall, 41% (22/54) of PF isolates encoded a full-length *inlA*, while 24% (13/54) had a 3-codon deletion in a.a. positions 738 to 740 (aspartic acid, threonine and serine), hereafter referred to as 3-codon deletion. Two isolates from retail possessed full-length *inlA*, however, a serotype 1/2a isolate (LR39) possessed eight non-synonymous and 26 synonymous mutations, while 19 non-synonymous and 63 synonymous mutations were observed in the 1/2b isolate (LR59).

Table 3-2. Distribution of *L. monocytogenes* (n=111) sero- and pulsotypes, and their *inlA* profiles, across different facilities (n=15).

Facility ID	Type	Number of isolates recovered	Isolate source[a]			Serotypes	Pulsotypes (PFGE)[b]	*inlA* genotype (positive/tested)	
			FPE	RUF	RTE			No PMSC[c]	With PMSC
Food processing facilities									
d5	Dairy	9	9	-	-	1/2a	F1, G2	3/3	-
d7	Dairy	1	1	-	-	1/2a	N1	1/1	-
d11	Dairy	3	3	-	-	1/2a	G1	1/1	-
f19	Fish	15	12	-	3	1/2c, 3a	H1, H4, K2, K3, K4, K5	-	10/10
f20	Fish	21	9	-	12	1/2a, 4b	A1, L1, M1	8/8	-
f21	Fish	10	6	-	4	1/2a, 4b	A2, K1, K2, K4, K6, K8, K9	1/4	3/4
f28	Fish	11	6	-	5	1/2a, 4b	B1, H3	6/7	1/7
f31	Fish	16	1	-	15	1/2a, 4b	B1, C1, C2, E1, I1, I2	10/10	-
m38	Meat	3	-	3	-	1/2a, 1/2c	H2, J1	1/2	1/2
m44	Meat	3	-	3	-	1/2a	H6	1/1	-
m46	Meat	3	3	-	-	1/2a	G3, G4	1/1	-
m49	Meat	6	6	-	-	1/2a, 1/2b	D1, D2, F2, K10	1/3	2/3
m50	Meat	4	-	4	-	1/2a, 1/2c	H5, K7	-	3/3
	Total	105							

Table 3-2. Continued.

Facility ID	Type	Number of isolates recovered	Isolate source[a]			Serotypes	Pulsotypes (PFGE)[b]	*inlA* genotype (positive/tested)	
			FPE	RUF	RTE			No PMSC[c]	With PMSC
Retail facilities									
rf7	Fish	3	-	-	3	1/2a	LMACI.0001 LMAAI.0001	1/1	-
rf11	Fish	3	-	-	3	1/2b	LMACI.0470 LMAAI.0584	1/1	-
	Total	6							

[a]FPE, food processing environment; RUF, raw, unprocessed food; RTE, ready-to-eat food.
[b]Pulsotype designation is based on *Apa*I and *Asc*I enzymes and Tenover et al. (1995) composite designation in the isolate population originating from food processing facilities, where an identical letter/number indicates indistinguishable pattern; same letter and different number indicate closely related isolates and different letters represent unrelated isolates.
[c]PMSC, premature stop codon.

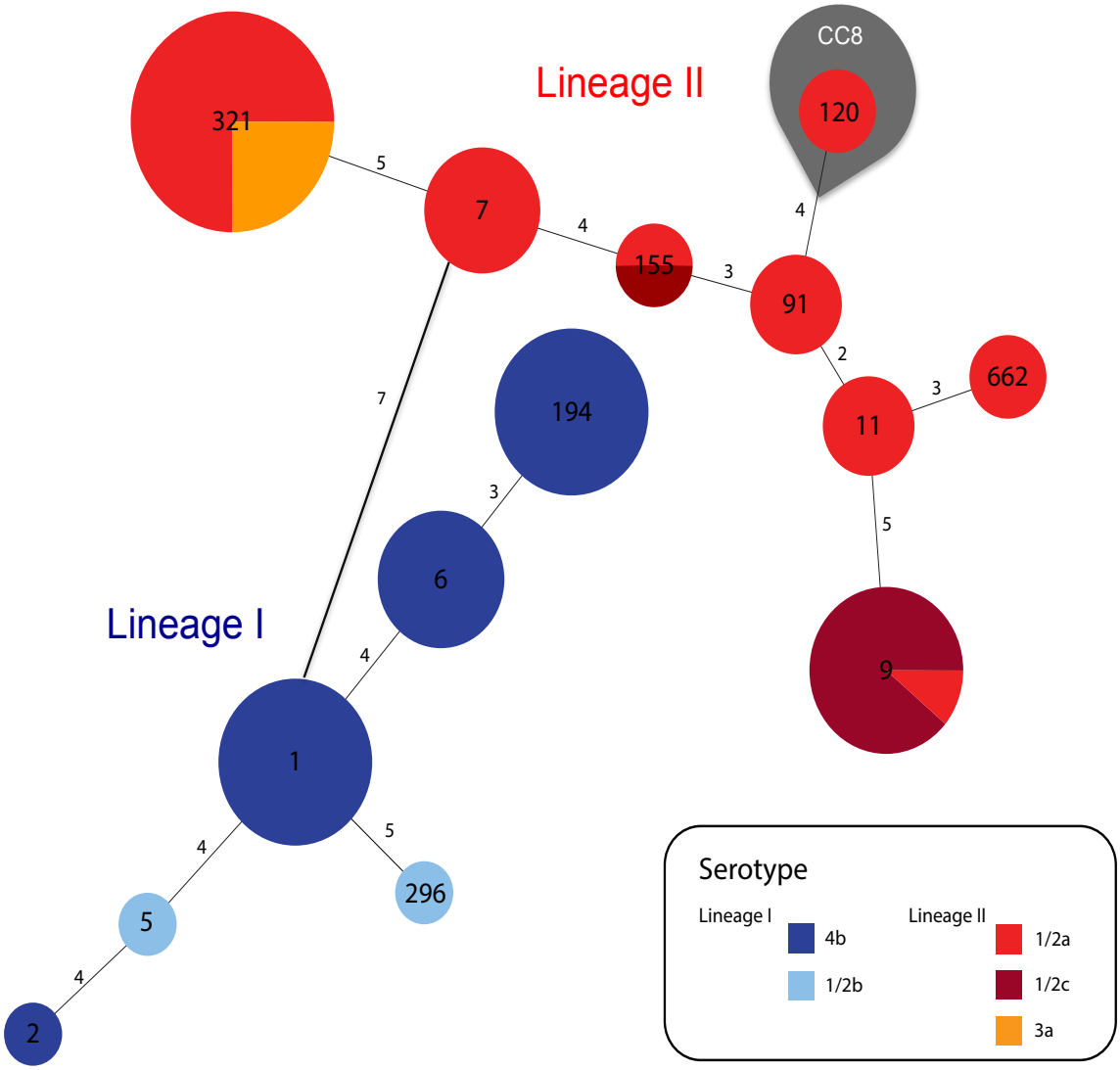


Figure 3-1. Minimum spanning tree of different serotypes of *L. monocytogenes* derived from the food chain, created using Bionumerics v6.5. Branch numbers indicate allele differences between connected sequence types; node diameter is in positive correlation to number of isolates; node color shows isolate serotype. Dark shading represents clonal complex 8 (CC8).

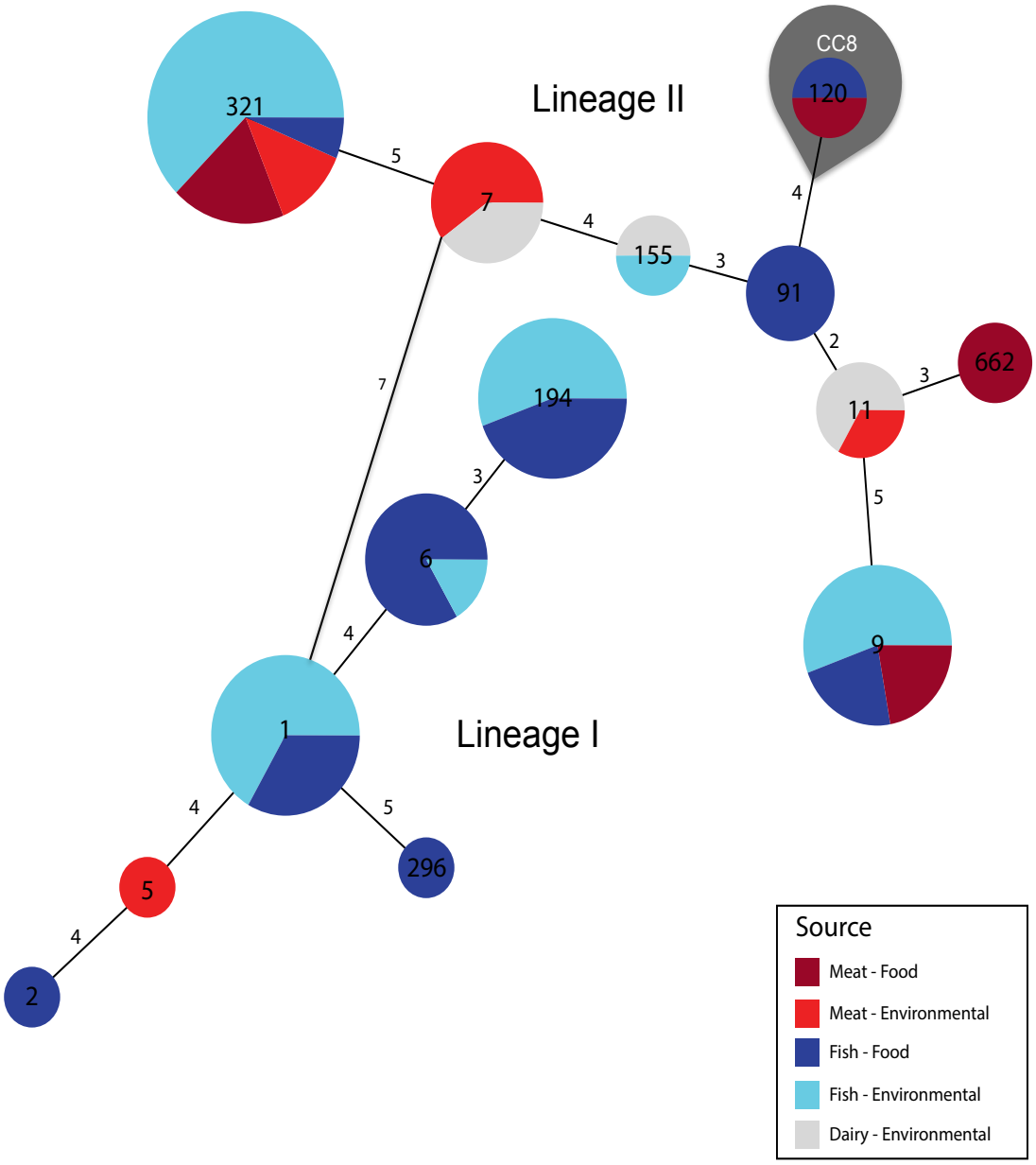


Figure 3-2**.** Minimum spanning tree of *L. monocytogenes* derived from different sources within the food chain, created using Bionumerics v6.5. Branch numbers indicate allele differences between connected sequence types; node diameter is in positive correlation to number of isolates; node color shows isolate source. Dark shading represents clonal complex 8 (CC8).

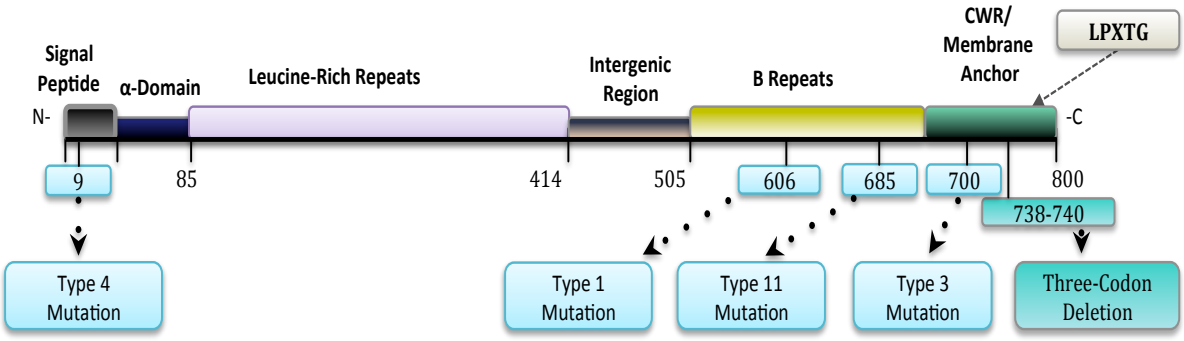


Figure 3-3. Full-length *inlA* illustration, with the scale below representing amino acid positions, and types of mutations that occur due to premature stop codons. CWR, cell wall spanning region; LPXTG, leucine-proline-variable-threonine-glycine peptidoglycan anchored protein.

Since *inlA* PMSCs have been frequently reported in serotype 1/2a strains, the mutability of 1/2a isolates compared to other serotypes, including 4b, was investigated. Point mutations occurring in the *rpoB* gene, encoding RNA polymerase beta subunit have been shown to afford resistance to RIF (RIF^R) (Wehrli, 1983). Irrespective of serotype, significantly more RIF^R colonies were observed in strains not possessing *inlA* PMSCs compared to those with *inlA* PMSC mutations (p=0.0015) (Figure 3-4A), suggesting that different environmental and/or clinical pressures influence the mutation rates in *inlA* and *rpoB* genes. Correspondingly, significantly more RIF^R colonies were observed for 4b serotype strains compared to 1/2a, 1/2c, and 3a strains (p=0.0002) (Figure 3-4B).

3.3.3 Distribution of *inlA* genotypes across different food processing facilities

No PMSCs were seen in *inlA* of *L. monocytogenes* isolates recovered from RF, or dairy facilities, while 33% and 60% of isolates from fish and meat facilities, respectively, had PMSCs. Of five fish facilities examined, three facilities had isolates lacking PMSCs, while two facilities had PMSCs in all (10/10) or 75% of recovered isolates (Table 3-2).

Table 3-3. Number of *L. monocytogenes* isolates[a] recovered from food processing environments (n=29), raw unprocessed (n=6) and ready-to-eat (n=21) foods with full-length *inlA*, *inlA* mutations resulting in premature stop codons (PMSC) or 3-codon deletions.

***inlA* Genotype**	**No. (%) environmental isolates**	**No. (%) food isolates**		**Serotype (No. of strains)**	**Facility ID**
		RUF	**RTE**		
Without PMSC[a]	16 (55)	2 (33)	19 (90)	1/2b (1), 1/2a (15), 4b (21)	d5, d7, d11, f20, f21, f28, m38, m44, m46, m49
With 3-codon deletion (a.a. 738 - 740)	4 (14)	0	9 (47)	1/2a (1), 4b (12)	f20, f31
With PMSC	13 (45)	4 (67)	2 (11)		
Type 1 (a.a. 606)	1 (3)	0	0	1/2b (1)	f49
Type 3 (a.a. 700)	7 (24)	2 (33)	1 (5)	1/2a (6), 3a (4)	f19, f21, f49, f50
Type 4 (a.a. 9)	5 (17)	0	1 (5)	1/2c (6)	f19
Type 11 (a.a. 685)	0	2 (33)	0	1/2c (2)	f38, f50

[a]Numbers do not add up, as isolates without PMSCs also include the isolates possessing 3-codon deletion.

More meat facilities (n=3) had *L. monocytogenes* isolates possessing *inlA* PMSCs than isolates without mutations (n=2). Two meat facilities had no PMSC mutations in their isolates, one had PMSCs in all isolates (n=3), and two facilities had 50% and 67% of isolates with mutations (Table 3-2). Type 3 mutations were found among isolates from fish and meat facilities, while type 4 was only seen in isolates from fish facilities, and types 1 and 11 only in *L. monocytogenes* from meat facilities. The most common type of mutation among fish isolates possessing PMSCs was type 3, followed by type 4 mutations. Similarly, type 3 mutations most commonly occurred in isolates from meat facilities, followed by type 11 and type 1 mutations (Appendix B, Table B-1).

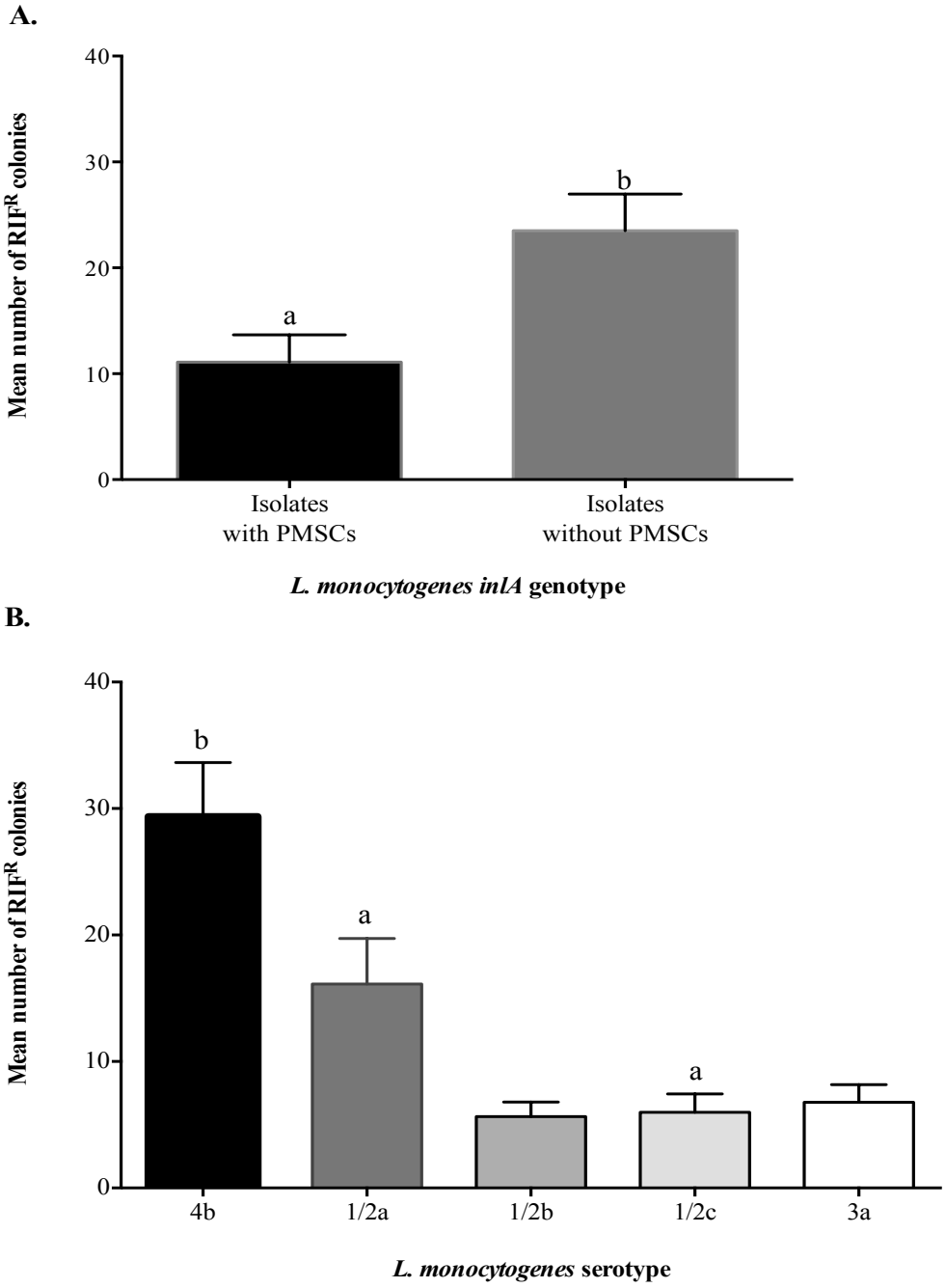


Figure 3-4. Mutability of different *L. monocytogenes inlA* genotypes (A) and serotypes (B) assessed by the number of rifampicin-resistant colonies after 48 h growth at 35°C in the presence of 100 µg/ml rifampicin. Mutability of each isolate was assayed in triplicate in each experiment, and two independent experiments were performed. Bars represent mean number of colonies, and error bars indicate standard error of the mean. Different letters above the bars represent significant differences ($p<0.05$) between geno- and serotype groups determined using the Mann-Whitney (A) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (B). Serotype 1/2b and 3a isolates were excluded from statistical analysis due to small number of isolates examined.

Isolates possessing the 3-codon deletion were observed in two fish (f20, f31), but not in dairy or meat facilities (Table 3-2). In one of the facilities (f20), all but one isolate (88%) had this deletion, while 60% of samples had the same codons missing in the other facility (f31). PFGE typing showed these isolates were not clonal. None of the isolates from facilities f20 and f31 possessed PMSCs.

3.3.4 *inlA* mutations within different serotypes and multilocus sequence types

PMSC mutations in *inlA* were observed in four of the five serotypes examined, including all 1/2c (n=8) and 3a (n=4) isolates, followed by 1/2a (30%; n=6), and one 1/2b (50%; n=2) isolate. Serotypes 1/2a and 3a carried only type 3 mutations while serotype 1/2b had only a type 1 mutation. The only serotype with more than one type of mutation (i.e. type 4 and 11) was 1/2c.

A 3-codon deletion was observed in 13 strains derived from fish processing facilities. With the exception of one 1/2a isolate, this deletion was seen in 4b serotype strains. Overall, 57% of 4b serotype isolates possessed this 3-codon deletion, though no *inlA* PMSC mutations were found in serotype 4b isolates.

Eleven different STs were observed among full-length *inlA* genotypes. ST5 and ST321 were seen exclusively in type 1 and type 3 *inlA* PMSC isolates, respectively. ST9 was associated with type 4 and 11 *inlA* mutations; however, full-length *inlA* genotypes were also observed in those STs. The majority of 3-codon deletion isolates were grouped into ST194 (6/13) and ST6 (6/13), while one isolate belonged to ST155. While ST155 and ST194 were also seen among isolates possessing the full-length *inlA*, ST6 was found exclusively in isolates possessing this 3-codon deletion.

3.3.5 Occurrence of *inlA* PMSCs in isolates recovered from different sources

Isolates with *inlA* PMSC mutations were seen more commonly in FPE samples than RUF and RTE foods (p=0.0068). Further, more isolates from RUF (4/6) carried *inlA* PMSCs compared to those isolated from RTE (2/21) foods (p=0.011). Nine of the 13, 3-codon deletion mutants were recovered from RTE foods (69%), with the remaining isolates being environmental. Isolates encoding a full-length InlA (i.e. excluding isolates with the 3-codon deletion) were observed predominantly in FPE samples (12/24), followed by RTE (10/24) and RUF (2/24) samples (Table 3-3).

3.3.6 Invasion of Caco-2 cells by *L. monocytogenes* strains possessing truncated InlA or 3-codon deletion

inlA PMSC-encoding isolates exhibited reduced Caco-2 cell invasion phenotypes (Figure 3-5). A 4b isolate (FF46-3) possessing wild type *inlA* was 2.2 times more invasive (p<0.0001) than a clinical isolate (08-5578) which caused 23 deaths during a 2008 deli meat listeriosis outbreak in Canada. This isolate was also 10.8 times more invasive (p<0.0001) than the laboratory control EGD-SmR strain (Figure 3-5). This phenomenon was observed for another 4b isolate (FF19-1) and a 1/2a strain (FE13-1) possessing the 3-codon deletion, both of which were 4.7 and 7.1 times more invasive (p<0.0001), respectively, compared to the control EDG-SmR strain. When compared to the Canadian deli meat outbreak strain 08-5578, FF19-1 and FE13-1 were as invasive, or 1.4 times more invasive (p=0.006), respectively (Figure 3-5).

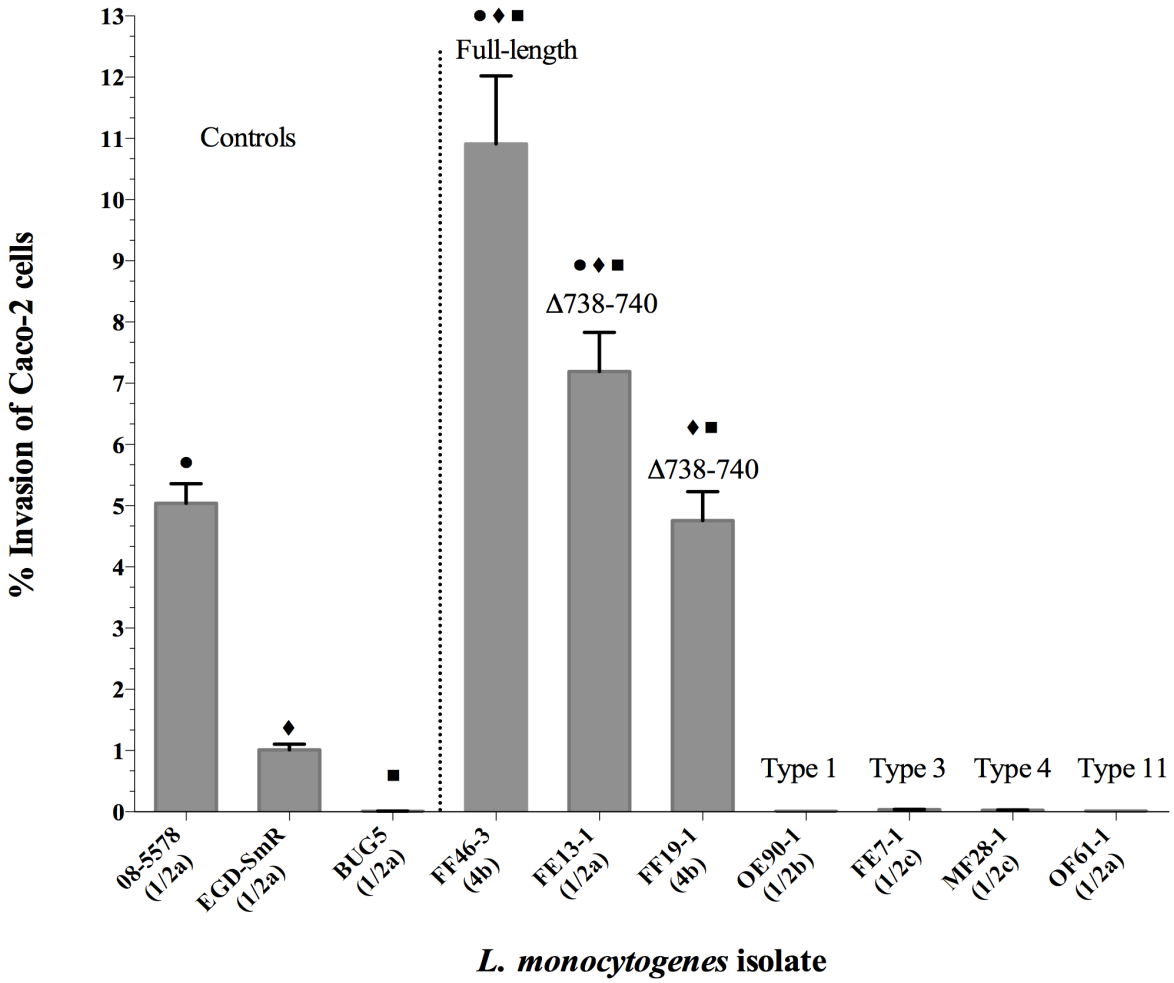


Figure 3-5. Invasion efficiency (% of bacteria recovered relative to initial inoculum) of *L. monocytogenes* isolates possessing *inlA* PMSC mutations (type 1, 3, 4 and 11) or a 3-codon deletion at amino acid position 738 to 740 (Δ738-740) compared to wild type clinical isolates (08-5578 and EGD-SmR) and a Tn*1545*-induced non-invasive *inlA* mutant of EGD-SmR (BUG5). Assays for each isolate were carried out in triplicate and repeated two times. Bars represent mean invasion efficiencies, and error bars indicate standard error of the mean. Different symbols above the bars indicate significantly higher invasion efficiency (p <0.05; *t*-test) when compared to controls 08-5578 (●), EGD-SmR (◆) or BUG5 (■).

3.3.7 Cold growth adaptation of strains from different serogroups and sources

Three cold growth categories were observed among 33 isolates assessed for their ability to adapt to 4°C following downshift from 37°C. The first category included fast adapting strains

(n=15) possessing a LPD less than 70 h. The second group was comprised of intermediate cold growth adaptors with LPD ranging between 70 to 200 h, and included the majority of strains (n=13). Finally, five strains adapted slowly to 4°C, possessing a LPD > 200 h.

Fast adapting strains included mainly RTE food-derived isolates, and to a lesser degree, FPE and RUF isolates, while intermediate cold growth adaptors were recovered predominantly from FPE, but also included isolates from RTE and RUF foods (Figure 3-6). Slow growing strains were seen only in FPE and RTE samples (Figure 3-6). No significant differences were observed in LPD or MGR between food and environmental isolates (Figure 3-7).

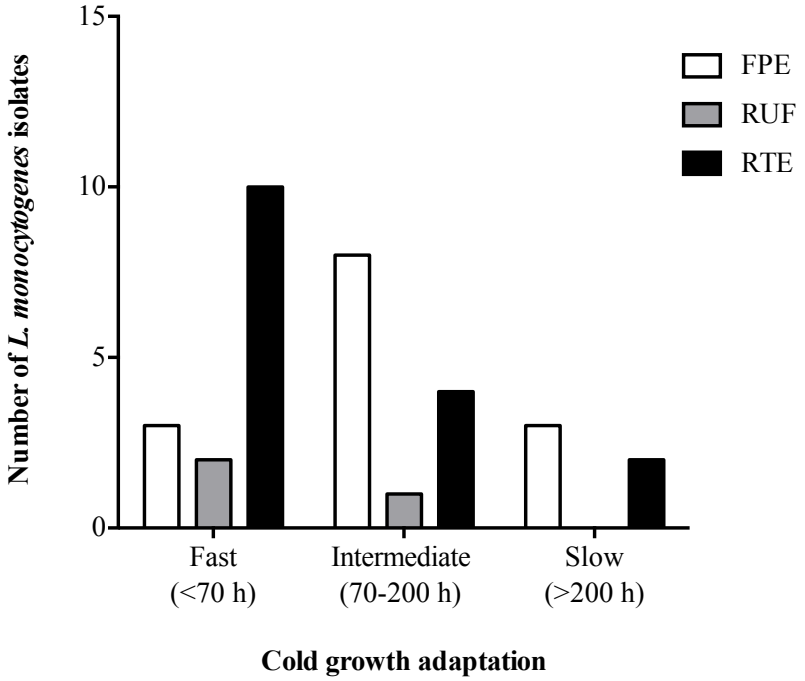


Figure 3-6. The distribution of *L. monocytogenes* isolates recovered from food processing environments (FPE), raw unprocessed (RUF), and ready-to-eat (RTE) foods, within three cold growth adapting groups, when grown at 4°C. Differences were not statistically significant (p>0.05, chi-square).

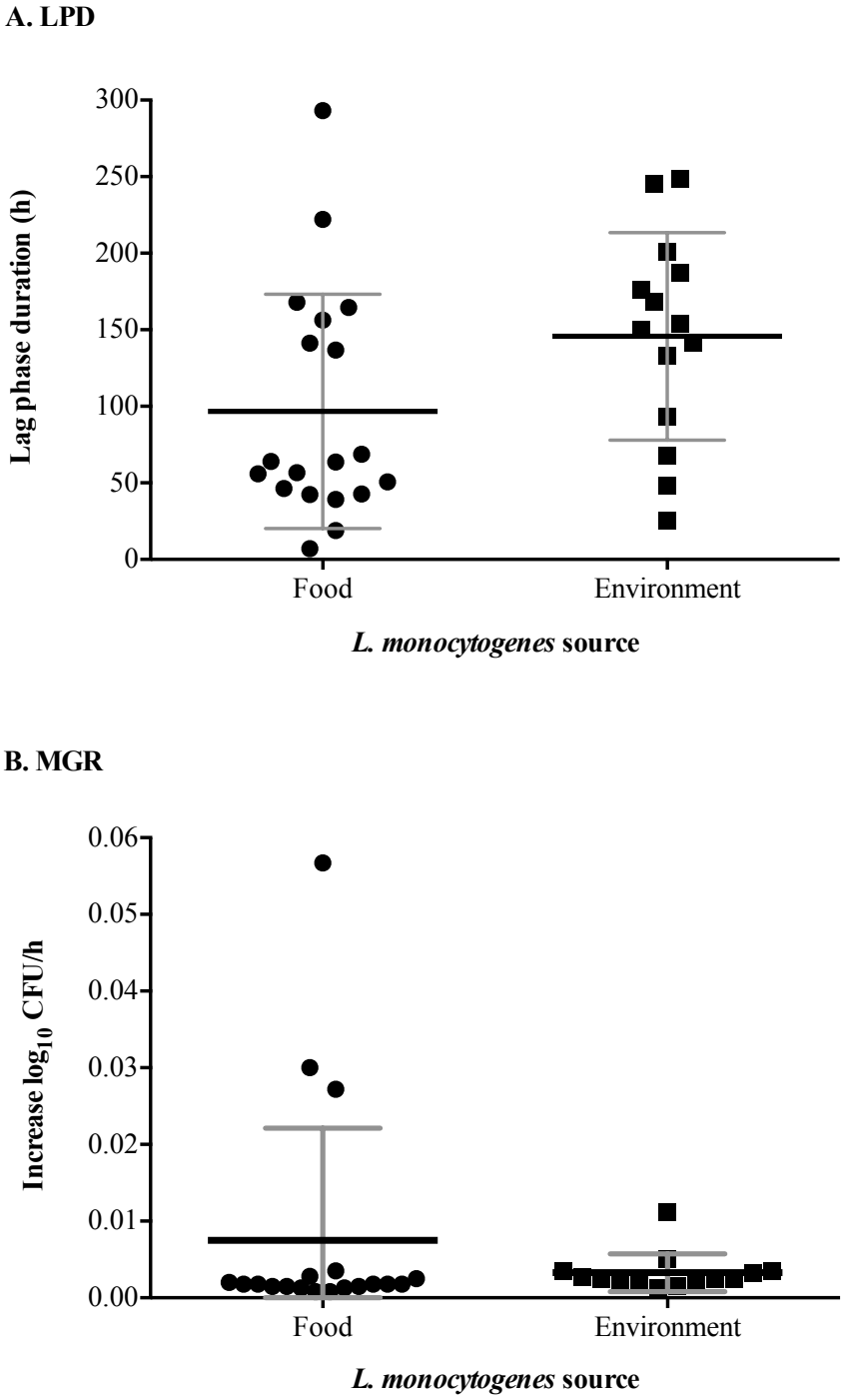


Figure 3-7. Lag phase duration (A) and exponential growth rate (B) of 33 *L. monocytogenes* isolates recovered from food and food processing environments following a down-shift from 37 to 4°C in BHI. Each isolate was assayed in duplicate, and two independent growth assays were performed. Middle horizontal lines represent mean values, with standard deviations.

The majority of fast adapting strains were of the serotype 4b (53%), followed by 1/2a (40%) and 1/2c (7%) serotypes. Intermediate cold-adaptors were represented predominantly by 1/2a strains (46%), followed by 1/2c, 4b, 3a and 1/2b serotypes, respectively. Of the five slow adapting strains, three were 1/2a and two 4b serotypes.

3.3.8 Cold growth adaptation of different *L. monocytogenes inlA* genotypes

Significant differences in the ability of different isolates to adapt and grow at 4°C, both with and without *inlA* PMSCs were observed (fast *vs.* intermediate, p=0.042). Overall, intermediate cold adapting strains more frequently possessed *inlA* PMSCs (70%) compared to fast (20%) and slow (10%) cold adaptors (Figure 3-8A). In contrast, with the exception of two isolates (serotypes 1/2c and 1/2a), fast adapting strains lacked *inlA* PMSCs (Figure 3-8B). Notably, isolates possessing a wild type *inlA* (i.e. full length InlA) or the 3-codon deletion comprised 57% of fast-adapting strains, followed by 26% intermediate, and 17% of slow growing strains (Figure 3-8A).

3.4 Discussion

Results obtained in these series of experiments demonstrated variability in *inlA* genotypes among *L. monocytogenes* isolates recovered from foods and processing environments in B.C. that were often unique within food processing facilities. Overall, 34% (19/56) of examined isolates possessed mutations in *inlA* due to PMSCs, which is lower than the rate reported for food-chain isolates in the United States (US) (45%) (Chen et al., 2011; Ward et al., 2010), but similar to levels reported in France (Jacquet et al., 2004). In addition to previously described *inlA* mutations, including types 1, 3, 4 and 11, *inlA* genotypes with a consecutive

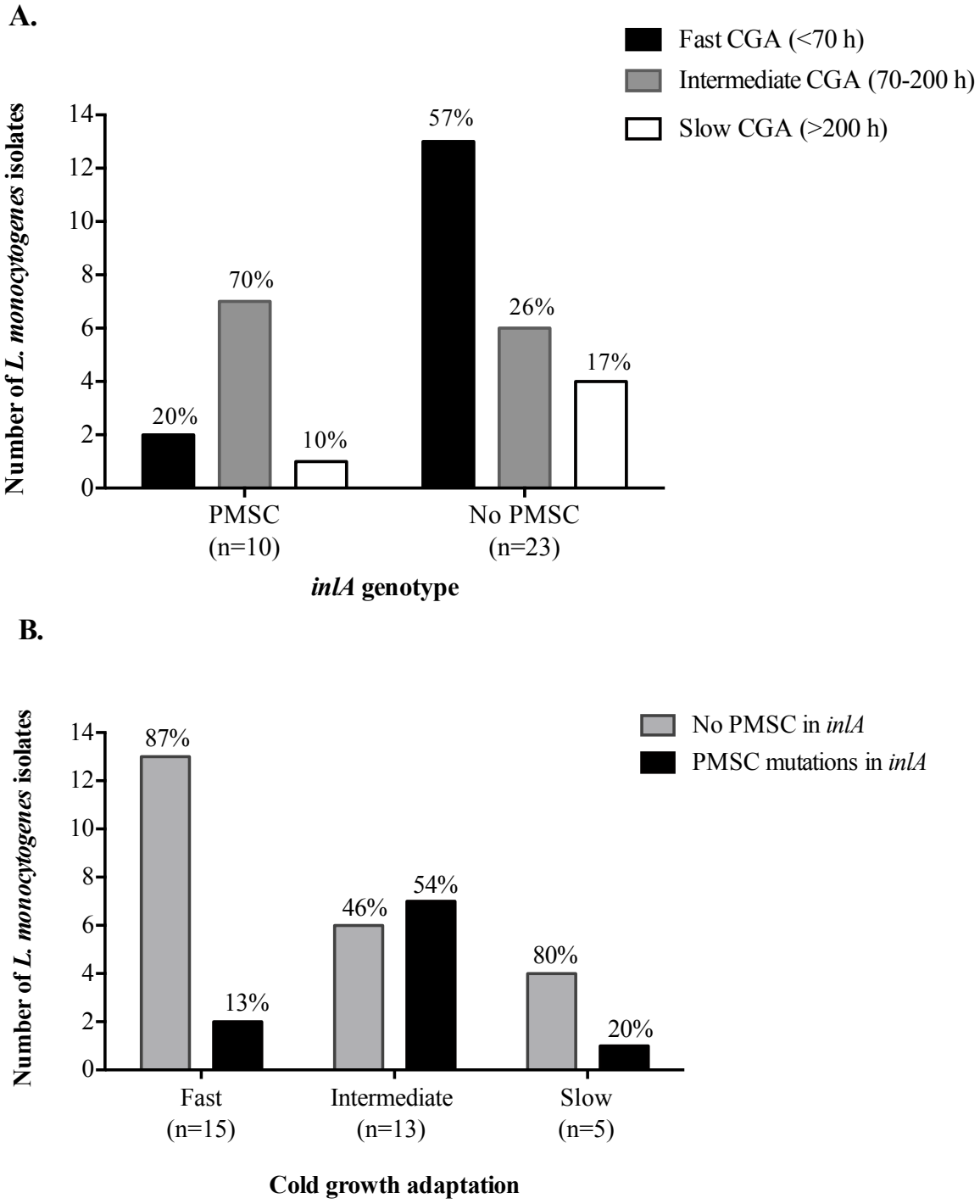


Figure 3-8. Identification of *L. monocytogenes* isolates with or without premature stop codons (PMSC) in *inlA* as fast (<70 h), intermediate (70-200 h) or slow (>200 h) cold growth adaptors (CGA), following a temperature down-shift from 37 to 4°C in BHI. Differences in cold growth adaptation between fast and intermediate *L. monocytogenes inlA* genotypes were significant (Fisher's exact, p=0.042). The percentage of isolates within geno- (A) and phenotypic groups (B) is indicated above bars.

3-codon deletion in the amino acid positions 738 to 740 were observed. To date, this phenomenon has been reported only in a single isolate from a meat facility in Portugal (Ferreira et al., 2011). It has been suggested that certain *inlA* PMSC mutations accumulate at the population level with notable differences in *inlA* PMSCs occurring in North America compared to European countries (Rousseaux et al., 2004; Van Stelten et al., 2010; Ward et al., 2010). Interestingly, type 11 (a.a. 685) *inlA* PMSC mutation was seen in B.C. isolates, which to date have not been reported outside of France (Chen et al., 2011; Felicio et al., 2007; Van Stelten et al., 2010; Ward et al., 2010).

MLST data showed grouping of isolates into 14 different STs, 13 of which have been reported worldwide (Chenal-Francisque et al., 2011; Roche et al., 2012) and one (ST662) unique to B.C. isolates. It has been suggested that certain clonal complexes (e.g., CC1, 2, 3 and 9) are widely distributed across continents, while some predominate in specific regions (e.g., CC288 in North America, CC6 in Europe). In agreement with this, CC1, 2, and 9 isolates occurred in the B.C. isolate collection. Interestingly, CC8 isolates have been reported to dominate amongst clinical isolates in Canada (Knabel et al., 2012). Specifically, Knabel et al. (2012) reported that 1/2a serotype isolates, with similar PFGE patterns, and belonging to CC8 have been recurring causes of sporadic and outbreak-linked listeriosis in Canada since 1980s (Knabel et al., 2012). A strain that caused a large listeriosis outbreak in the summer of 2008 and led to 23 deaths of Canadian consumers of deli meat belonged to CC8 (Gilmour et al., 2010; Knabel et al., 2012). Two ST120 isolates, belonging to CC8, were found in the *L. monocytogenes* collection examined here (OF28-1 and LR39-1). One of the isolates was recovered from a raw meat sample (OF28-1) at a meat facility (m44), while the other originated from a RTE fish sample collected at retail (rf7). Although only two isolates

belonging to CC8 were recovered in the current study, considering the link with clinical strains, the presence of CC8 in the food chain-derived isolates is of concern.

While no particular ST has been associated with wild type *inlA* genotypes, ST9 isolates have been reported to commonly carry *inlA* mutation types 11, 12, and 14 (Roche et al., 2012). Type 11 *inlA* mutations were also seen in two of the B.C. ST9 isolates, while six of the isolates possessed type 4 mutations. In addition, ST6 and ST194 carried a 3-codon deletion at amino acid position 742 in French isolates from foods and food processing environments (Roche et al., 2012). Interestingly, ST6, ST194, and ST155 isolates from B.C. possessed a similar 3-codon deletion but at a different amino acid position (738 to 740).

It is well established that frameshift and transition/transversion mutations in *inlA* can lead to PMSCs, resulting in a truncated or non-secreted InlA. Strains possessing these genotypes are associated with attenuated virulence (Bonazzi et al., 2009; Jonquieres et al., 1998; Nightingale et al., 2005) and are predominantly seen in *L. monocytogenes* adapted to environmental and food processing niches (i.e. 1/2a serotype strains), and to a lesser degree in clinical strains overrepresented by 4b serotypes (Jacquet et al., 2004; Ward et al., 2010). In-line with this, isolates in the present study belonging to 1/2a, 1/2c, and 3a serotypes possessed *inlA* PMSC mutations. In general, 4b strains are typically more conserved in their genetic content, exhibit lower recombination rates, and are less likely to possess plasmids and extra-chromosomal elements (Orsi et al., 2011; Orsi et al., 2007; Ragon et al., 2008). Interestingly, when different serotypes were compared in their ability to acquire point mutations leading to RIF^R, 4b strains were significantly (p=0.002) more likely to gain mutations conferring resistance than all other serotypes. Considering that 1/2a serotype strains are known to possess mutations in several virulence loci, including *actA*, *inlA*, and

prfA [reviewed by Orsi et al. (2011)], it was expected 1/2a serotype strains would have higher mutability in this assay. In fact, the opposite was observed, though reasons for this are not clear. This is particularly interesting since positive selection, resulting from the acquisition of advantageous mutations, has been reported to contribute to the evolution of numerous genes in 1/2a strains but is less often reported for 4b serotypes (Dunn et al., 2009; Orsi et al., 2008). Although this assay has not previously been used to examine mutability in *L. monocytogenes*, it has been used to examine mutation rates in Enterobacteriaceae (Allen and Poppe, 2002; LeClerc et al., 1996) in which comparison to reference strains was made to identify hyper-mutability. Results reported here represent the relative ability of strains to acquire mutations leading to RIF^R. Although further work is needed to explore this phenomenon, results obtained for the B.C. population suggest serotype 4b strains may acquire RIF^R mutations more readily than 1/2a strains. Interestingly, the opposite is true for the *inlA* mutations, suggesting different environmental and/or clinical pressures influence the mutation rates in *inlA* and *rpoB* genes. Indirectly, these data suggest that there may be a yet unidentified selection pressure for the maintenance of *inlA* genes encoding a full-length InlA in isolates belonging to 4b serotype.

Highly invasive isolates possessing a 3-codon deletion in *inlA* (a.a. 738-740) were observed within the B.C. collection, which is contrary to a previous report (Ferreira et al., 2011). These isolates exhibited invasion efficiencies equivalent to or surpassing that of the deli meat outbreak strain (08-5578) that contributed to the deaths of 23 individuals in 2008 (Weatherill, 2009) and the EGD-SmR strain (Figure 3-5). In recent years, strains possessing truncated InlA proteins have been identified as strains with lower invasiveness and, accordingly, have been suggested to present reduced public risk (Nightingale et al., 2005;

Nightingale et al., 2008). Although this seems prudent for most *inlA* genotypes in which PMSCs lead to truncated proteins, this characteristic does not apply to the 3-codon deletion observed amongst B.C. isolates recovered in the present study. In contrast, although the InlA protein is truncated by three amino acids, isolates possessing it remain equally, or more, invasive than control strains, thus indicating they are of considerable risk and should be considered a public health concern. Considering 12 of 13 3-codon deletions were serotype 4b, it would be of interest to compare the internalin gene complement of the single 1/2a isolate with this deletion to the 4b strains harboring the same *inlA* genotype. It has been shown experimentally that the deletion of a.a. 714 to 766 corresponding to the pre-anchor region did not reduce the invasiveness when the modified *inlA* gene was transferred to *L. innocua* (Lecuit et al., 1997). However, the impact of a consecutive deletion of aspartic acid, threonine and serine in positions 738 to 740 in naturally occurring *L. monocytogenes* strains has not been described before. It is possible that this deletion may affect protein folding in a manner that would enhance bacterial interaction with its human cell surface receptor E-cadherin. However, it is also possible that other virulence-related factors are contributing to invasion. In particular, a host of other internalin genes (*inlB*, *inlC2*, *inlD*, *inlE*, *inlF*, *inlG*, *inlH*) have been implicated in invasive behavior (Orsi et al., 2011; Raffelsbauer et al., 1998; Tsai et al., 2006). Of these, *inlC2*, *inlD*, *inlE*, and *inlJ* are common to lineage I and II strains, while *inlF*, *inlG*, and *inlH* have only been observed in lineage II (Orsi et al., 2011; Tsai et al., 2006).

In general, it has been proposed that over-representation of serotype 1/2a (lineage II) in isolates that originate from food and FPEs, is a result of their enhanced capacity to survive food chain conditions, though data substantiating this assertion are often conflicting and

limited (Orsi et al., 2011). It has been reported, however, that 4b strains incubated at 4°C for four weeks and subsequently up-shifted to 37°C, indeed possessed shorter LPD than 1/2a isolates (Buncic et al., 2001). This implies 4b strains present in foods may quickly adapt to host temperature, and correspondingly may be more likely to cause disease. The ability of various serotypes recovered from B.C. to adapt and subsequently grow at 4°C following a downshift from 37°C was investigated here. When the 33 strains were examined, three cold-adapting groups were observed, similar to reports previously made among *L. monocytogenes* derived from different origins (Arguedas-Villa et al., 2010). When sample origin was examined, the majority of fast-adapting strains from B.C. were recovered from RTE foods, though differences were not significant. It is tempting to speculate that wild type *inlA* genotypes (i.e. full-length) may be an indicator of food chain strain fitness. Support for this stems from observations centering on the absence/presence of *inlA* PMSCs in respective cold-growth groups. Significantly more intermediate cold adaptor isolates possessed *inlA* PMSCs (70%) compared to fast-adapting isolates (p=0.042), with only two fast-adaptor isolates shown to encode a PMSC. This observation lends support to the use of *inlA* as a suitable biomarker to identify high-risk strains, though in this light it may be used as an indicator of increased ability to adapt and grow at refrigeration temperatures. Considering cold temperatures are used in RTE food processing facilities and are relied on to ensure product quality and safety throughout the food supply chain, these strains may possess enhanced ability to persist in FPE. Furthermore, if present in food, *L. monocytogenes* strains having an *inlA* gene, which will produce a full-length InlA, may have increased ability to grow to potentially dangerous levels during cold storage, particularly if abusive temperature conditions are encountered.

3.5 Conclusions

In summary, *inlA* mutations were observed in four *L. monocytogenes* serotypes recovered from the B.C. food continuum. Notably, when the adaptive mutability of isolates to rifampicin was examined, serotype 4b isolates acquired mutations more frequently than all other serotypes. The opposite, however, was true for the *inlA* mutations. None of the examined 4b serotype strains possessed PMSC mutations in *inlA*. Interestingly, isolates with the 3-codon *inlA* deletion (a.a. 738-740) exhibited highly invasive phenotypes, suggesting this *inlA* genotype may be of public health concern. When the ability of *L. monocytogenes* isolates to adapt to cold temperature was examined, isolates possessing rapid cold adaption were more likely to encode an *inlA* gene lacking PMSCs. These results substantiate, in new ways, the assertion that isolates lacking *inlA* PMSCs are a significant concern. *Listeria monocytogenes* lacking *inlA* PMSCs were more commonly recovered from RTE food. Since those isolates adapted more rapidly to refrigeration temperature than isolates with PMSCs, they represent *L. monocytogenes* of significant concern to food processors and public health authorities.

Chapter 4: Antimicrobial resistance and co-selection phenomenon in *Listeria* spp. recovered from B.C. food and food processing environments

4.1 Introduction

Listeria spp. may be recovered from a variety of animals (Fenlon, 1999; Müller, 1988) and environments, including aquatic and non-aquatic sources (Colburn et al., 1990; Fenlon, 1999; Lyautey et al., 2007; Schaffter and Parriaux, 2002; Weis and Seeliger, 1975). This ubiquity challenges the ability of food processors to effectively exclude *Listeria* from food production environments and, ultimately, food. Of the 15 *Listeria* species, *Listeria monocytogenes* is the only species that has been routinely associated with human infections, though *L. ivanovii* also occasionally causes human disease (Guillet et al., 2010). The recovery of *Listeria* spp. in food processing facilities is of serious concern to processors since 99% of listeriosis infections are linked to contaminated food (Farber and Peterkin, 1991; Pinner et al., 1992; Scallan et al., 2011; Schuchat et al., 1991). Consumption of *L. monocytogenes* may lead to a symptomatic infection that varies in severity based on host status. In healthy adults, listeriosis is limited to the gastrointestinal environment and is characterized by mild enteritis and/or influenza-like symptoms (Swaminathan and Gerner-Smidt, 2007). In susceptible populations, including young children, the elderly, and immunocompromised individuals, infections may become invasive (Schlech, 2000). This form of listeriosis often results in severe clinical outcomes, with mortality rates ranging between 20 and 40% (Farber and Peterkin, 1991; Hof et al., 1997; Weatherill, 2009). Clinical presentation of invasive listeriosis may include encephalitis, meningitis, septicemia, and spontaneous abortions during the last trimester of pregnancy (McLauchlin et al., 2004).

Considering the morbidity and high mortality rates linked to invasive listeriosis, antibiotic chemotherapy is required to improve clinical outcomes. With the exception of cephalosporins, fosfomycin, and early quinolones, to which innate resistance has been reported (Hof et al., 1997; Morvan et al., 2010), *L. monocytogenes* is generally considered sensitive to most clinically relevant antibiotics (Hof et al., 1997; Troxler et al., 2000). The treatment course for invasive listeriosis is typically comprised of an aminopenicillin (e.g., ampicillin or amoxicillin) in combination with an aminoglycoside, such as gentamicin (Boisivon et al., 1990; Hof, 2004; Temple and Nahata, 2000). In cases where reduced sensitivity or resistance to beta-lactams is encountered, a number of agents active against Gram positive bacteria may be used, though cotrimoxazole is generally regarded as the second-choice therapeutic option (Boisivon et al., 1990; Hof, 2004; MacGowan, 1990; Temple and Nahata, 2000).

The first report of antibiotic resistant *L. monocytogenes* was made in 1988. A clinical isolate from a patient with meningitis possessed resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline (Poyart-Salmeron et al., 1990). Although acquired antimicrobial resistance (AMR) is thought to be rare in *L. monocytogenes*, the aforementioned AMR was encoded on a mobile plasmid shown to be transmissible to other *L. monocytogenes* strains, enterococci, and *Staphylococcus aureus*. Since then, increased reports of AMR in *L. monocytogenes* and other *Listeria* spp. have been made (reviewed in Charpentier and Courvalin, 1999; Lungu et al., 2011), though the body of literature remains limited compared to other notable foodborne pathogens. In particular, increasing trends of reduced sensitivity to fluoroquinolones and tetracycline were observed in a large collection of clinical isolates (Morvan et al., 2010). Resistance to fluoroquinolones (e.g., ciprofloxacin)

has been attributed to an efflux pump, Lde, belonging to the major facilitator superfamily (Godreuil et al., 2003). Notably, repeated exposure to sub-lethal concentrations of benzalkonium chloride (BAC) or ciprofloxacin produced derivative strains that possessed an increased tolerance to respective selective agents (Rakic-Martinez et al., 2011). In these strains, tolerance to gentamicin and other toxic compounds increased as well, thus indicating a co-selection phenomenon (Rakic-Martinez et al., 2011). This observation has significant implications as quaternary ammonium compounds (QACs) are routinely used for the disinfection of food production environment surfaces (McDonnell and Russell, 1999; Merianos, 1991; Rakic-Martinez et al., 2011). As a result, the frequent use of QAC antimicrobials may result in selection for strains possessing reduced susceptibility to a key therapeutic agent.

In British Columbia (B.C.), a survey of dairy, fish, and meat sectors revealed a high prevalence of *L. monocytogenes* and other listeriae primarily recovered from fish processing facilities (Kovačević et al., 2012a). Furthermore, examination of fish and meat products available at retail in B.C. showed that contaminated fish products possessed strains of considerable public risk (Kovačević et al., 2012b). At this time, a paucity of data describing AMR in *L. monocytogenes* and other listeriae in Canada exists, with the most recent publication examining resistance in isolates originating from animal feces rather than the food supply (Lyautey et al., 2007). Considering the link between contaminated food, food processing environments, and listeriosis, generating data that will further describe AMR in food-related isolates seems prudent. Therefore, the objective of this research was to determine whether *Listeria* spp. recovered from the B.C. food supply possessed resistance to clinically relevant antibiotics. In addition, the possibility of whether a similar relationship

between reduced susceptibility to ciprofloxacin and antibiotics used in the treatment of listeriosis (e.g., gentamicin), as reported (Rakic-Martinez et al., 2011), also existed within food chain-derived *L. monocytogenes* isolates was researched. Two hypotheses were structured for investigation: (H1) *Listeria* spp. recovered from B.C. food chain possess resistance to clinically relevant antimicrobials; (H2) The adaptation of food chain-derived *L. monocytogenes* to high concentrations of ciprofloxacin leads to resistance to BAC.

4.2 Materials and methods

4.2.1 Bacterial isolates

Listeria spp. (n=111) used in this study were recovered from FPE swabs (n=53), raw unprocessed food (RUF; n=18) and ready-to-eat (RTE) foods (n=40) collected from dairy, fish, and meat processing facilities, and retail facilities in B.C. as part of the survey described in Chapter 2. *Listeria* species in this study included: *L. innocua* (n=22), *L. monocytogenes* (n=56), *L. seeligeri* (n=12), and *L. welshimeri* (n=21), with isolate origins and serotyping data for *L. monocytogenes* described in Table 4-1 (more details for *L. monocytogenes* isolates are given in Table B-1, Appendix B). Bacterial cultures were maintained in peptone with 20% glycerol at -80 °C. Prior to experiments, isolates were grown overnight on tryptic soy agar (TSA; Difco, Becton Dickinson Diagnostics, Mississauga, ON, Canada) at 35°C.

4.2.2 Antimicrobial resistance screening

AMR was assessed by disc diffusion assay. Briefly, single colonies of *Listeria* isolates were inoculated into 10 ml of tryptic soy broth (TSB; Difco) and incubated at 35°C for 18 ± 2 h, with shaking (200 rpm). Following incubation, cultures were diluted to 1 x 10^7 CFU/ml in

tempered 0.75% agar (45°C; Difco), mixed gently, and poured onto Mueller-Hinton agar (MHA; Difco). Once solidified, antimicrobial susceptibility test discs (BBL™ Sensi-Disc™, BD Diagnostics, Sparks, MD, USA) were applied and plates incubated at 35 °C for 24 h. A panel of 18 antimicrobials comprising 11 classes of antibiotics were used: amikacin (AMK; 30 μg), ampicillin (AMP; 10 μg), cefoxitin (FOX; 30 μg), chloramphenicol (CHL; 30 μg), ciprofloxacin (CIP; 5 μg), clindamycin (CLI; 2 μg), erythromycin (ERY; 15 μg), gentamicin (GEN; 10 μg), imipenem (IPM; 10 μg), kanamycin (KAN; 30 μg), linezolid (LZD; 30 μg), nalidixic acid (NAL; 30 μg), rifampin (RIF; 5 μg), streptomycin (STR; 10 μg), cotrimoxazole (SXT; 10 μg), tetracycline (TET; 30 μg), trimethoprim (TMP; 5 μg), and vancomycin (VAN; 10 μg). Zones of inhibition were measured to the nearest millimeter at 24 h. Since no resistance criteria exist for *Listeria* susceptibility testing in Clinical and Laboratory Standards Institute (CLSI) guidelines for the tested AMs other than AMP and SXT, CLSI criteria for staphylococci were applied (Clinical and Laboratory Standards Institute, 2011). *Escherichia coli* K-12 MG1655 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains.

Table 4-1. *Listeria* isolates (n=111) recovered from food processing environments, raw unprocessed food, or ready-to-eat foods used in the study.

Source	***L. monocytogenes* Serotypes**						**Other *Listeria* spp.**[a]			
	Total	**1/2a**	**1/2b**	**1/2c**	**3a**	**4b**	**Total**	***Li***	***Ls***	***Lw***
Processing environment	29	11	1	5	4	8	24	8	12	4
Food[b]										
RUF	6	4	0	2	0	0	12	2	0	10
RTE	21	6	1	1	0	13	19	12	0	7
Total	56	21	2	8	4	21	55	22	12	21

[a]*Li, Listeria innocua*; *Ls, Listeria seeligeri*; *Lw, Listeria welshimeri.*
[b]RUF, raw unprocessed food; RTE, ready-to-eat food products.

4.2.3 Plasmid profiling of *L. monocytogenes* isolates with antimicrobial resistance

In *L. monocytogenes*, plasmid-mediated AMR has been reported for strains displaying CHL, CLI, ERY, STR and TET resistance (Hadorn et al., 1993; Poyart-Salmeron et al., 1990). As such, PCR was used to screen for plasmids in seven strains displaying resistance or reduced susceptibility (RSC) to CLI, TET, STR and LZD. Template DNA was isolated from overnight cultures grown in BHI (10 ml) at 37°C, with shaking (200 rpm). Following incubation, 1 ml of culture was spun (10,000 x *g*; 1 min), resuspended in 500 µl of deionized water, boiled for 10 min, spun for 10 min at 10,000 x *g* (4°C), and supernatant was placed in a new microfuge tube. Oligonucleotide primers (Table 4-2) were designed to amplify putative plasmid genes that were annotated following whole-genome sequencing (Sagert, 2013). PCR reactions included 1.0 µM of each oligonucleotide, 50-100 ng of DNA, and 1 U of Platinum *Taq* polymerase (Invitrogen Canada Inc., Burlington, ON). All reactions were cycled as follows: one cycle at 95°C for 5 min; followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min; and a final extension at 72°C for 7 min. Amplicons were visualized (ChemiDoc™ XRS+ System, Bio-Rad, Hercules, CA, USA) on a 1% Tris-Borate-EDTA (TBE; 89 mM Tris-Borate and 2 mM EDTA, pH 8.3, Sigma-Aldrich) agarose gel following electrophoresis for 60 min at 120 V (Bio-Rad Horizontal Electrophoresis System). For strains that were putatively positive for plasmids using the PCR screen, plasmids were isolated using the alkaline extraction method developed by Birnboim and Doly (1979). *Listeria monocytogenes* 08-5578 was used as a control strain (Gilmour et al., 2010). Isolated plasmids were visualized using pulsed-field gel electrophoresis (PFGE). Briefly, samples were run for 7.5 h in a 1% TBE-agarose gel using an initial switch time of 2.2 sec and final switch time of 63.8 sec with recirculation of 4°C running buffer.

Table 4-2. Oligonucleotide primers used for plasmid screening.

Primer Name	Sequence (5' - 3')	Product Size (bp)
p02-*mcoF*	GGTTAAACAAGAGGCTGCTA	442
p02-*mcoR*	TTCTTTTCTTGCTGAAGGAG	
p17-*umuCF*	ATGTACCGGAAACTGTATGG	467
p17-*umuCR*	TTCAAGAAAGTAGGGGACAA	
p50-*traGF*	ATTCTGGTACGGCAAAACTA	504
p50-*traGR*	TATCAATCCGCTTGTCTCTT	
p62-*parF*	GAACAAGCCTTTGCTTATGT	509
p62-*parR*	TCTACCCGTTCTTTTTCTTG	
p74-*cadAF*	CCGGATAGAGAGCAAGTATG	508
p74-*cadAR*	TGTACTGAAGGCTGAAGGTT	
p08-*ydhKF*	AGCTTGTTCAACTGGTAACGAAG	480
p08-*ydhKR*	TCGTCAGTTGTCATCCATTTATG	
p33-*yfiSF*	ATTGCCAGCGCTGCTTATAG	630
p33-*yfiSR*	TACCACAAGCCCTTGTTGTTC	
p63-*bcrB*[a]	CGTGTCAGCAGATCTTTGATTAAG	637
p64-*bcrC*[a]	TTGGCGCAATCTTATTTGAAG	

[a]Primers previously published as p63-*qacF* (*bcrB*), and p64-*ebrB* (*bcrC*) (Kovacevic et al., 2013b).

4.2.4 Investigation of ciprofloxacin resistance

To investigate the nature of CIP resistance, *L. monocytogenes* isolates (n= 29) with RSC or resistance were grown in the presence of reserpine (20 µg/ml; Sigma Aldrich Canada Co., Oakville, ON), a known efflux pump inhibitor (Godreuil et al., 2003). Briefly, isolates were grown in TSB at 35°C with shaking (200 rpm) for 20 ± 2 h. CIP (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and sterile deionized water, and added (100 µl) to 96-well microtitre plates (CellTreat Scientific Product, Shirley, MA, USA) containing 95 µl of sterile TSB with or without reserpine and 5 µl of overnight culture (1:100 dilution; final concentration was 2 to 4 x 10^5 CFU/ml). Final concentrations of CIP tested were 0.25, 0.5, 1, 2, and 4 µg/ml. Microtitre plates were incubated at 35°C with shaking (150 rpm) for 20 ± 2 h

and optical densities measured at 595 nm (OD_{595}; BioRad iMark™ Microplate Absorbance Reader, Hercules, CA, USA). Additionally, a loopful from each well was applied onto TSA plates and incubated overnight at 35°C to confirm the presence or absence of bacterial growth. Sterility and positive controls were included in each microtitre plate. All assays included two biological and two technical replicates for each isolate.

4.2.5 Gentamicin and benzalkonium chloride resistance of CIP resistant *L. monocytogenes* isolates

Eight *L. monocytogenes* isolates (four 1/2a and four 4b serotype strains) were experimentally adapted to different concentrations of CIP ranging from 0.25 to 240 µg/ml. Briefly, isolates were grown overnight at 35°C in TSB containing CIP (i.e. maximum tolerated concentration for respective strains), with shaking (200 rpm), and using spread plate method applied onto TSA containing 2-fold higher concentration of CIP than that of the TSB culture. Plates were incubated at 35°C for 24 to 48 h. Up to three resistant colonies were picked, applied onto TSA containing one and a half times higher concentration of CIP than the previous passage, and incubated at 35°C for 24 to 48 h. Following incubation, colonies were transferred to TSB with the same concentration of CIP, and incubated at 35°C for 24 h. The process was terminated once strains achieved tolerance to 240 µg/ml of CIP.

GEN (Sigma-Aldrich) and benzalkonium chloride (BAC; 60% benzyl-dimethyl-dodecyl-ammonium chloride, and 40% benzyl-dimethyl-tetradecyl-ammonium chloride, Sigma-Aldrich) resistance of isolates displaying increased CIP tolerance was determined using microbroth dilution. In short, isolates were grown in TSB containing appropriate concentration of CIP at 35°C with shaking (200 rpm) for 20 ± 2 h. GEN was added (100 µl)

to 96-well microtitre plates containing 95 µl TSB and 5 µl of overnight grown culture (1:100 dilution; final concentration corresponding to 2 to 4 x 10^5 CFU/ml). Final concentrations of GEN were 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µg/ml. The same method was followed for BAC resistance testing, with final concentrations of BAC being 0.31, 0.63, 1.25, 2.5, 5, 7, 10, 20, and 40 µg/ml, as previously described (Rakic-Martinez et al., 2011). Microtitre plates were incubated at 35°C with shaking (150 rpm) for 20 ± 2 h and with OD_{595nm} measured (BioRad). Additionally, a loopful from each well was applied onto TSA plates and incubated overnight at 35°C to confirm the presence or absence of bacterial growth. Sterility and positive controls were included in each microtitre plate. All assays included two biological and two technical replicates for each isolate.

Additionally, following adaptation to high CIP concentrations, AMR disc diffusion assays were repeated for the eight isolates.

4.2.6 Statistical analysis

Fisher's exact test was used to assess the differences among *L. monocytogenes* 1/2a and 4b serotypes exhibiting resistance or reduced susceptibility to CIP. Results were considered significant when p was <0.05. Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, California, USA) software.

4.3 Results

4.3.1 Antimicrobial resistance of *Listeria* spp., and *L. monocytogenes* serotypes

All examined strains were sensitive to AMK, AMP, ERY, GEN, IPM, KAN, TMP, SXT and VAN, and resistant to NAL (Figure 4-1). Additionally, *Listeria* species other than

L. monocytogenes were sensitive to LZD, RIF, and STR (Figure 4-1A, B, D). Resistance of *L. monocytogenes* and other *Listeria* isolates, respectively, to FOX (98% *vs.* 89%), CIP (7% *vs.* 4%), CLI (36% *vs.* 60%), and TET (5% *vs.* 7%) was observed, as was RSC to CIP (68% *vs.* 60%) and CLI (63% *vs.* 40%). Reduced susceptibility in *L. monocytogenes* was also seen for LZD (5%), RIF (2%) and STR (5%) (Figure 4-1C), and to CHL for 7% of other listeriae (Figures 4-1A, B, D). FOX resistance was observed among all four species, while CIP resistance was observed only in *L. monocytogenes* and *L. seeligeri,* and TET resistance in *L. innocua* and *L. monocytogenes*.

Whereas similar AMR profiles for 1/2a and 4b serotypes were observed for LZD and STR, *L. monocytogenes* belonging to 1/2a were more frequently resistant or exhibited RSC to CIP (19/21) compared to 4b (11/21) (p=0.015); no significant differences were observed when 1/2a was compared to other serotypes. CLI, FOX, and NAL resistance was similar among all serotypes, while RSC to RIF was seen only in a single 1/2c isolate (Table 4-3).

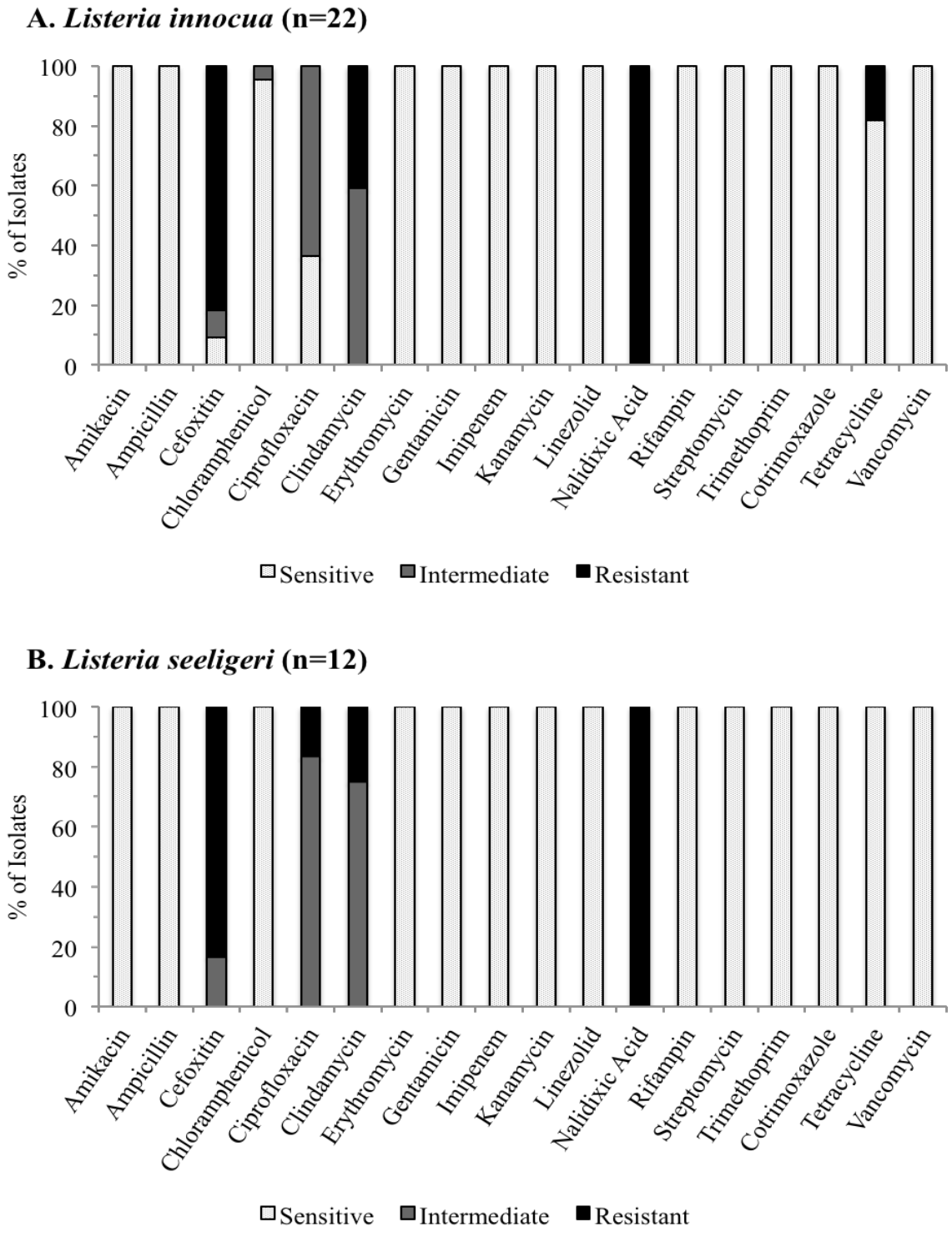
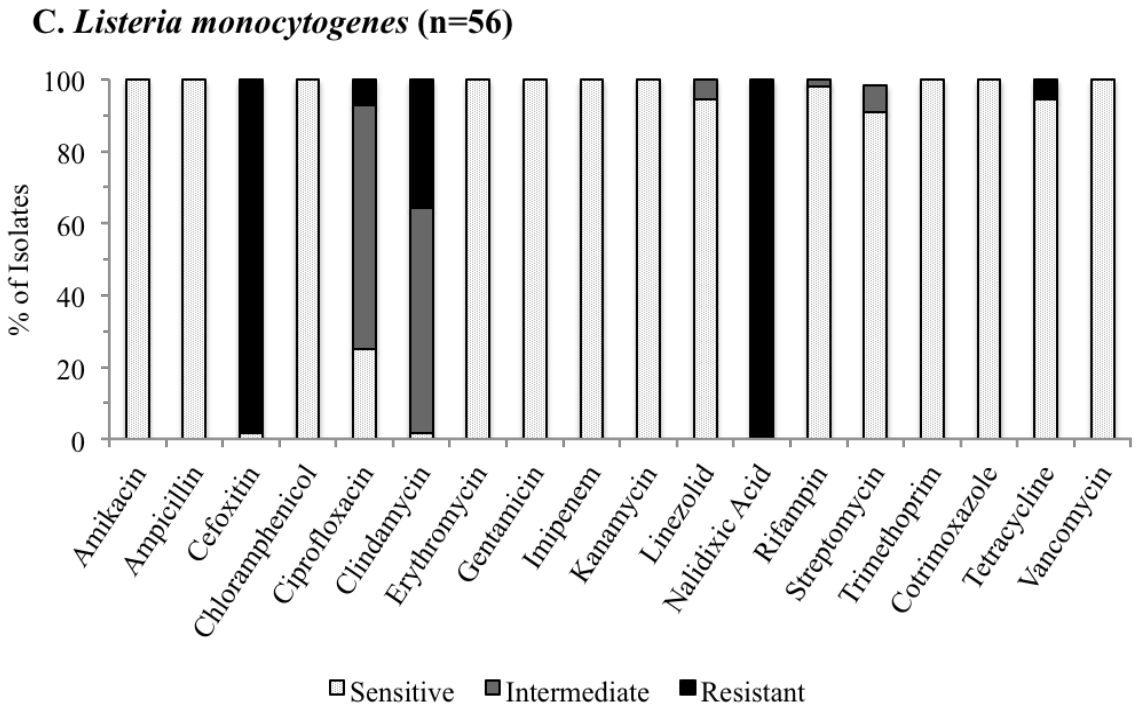


Figure 4-1. Antimicrobial resistance of (A) *L. innocua*, (B) *L. seeligeri*, (C) *L. monocytogenes*, and (D) *L. welshimeri* isolated from foods and food processing environments in British Columbia.



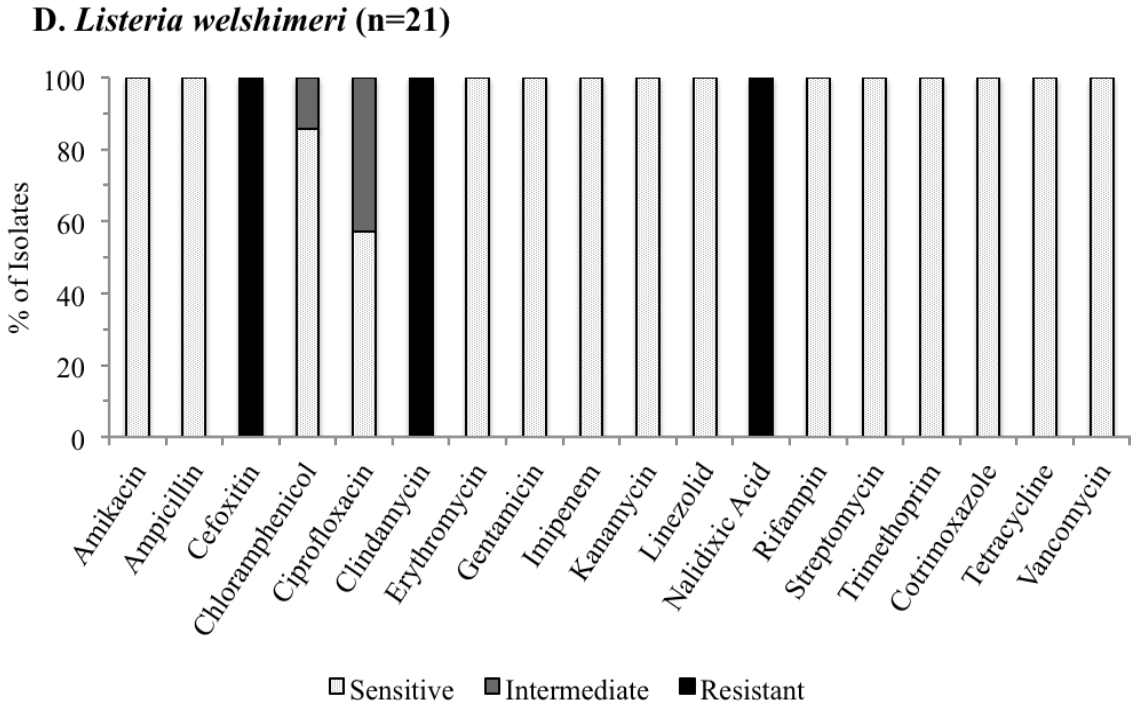


Figure 4-1. Continued.

Table 4-3. Breakdown of *L. monocytogenes* isolates of different serotypes, resistant and with reduced susceptibility to antimicrobial agents. Numbers in brackets represent percentage of isolates within each serotype group resistant or with reduced susceptibility to respective antibiotics.

Antimicrobial Agents	Number of *L. monocytogenes* (%)				
	1/2a (n=21)	1/2b (n=2)	1/2c (n=8)	3a (n=4)	4b (n=21)
Resistant					
Ciprofloxacin	3 (14)	0	0	0	1 (5)
Clindamycin	11 (52)	2 (100)	1 (13)	0	6 (29)
Cefoxitin	20 (95)	2 (100)	8 (100)	4 (100)	21 (100)
Nalidixic Acid	21 (100)	2 (100)	8 (100)	4 (100)	21 (100)
Tetracycline	3 (14)	0	0	0	0
Reduced Susceptibility					
Ciprofloxacin	16 (76)	2 (100)	8 (100)	2 (50)	10 (48)
Clindamycin	10 (48)	0	7 (88)	4 (100)	14 (67)
Linezolid	1 (5)	0	0	0	2 (10)
Rifampin	0	0	1 (13)	0	0
Streptomycin	1 (5)	0	0	0	2 (10)

4.3.2 Antimicrobial resistance of *L. monocytogenes* from different sources

Resistance to FOX, CLI, and NAL was observed for *L. monocytogenes* isolates regardless of whether they were recovered from food or the processing environment. Additionally, among isolates recovered from FPE and RTE food, respectively, 7% and 10% were resistant to CIP. With respect to TET, 14% of RTE food isolates were resistant, and no resistance was observed in *L. monocytogenes* from FPE. Interestingly, a number of TET resistant *L. monocytogenes* isolates from smoked fish samples originating from a single fish processing facility (f31) were recovered. PFGE analysis (Figure A-1, Appendix A) indicated that the isolates were not clonal in origin, and that they did not possess plasmids that would support

possible plasmid-mediated horizontal gene transfer. At this time, reasons for selection and/or maintenance of TET resistance within this facility remain unclear.

With regards to RIF, LZD, and STR, RSC was observed for *L. monocytogenes* in RUF and RTE foods more commonly than in processing environment samples. Reduced susceptibility to both RIF and STR was only observed in food samples, while isolates with RSC to LZD were recovered from FPE (3%) and RTE foods (10%).

4.3.3 Presence of plasmids in *L. monocytogenes* isolates

Using whole genome sequencing data (Gilmour et al., 2010), primers were designed to genes encoded on plasmids but were absent from the *L. monocytogenes* chromosome. Of the seven strains screened, the presence of *umuC*, a putative DNA polymerase V and *par* in three *L. monocytogenes* isolates exhibiting resistance or reduced susceptibility to TET, STR, and LZD (Table 4-4) were detected. Plasmids of approximately 13 and 60 or 70 kb were recovered in these strains (Figure 4-2).

In addition, one of the isolates (FF11-1) possessed plasmid mediated *bcrB* and *bcrC* genes linked to a BAC resistance cassette (Elhanafi et al., 2010) (Table 4-4).

Table 4-4. Plasmid screening of selected *L. monocytogenes* strains possessing reduced susceptibility or resistance to clinically relevant antibiotics.

Strain ID	Sero-type	Origin	AMR[a] Profile	Plasmid	p02 *mco*	p17 *umuC*	p50 *traG*	p62 *par*	p74 *cadA*	p08 *ydhK*	p33 *yfiS*	*bcrB-bcrC*
08-5578[b]	1/2a	Clinical	CIP^I, CLI^R, FOX^R, NAL^R	Yes	+	+	+	+	+	+	-	-
FF11-1	1/2a	Cold-SS[c]	CIP^I, CLI^I, FOX^R, NAL^R, STR^I	Yes	-	+	+	+	-	-	-	+
FF14-1	4b	Hot-SS	CIP^I, CLI^I, FOX^R, NAL^R, LZD^I	No	-	-	-	-	-	-	-	-
FF45-1	4b	Spring wood SS	CIP^I, CLI^I, FOX^R, NAL^R, LZD^I	No	-	-	-	-	-	-	-	-
FF63-1	1/2a	Salmon jerky	CIP^I, CLI^I, FOX^R, NAL^R, TET^R	No	-	-	-	-	-	-	-	-
FF65-1	1/2a	Shrimp meat	CLI^R, FOX^R, NAL^R, TET^R	No	-	-	-	-	-	-	-	-
FF66-1	4b	Hot-SS	CIP^I, CLI^R, FOX^R, NAL^R, STR^I	Yes	-	+	-	+	-	-	-	-
FF67-1	4b	Hot-SS	CLI^R, FOX^R, NAL^R, STR^I	Yes	-	+	-	+	-	-	-	-

[a]Antimicrobial resistance, I – intermediate, R – resistant.
[b]Control strain from a clinical isolate, described by Gilmour et al. (2010).
[c]SS, smoked salmon.

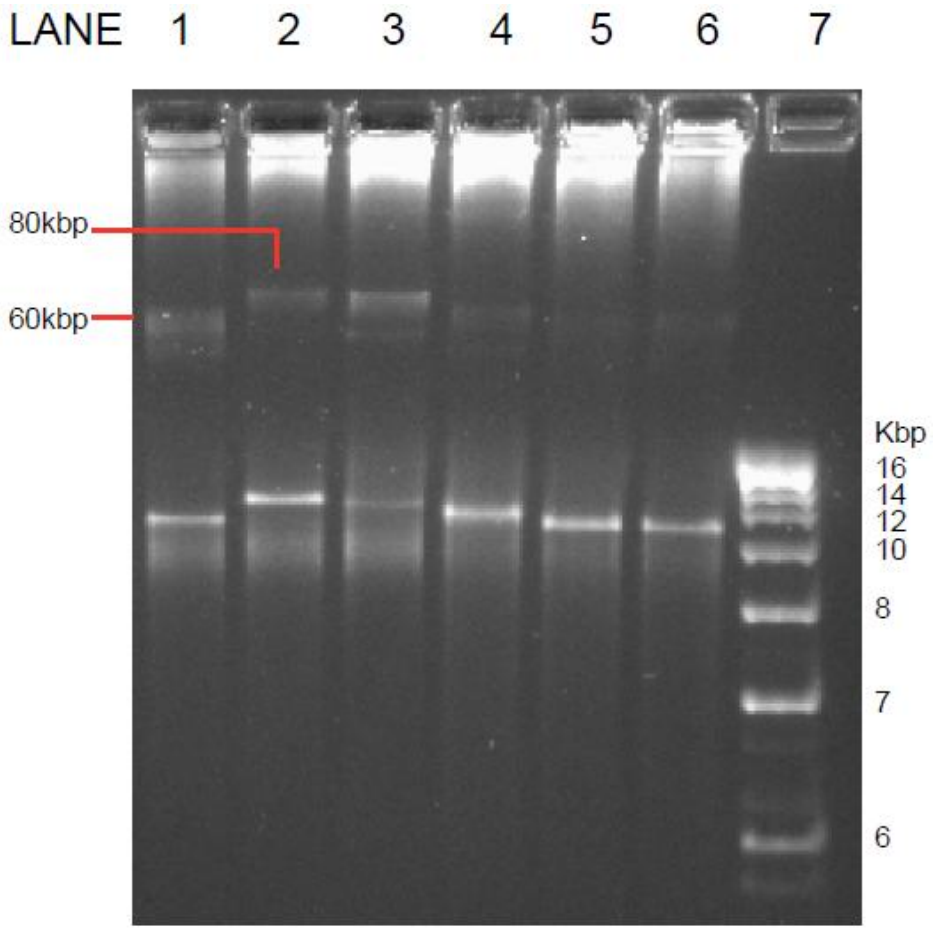


Figure 4-2. Plasmid profiles of three *L. monocytogenes* isolates possessing resistance or reduced susceptibility to TET, STR, and LZD, separated using pulsed-field gel electrophoresis. Lanes 1, 2, and 3 are *L. monocytogenes* isolates possessing pLM0812 (60 kb), pLM5026 (80 kb), and pLM5578, respectively; Lanes 4, 5, and 6 are isolates FF11-1, FF66-1, and FF67-1, respectively; and Lane 7 is a supercoiled DNA ladder.

4.3.4 Efflux-mediated resistance to ciprofloxacin among *L. monocytogenes* strains

The addition of reserpine, a known inhibitor of efflux pumps, to TSB containing different concentrations of CIP resulted in 0 to 4-fold changes in MICs for wild type (WT) *L. monocytogenes* strains (Figure 4-3). While all 4b isolates had lower MICs in the presence of reserpine, the MICs of four 1/2a isolates were not affected, which suggests CIP resistance was mediated by a mechanism(s) other than active efflux.

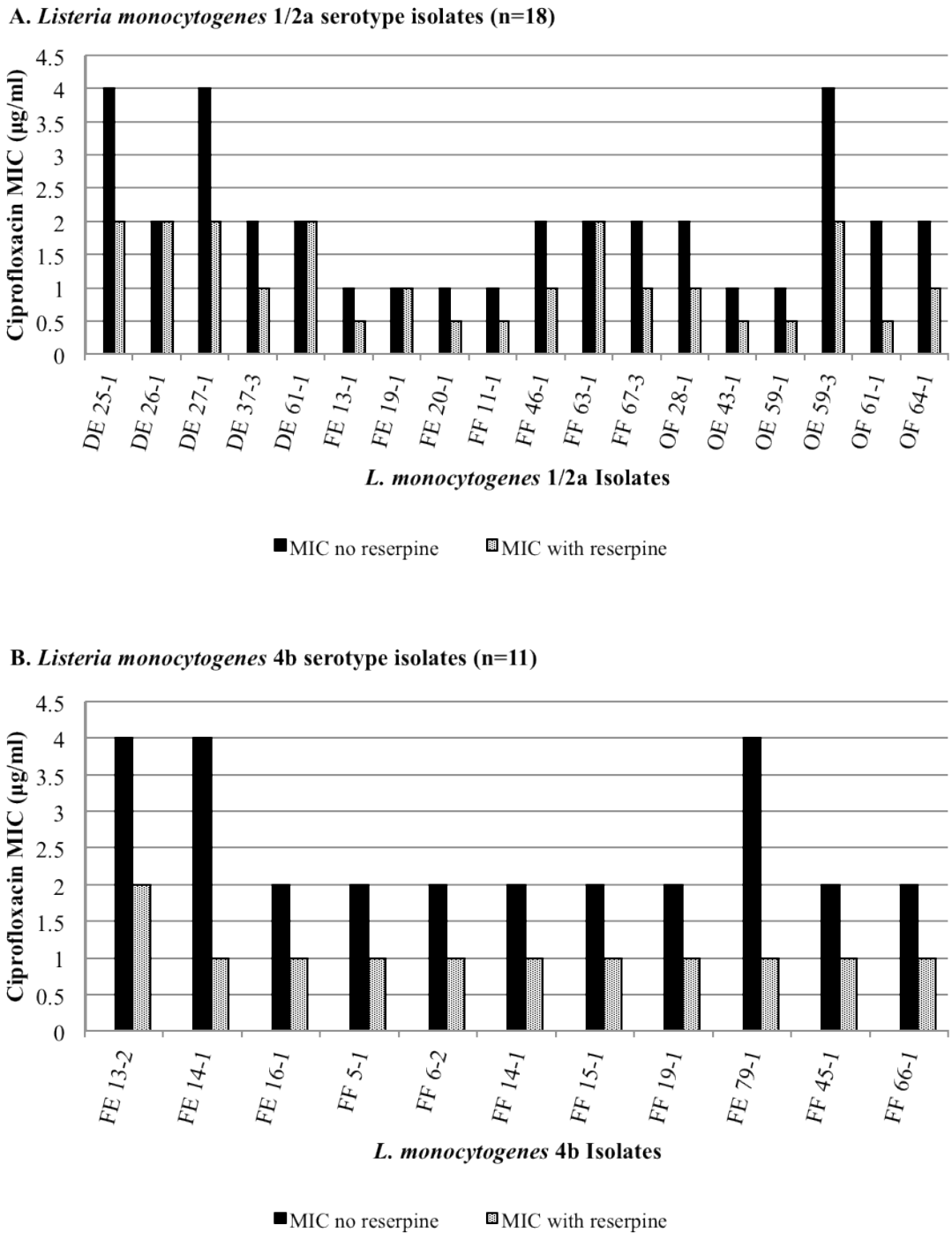


Figure 4-3. The effect of reserpine on *L. monocytogenes* 1/2a (A) and 4b (B) isolates possessing reduced susceptibility or resistance to ciprofloxacin.

For eight isolates that were experimentally adapted to high concentrations of CIP (240 μg/ml), a 4 to 30-fold decrease in MICs was observed in the presence of reserpine when compared to respective WT parental strains, therefore indicating that increased resistance to CIP was primarily attributed to increased efflux (Table 4-5).

Table 4-5. Minimum inhibitory concentration of gentamicin (GEN), benzalkonium chloride (BAC), and ciprofloxacin (CIP) in the absence or presence of reserpine in wild type (WT) *L. monocytogenes* strains recovered from food processing environments and their respective ciprofloxacin adapted strains (CIP^R).

Strain ID	Type[a]	Minimum Inhibitory Concentration (μg/ml)				
		GEN	BAC	CIP	CIP with reserpine	CIP fold-change
DE25-1	WT	4	7	4	2	2
	CIP^R	4	10	240	30	8
DE26-1	WT	4	5	2	2	0
	CIP^R	4	7	240	60	4
DE27-1	WT	4	5	4	2	2
	CIP^R	4	10	240	30	8
FE13-2	WT	4	5	4	2	2
	CIP^R	4	5	240	15	16
FE16-1	WT	4	5	2	1	2
	CIP^R	4	5	30	3.75	8
FF5-1	WT	4	5	2	1	2
	CIP^R	4	5	240	7.5	32
FE79-1	WT	2	2.5	4	1	4
	CIP^R	4	5	120	15	8
FF46-1	WT	4	2.5	2	1	2
	CIP^R	8	5	240	15	16

[a]WT, wild type; CIP^R, adapted to high concentrations of CIP.

4.3.5 Antimicrobial profiles of *L. monocytogenes* strains adapted to high concentrations of ciprofloxacin

Following adaptation to CIP (240 μg/ml), antibiograms were examined and subsequently compared to respective parental strains. All WT strains exhibited RSC to CIP and CLI, with the exception of FF46-1 isolate which was resistant to CIP, and DE27-1, which possessed resistance to CLI. Following CIP adaptation, all strains became resistant to CLI, and one of the strains (DE27-1) developed RSC to LZD (Table 4-5).

4.3.6 Gentamicin and benzalkonium chloride resistance of *L. monocytogenes* strains adapted to high concentrations of ciprofloxacin

GEN and BAC resistance was also investigated in the eight CIP-adapted strains. No difference in MICs for GEN was observed in six of the adapted strains (Table 4-5). Two strains, however, had a 2-fold increase in the MIC of GEN (Table 4-5). Moreover, increases in MIC (ranging from 1.4 to 2 times) of BAC were observed for five CIP-adapted strains compared to respective parental strains (Table 4-5).

4.4 Discussion

In this study, no resistance to AMP and GEN in different *Listeria* strains was observed; however, the results showed that a large proportion of *Listeria* isolates recovered from the B.C. food chain possessed resistance or increased tolerance to antimicrobials that are typically effective against listeriae (Safdar and Armstrong, 2003; Troxler et al., 2000). In addition to AMR to antibiotics for which innate resistance of *Listeria* has been reported previously (Charpentier and Courvalin, 1999; Hof et al., 1997; Troxler et al., 2000), high

levels of CIP and CLI tolerance, especially among isolates recovered from food, were observed. With the widespread usage of antimicrobials in clinical and animal production settings, the recovery of listeriae originating from the food chain that possess concerning levels of AMR may not be surprising (Lungu et al., 2011; Poyart-Salmeron et al., 1992). In fact, the emergence of AMR in *Listeria* spp. has been anticipated (Poyart-Salmeron et al., 1992). One popular theory is that enterococci and streptococci may transmit mobile plasmids encoding AMR determinants to *Listeria* spp. in farming environments (Charpentier et al., 1995; Lemaître et al., 1998; Poyart-Salmeron et al., 1992). Furthermore, within FPEs, frequent exposure of persistent strains to biocides may promote strains with increasing tolerance to a spectrum of clinically relevant therapeutic agents (Christensen et al., 2011; Rakic-Martinez et al., 2011).

In general, resistance or RSC to FOX and NAL was not unexpected, as *Listeria* spp. have been found to be inherently resistant to cephalosporins, and NAL is used as a selective agent during *Listeria* isolation (Hof et al., 1997; Lyon et al., 2008; Troxler et al., 2000; Wieczorek et al., 2012). Whereas resistance to cephalosporins has been ascribed to low-binding affinity to penicillin-binding proteins present in *Listeria* spp. cytoplasmic membranes (Hof et al., 1997; Troxler et al., 2000), mechanisms underlying NAL resistance remain difficult to determine. Alterations of the amino acid sequence in the quinolone resistance-determining regions (QRDR) of *gyrA*, and a no observable impact on MICs following exposure to reserpine suggest mutations in DNA gyrase, or other QRDR regions, contribute to the complex mechanism behind NAL resistance in *Listeria* spp. (Lampidis et al., 2002).

In contrast, RSC to CHL, LZD, RIF and STR was surprising, as listerial strains possessing AMR and RSC are rarely encountered (Charpentier et al., 1995; Morvan et al., 2010; Walsh et al., 2001). Conter et al. (2009) found 3.2% and 1.6% of *L. monocytogenes* from food and FPEs resistant to LZD and RIF, respectively. In the current study, 5 and 2% of *L. monocytogenes* tested exhibited RSC to LZD and RIF, respectively, while two isolates (*L. innocua*, *L. welshimeri*) originating from the same fish processing facility possessed RSC to CHL. Reasons for CHL, LZD, and RIF RSC in food chain isolates are not clear at this time. However, it is notable that when strains were adapted to high concentrations of CIP, one isolate (DE27-1) originally sensitive to LZD developed RSC.

With regards to CIP, CLI, and TET resistance, different incidences have been reported among *Listeria* spp., as well as among isolates derived from human clinical and food processing sources. Specifically, no resistance to CIP for *L. innocua*, low levels among *L. monocytogenes* (2.2%), and high resistance for *L. welshimeri* (100%) strains, in all cases of diverse origins (i.e. clinical, animal, food, and environmental), was reported by Davis and Jackson (2009). In contrast, Troxler et al. (2000) grouped *L. monocytogenes* and *L. welshimeri* as naturally sensitive to CIP, with only two of 32 (6.3%) strains exhibiting resistance and seven (21.9%) with RSC. Reduced susceptibility to CIP was reported for 80% of *L. innocua*, *L. seeligeri*, and *L. ivanovii*, and 16% were resistant (Troxler et al., 2000). In a recent study from Poland (Korsak et al., 2012), only one of 471 (0.2%) *L. monocytogenes* from a food processing plant possessed resistance to CIP, while United States (US) (Safdar and Armstrong, 2003) and French (Morvan et al., 2010) studies found resistance in 0 and 0.4% of isolates, respectively. In the current study 7% of *L. monocytogenes* and 17% of *L. seeligeri* isolates possessed resistance to CIP, while RSC was highest among *L. seeligeri*

(83%), followed by *L. monocytogenes* (67%), *L. innocua* (64%) and *L. welshimeri* (43%). Intriguingly, RSC or resistance to CIP in *L. monocytogenes* was more common in 1/2a compared to strains belonging to serotype 4b (p=0.015), a phenomenon that has not been reported or explained elsewhere. Resistance to CIP in *L. monocytogenes* has been attributed almost exclusively to active export of the drug via efflux-pumps. In particular, the *lde* gene encoding an efflux pump (Lde) contributes to fluoroquinolone resistance (Godreuil et al., 2003; Lismond et al., 2008). Although *lde* has also been found in CIP sensitive isolates, resistance is believed to result from increased gene expression (Godreuil et al., 2003; Morvan et al., 2010). Interestingly, QRDR-based mutations do not significantly impact tolerance of *L. monocytogenes* to CIP (Lampidis et al., 2002).

To investigate whether efflux activity contributed to CIP resistance, cells were exposed to reserpine. Notably, reserpine only marginally decreased the MICs of CIP in WT *L. monocytogenes* strains, suggesting the efflux pumps were not the sole mechanism conferring CIP tolerance in this experiment. However, when strains were adapted to higher concentrations of CIP, reserpine treatment led to a more pronounced increase in CIP sensitivity, indicating efflux-mediated resistance was the primary means by which high-level resistance was generated (Figure 4-3). Interestingly, MICs of experimentally adapted strains in the presence of reserpine were still above that of respective WT parental strains, suggesting another mechanism(s) contributed to increased CIP resistance. This is supported by the observation that four of 14 1/2a strains did not use active efflux for resistance (Figure 4-3A). Rakic-Martinez et al. (2011) noted that *L. monocytogenes* strains with high CIP MICs exhibited more significant reduction in MICs in the presence of reserpine compared to strains

with lower CIP MICs. As such, increased tolerance to CIP may be largely attributed to efflux pump activity, but additional mechanisms of resistance should be investigated.

Resistance to CLI was observed, particularly in *L. welshimeri* (100%) (Figure 4-1). Similar to CIP, other studies report varying degrees of CLI resistance across *Listeria* spp. (Chen et al., 2010; Davis and Jackson, 2009; Troxler et al., 2000). While Chen et al. (2010) observed 85% of *Listeria* spp. from catfish fillets and processing environments in the US were resistant to CLI, another US study examining strains from human, food, animal, and environmental sources found considerably lower (28%) frequency of resistance (Davis and Jackson, 2009). Clindamycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. This mechanism is similar to those linked to reduced ERY and CHL efficacy, which raises concerns about the potential for cross-resistance to differing antibiotic classes through a common mechanism (Depardieu et al., 2007). While no resistance to ERY was observed in the B.C. isolates tested here, RSC to CHL was observed in two strains (Figure 4-1). Interestingly, when eight strains of *L. monocytogenes* with RSC to CLI were adapted to high concentrations of CIP, they concomitantly became resistant to CLI, for reasons not fully understood. A 2-fold reduction in MICs to ERY and CLI was demonstrated by Mata et al. (2000), when the *mdrL* gene was disrupted. This gene encodes for a multidrug efflux transporter in *Listeria*, suggesting this mechanism of resistance may be shared across antimicrobial classes.

While CIP and CLI resistance have been ascribed to efflux-pumps and 23S ribosomal RNA modifications, resistance to TET stems from the acquisition of *tet* genes conferring resistance through ribosomal protection or efflux (Charpentier et al., 1995; Poyart-Salmeron et al., 1990; Poyart-Salmeron et al., 1992). Strains resistant to TET in the current study were

encountered only among *L. innocua* (4/22) and *L. monocytogenes* (3/56) isolates. Charpentier et al. (1995) found TET resistance among 6% of *L. innocua* and *L. monocytogenes* strains of clinical, food, and environmental sources isolated worldwide. In the current study, all food *L. monocytogenes* isolates possessing TET resistance originated from a single fish processing facility. Although this suggests that horizontal gene transfer or clonal dissemination occur within this environment, a lack of plasmids and differing pulsotypes do not support this hypothesis. No TET resistant phenotypes were recovered from environmental sources in this facility. Environmental isolates other than *L. monocytogenes* resistant to TET were, however, recovered in dairy and meat facilities.

The presence of plasmids in *L. monocytogenes* has been shown to confer resistance to cadmium and BAC. However, differing reports exist on the role of plasmids in AMR (Earnshaw and Lawrence, 1998; Gilmour et al., 2010; Lemaître et al., 1998; Rakic-Martinez et al., 2011; Romanova et al., 2006). Plasmid-mediated resistance to CHL, ERY, STR and TET in *L. monocytogenes* has been described by Poyart-Salmeron et al. (1990), as well to TET (Slade and Collins-Thompson, 1990) in *L. innocua* from raw milk, and different species of *Listeria* by Charpentier et al. (1995). Among the B.C. isolates examined in this study, three *L. monocytogenes* strains harbored plasmids exhibiting RSC to STR, but did not possess resistance to TET, CHL or ERY. Additionally, their MICs for BAC were comparable to *L. monocytogenes* strains not carrying plasmids.

Recently, common mechanisms of listerial resistance to CIP and BAC have been proposed (Rakic-Martinez et al., 2011). To investigate the effect of adaptation to high concentrations of CIP on BAC resistance, eight B.C. *L. monocytogenes* isolates were examined. These included four 1/2a and four 4b serotypes. Following CIP-adaptation, MICs

for BAC were up to two times higher in experimentally adapted strains. This is in accordance with findings reported by Rakic-Martinez et al. (2011), where *L. monocytogenes* strains selected on CIP (2 μg/ml) exhibited up to three times higher MICs of BAC compared to parental strains. The same phenomenon was seen for *L. monocytogenes* strains selected on BAC (10 μg/ml), with 4- to 8-fold increases in resistance to CIP (Rakic-Martinez et al., 2011). This could explain in part the differences in CIP susceptibility of clinical isolates described by Safdar and Amstrong (2003) and Davis and Jackson (2009) when compared to *Listeria* spp. derived from the food production sector described herein. As quaternary ammonium compounds, such as BAC, are commonly employed as sanitizers in food processing facilities, bacterial exposure to sub-lethal sanitizer concentrations is a possibility (e.g., persistent strains). This continuous exposure may lend to selection for progeny possessing increasing sanitizer tolerance, and through similar mechanisms of resistance potentially lead to increased tolerance to clinically relevant antibiotics (Rakic-Martinez et al., 2011).

While Rakic-Martinez et al. (2011) also reported increases in GEN MICs from 8 to 64 μg/ml in strains selected on CIP, the same phenomenon was not observed in the experiments described in this chapter. Of the eight CIP adapted strains, MICs for GEN remained the same for six isolates; only two adapted strains (FE79-1, FF46-1) had increased MICs for GEN (Table 4-5). One notable difference between strains examined by Rakic-Martinez et al. (2011) and the B.C. strains described herein, is that the former study examined strains of clinical origin while in the present study CIP adapted strains originated from dairy and fish FPEs and RTE fish samples. These factors cannot be excluded as contributors to the observed differences in AMR phenotypes resulting from increasing CIP

exposure (Buncic et al., 2001; Ragon et al., 2008). Furthermore, two strains examined by Rakic-Martinez et al. (2011) possessed plasmid-mediated resistance to cadmium (*cadAC*) and BAC (*bcrABC*). Although no difference in GEN resistance was seen between WT plasmid-harboring and cured strains when adapted to CIP, lower MICs of GEN were observed in plasmid-cured strains than those possessing the plasmid. This observation suggests that plasmid encoded factors contribute to GEN resistance (Rakic-Martinez et al., 2011). Strains used in the current study for CIP-adaptation did not harbor plasmids.

4.5 Conclusions

In summary, *Listeria* isolates recovered from the B.C. food chain showed resistance or RSC to antimicrobials to which *Listeria* have been historically sensitive. Resistance to antimicrobials commonly used in the treatment of listeriosis (e.g., AMP, GEN, SXT) was not observed. However, it is of concern that a high proportion of food chain-derived *Listeria*, including 1/2a and 4b *L. monocytogenes* isolates which are frequently linked to listeriosis, possessed reduced susceptibility and resistance to CLI and CIP. Furthermore, a co-selection of strains with increasing tolerance to BAC, CLI and GEN was demonstrated among B.C. strains. These findings highlight current concerns regarding co-selection phenomena associated with different classes of antimicrobial agents used in clinical and food processing settings. Considering the environmental ubiquity of *Listeria* in nature, its inevitable occurrence in food processing environments, combined with repeated exposure to biocides, there is a need to improve our understanding of potential pressures that may contribute to co-selection of antibiotic and biocide resistance mechanisms. Knowledge of these phenomena, along with their potential for transmissibility, will aid in developing mitigation strategies

permitting effective control of *Listeria* spp. in food processing environments. This in turn will minimize the potential for development of resistance to clinically relevant antibiotics among food chain-derived *L. monocytogenes*.

Chapter 5: The role of *Listeria* genomic island 1 (LGI1), in the tolerance of *Listeria monocytogenes* to antimicrobials and other stresses encountered in the food processing chain

5.1 Introduction

Despite efforts made by both the food industry and food safety authorities to prevent microbiological contamination of food, pathogenic microorganisms continue to enter the food supply. In the production of ready-to-eat (RTE) foods, *Listeria monocytogenes* is of particular concern. This foodborne pathogen tolerates various extrinsic and intrinsic parameters that normally minimize bacterial survival and their proliferation in foods (Farber and Peterkin, 1991). The presence of *L. monocytogenes* in RTE products is particularly troublesome for vulnerable populations that include pregnant women and their fetuses, the young and the elderly, and people with impaired immune systems. In fact, 20 to 40% of foodborne listeriosis infections that occur in high-risk individuals can lead to fatalities (Clark et al., 2010). Evidence suggests the risk to vulnerable populations may be even higher if virulent strains of *L. monocytogenes* are encountered in RTE foods (Chen et al., 2006; Orsi et al., 2011).

While some *L. monocytogenes* strains have enhanced abilities to adapt, grow and persist in the food chain, some have evolved to more effectively evade host immune responses (Orsi et al., 2011; Orsi et al., 2007). Genetic variations among different strains are associated with enhanced bacterial survival in food processing and increased, or attenuated virulence (Orsi et al., 2011). For instance, genetic variations in the *inlA* gene that encodes for a protein involved in the initial invasion and colonization of the gastrointestinal environment,

have been shown to affect virulence of *L. monocytogenes* (Orsi et al., 2011). In particular, attenuated virulence has been reported for isolates with premature stop codons that lead to formation of truncated InlA proteins (Felicio et al., 2007; Handa-Miya et al., 2007; Jonquieres et al., 1998; Nightingale et al., 2005; Rousseaux et al., 2004). Interestingly, these *inlA* mutations are not associated with serotype 4b isolates recovered from food processing or clinical isolates, but can be frequently seen among lineage II serotypes (i.e. 1/2a, 1/2c, 3a, 3c) that originate from food processing environments (Felicio et al., 2007; Nightingale et al., 2005; Nightingale et al., 2008; Rousseaux et al., 2004). In addition, serotype 4b isolates have been more frequently associated with meningoencephalitis cases than with blood stream infections (Swaminathan and Gerner-Smidt, 2007), and have been the most common serotype causing pregnancy-associated listeriosis (Clark et al., 2010; McLauchlin, 1990). As such, serotype 4b isolates are considered to be the most virulent among the 13 serotypes of *L. monocytogenes* (Swaminathan and Gerner-Smidt, 2007).

Differences in the ability of isolates to survive stresses encountered in food processing have also been demonstrated (Arguedas-Villa et al., 2014). *Listeria monocytogenes* isolated from human clinical samples, foods, and processing environments differ in relative abilities to adapt to cold temperatures, although precise mechanisms that result in different adaptation rates have not been described (Arguedas-Villa et al., 2014; Arguedas-Villa et al., 2010; Tasara and Stephan, 2006). Differences in tolerance and resistance of *L. monocytogenes* to antibiotics and sanitizers commonly used in food processing environments have also been noted (Jeyasekaran et al., 2000; Lemaître et al., 1998). However, many of these properties are both isolate- and situation-specific. As such, they are not linked to a particular serotype or groupings based on molecular sub-typing (e.g.,

clonal complexes defined by multilocus sequence typing, virulence types determined by multi-virulence-locus sequence typing). In fact, data that describe molecular properties of *L. monocytogenes* which would explain serotype differences in their disease causing potential and the severity, as well as their capacity to survive food processing stresses are presently limited (Swaminathan and Gerner-Smidt, 2007).

In Canada, the majority of human listeriosis cases that were reported between 1994 and 2004 were caused by *L. monocytogenes* isolates belonging to 1/2a serotype (Clark et al., 2010). In particular, a predominant clone (i.e. 1/2a serotype, CC8, single-locus variants of sequence type 120, PFGE profile LMACI.0001/ LMAAI.0001) has caused outbreaks and sporadic cases of listeriosis across Canada for more than two decades (Knabel et al., 2012). Examination of 1,061 *L. monocytogenes* isolates collected from 1995 to 2010 revealed the presence of this clone in 22.3% of isolates, with the nationwide distribution believed to have occurred by the mid-1990s. This particular PFGE clone was also linked to RTE deli meats implicated in the nationwide outbreak of listeriosis in 2008 (Gilmour et al., 2010). Isolates associated with this outbreak also possessed a previously unreported genomic island, LGI1 (Figure 1-1, Chapter 1) (Gilmour et al., 2010). Subsequent testing of the 71 human clinical *L. monocytogenes* isolates collected between 1988 and 2010, revealed the presence of LGI1 in 61% of isolates. However, the prevalence of the island among *L. monocytogenes* isolates recovered from the food chain is presently unknown.

This 50 kb island was shown to encode a combination of putative antimicrobial resistance, stress response, and virulence genes, thereby possibly enhancing the capacity of *L. monocytogenes* to survive in the food chain and cause human listeriosis (Gilmour et al., 2010). Ziegler (2012) observed an increase in minimum inhibitory concentrations (MIC) of

benzalkonium chloride (BAC) and benzethonium chloride in three isolates possessing LGI1, which suggested a potential role in *L. monocytogenes* resistance to sanitizers. Furthermore, the presence of genes that are typically involved in stress response, such as a two-component signal transduction system possessing a response regulator (locus 1851) and a sensor histidine kinase (locus 1852), and a putative small RNA polymerase sigma-24 subunit (locus 1859), has indicated that strains possessing LGI1 may be better equipped to survive environmental and/or food processing stresses (Gilmour et al., 2010; Ziegler, 2012). It is also tempting to speculate that the island contributes to virulence, considering that it was found in a number of clinical isolates examined over more than two decades (Knabel et al., 2012). The presence of genes homologous to type IV secretion-like systems (e.g., *virB4*, *virD4*, *cpa* and *tad*), as well as putative adhesin (i.e. *sel1*) further supports this idea (Gilmour et al., 2010; Ziegler, 2012); albeit evidence of increased virulence due to LGI1 is currently lacking. In fact, the function of genes located on LGI1, and their contribution to fitness and/or virulence of *L. monocytogenes* have not yet been confirmed.

Considering the genomic content of LGI1, and its prevalence among human clinical isolates in Canada, the overall objective of this research was to elucidate the contribution of LGI1 to virulence, and the potential role it has in the survival of *L. monocytogenes* under food chain-relevant conditions. To meet the objectives, research in this thesis chapter was carried out in two phases. Firstly, *L. monocytogenes* isolates recovered from B.C. foods and food processing environments were screened for the presence of LGI1. The second part of the research investigated the role of specific genes located on LGI1 in the survival of *L. monocytogenes* under stress-induced conditions (e.g., cold temperatures, acidic and saline conditions, sanitizers), and the adherence to and invasion of human cell lines. The research

hypotheses generated are based on the putative functions of the three selected genes. These included:

(H1) *emrE* contributes to enhanced tolerance of *L. monocytogenes* to antibiotics;

(H2) *emrE* contributes to enhanced tolerance of *L. monocytogenes* to quaternary ammonium compounds (QAC) and a cationic dye, acriflavine;

(H3) regulator gene *lmo1851* contributes to enhanced survival of *L. monocytogenes* in acidic pH;

(H4) regulator gene *lmo1851* contributes to enhanced survival of *L. monocytogenes* in cold temperature;

(H5) regulator gene *lmo1851* contributes to enhanced survival of *L. monocytogenes* in high salt environments; and

(H6) *sel1* contributes to the increased adherence and invasiveness of *L. monocytogenes in vitro*.

5.2 Materials and methods

5.2.1 Bacterial strains

The list of *L. monocytogenes* (n=56) strains used for LGI1 screening is presented in Table B-1 (Appendix B), and strains are described in detail in Chapter 3.

All LGI1 deletion mutants were generated in *L. monocytogenes* 08-5578, a clinical strain responsible for the Canadian deli meat listeriosis outbreak in 2008 which does not possess genes that encode previously described small efflux pump systems (e.g., *bcrABC* and *qacH*) (Gilmour et al., 2010). Bacterial strains and plasmids used for mutant generation are listed in Table 5-1. Additionally, when phenotype changes in mutants were observed, other

strains possessing LGI1 ($CC8^+/LGI1^+$, n=8), strains from clonal complex 8 (CC8) that do not possess LGI1 ($CC8^+/LGI1^-$, n=4), and strains belonging to serotype 1/2a, but that are not part of CC8 and do not possess LGI1 (1/2a $CC8^-/LGI1^-$, n=2) were exposed to identical conditions to investigate whether the same growth behavior was seen across unrelated strains possessing LGI1 (Table 5-1). *Listeria monocytogenes* EGD-SmR (Gaillard et al., 1986) and 81-0861 (Knabel et al., 2012) strains were used as negative LGI1 serotype 1/2a and 4b controls, respectively.

All media used were from Difco (Difco, Becton Dickinson, Sparks, MD) unless otherwise indicated. Strains and transformants were stored long-term at -80°C in tryptic soy broth (TSB; Acumedia, Neogen, Lansing, MI, US) supplemented with 20% (wt/vol) glycerol (*L. monocytogenes*), or Luria-Bertani (LB; Difco) broth with 20% glycerol (*Escherichia coli*). Prior to use, *L. monocytogenes* strains were recovered from a frozen stocks on tryptic soy agar (TSA; Acumedia) that was supplemented with 0.6% yeast extract (YE; Thermo Fisher Scientific, Ottawa, ON), while *E. coli* strains were recovered on LB agar, followed by 24 h incubation at 37°C. With the exception of specific sanitizer stress survival studies, which were performed in TSB, brain heart infusion (BHI; Difco) broth was used to grow *L. monocytogenes* strains prior to stress experiments. Specific conditions are described below for each stress treatment. Recovery of survivors following stress conditions was performed on TSA-YE, incubated at 37°C for 24 to 48 h.

Table 5-1. Bacterial strains and plasmids used in experiments.

Strains/ plasmids used	Description	LGI1[a]	Reference
Strains used in mutant constructs			
L. monocytogenes			
08-5578	Wild type, clinical strain (Ontario), serotype 1/2a, CC8	+	Gilmour et al. (2010)
08-5578:Δ*lmo1851*	08-5578 with 408 bp in-frame deletion within *lmo1851* gene	Δ*lmo1851*	This study
08-5578:Δ*emrE*	08-5578 with 240 bp in-frame deletion within *emrE* gene	Δ*emrE*	This study
08-5578:Δ*sel1*	08-5578 with 2598 bp in-frame deletion within *sel1* gene	Δ*sel1*	This study
E. coli			
DH5-α	DH5-α with pKSV7	-	Ziegler (2012)
One Shot® TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G	-	Invitrogen
TOP10:Δ*lmo1851*	TOP10 carrying pKSV7 with 08-5578 *lmo1851* insert with deletion	Δ*lmo1851*	This study
TOP10:Δ*emrE*	TOP10 carrying pKSV7 with 08-5578 *emrE* insert with deletion	Δ*emrE*	This study
TOP10:Δ*sel1*	TOP10 carrying pKSV7 with 08-5578 *sel1* insert with deletion	Δ*sel1*	This study
Plasmids used for mutant constructs			
pKSV7	Gram-positive and Gram-negative temperature sensitive shuttle vector; CHL^R (*L.* monocytogenes); AMP^R (*E. coli*) multiple cloning sites; *lacZ*; β-lac; *cat*, pE194ts[b]		Smith and Youngman (1992)
pKSV7:Δ*lmo1851*	pKSV7 with 08-5578 *lmo1851* insert with deletion		This study
pKSV7:Δ*emrE*	pKSV7 with 08-5578 *emrE* insert with deletion		This study
pKSV7:Δ*sel1*	pKSV7 with 08-5578 *sel1* insert with deletion		This study
Other *L. monocytogenes* strains used in stress experiments			
01-1465	Clinical, human blood (Ontario); 1/2a serotype, CC8[c]	+	Knabel et al. (2012)
01-2417	Clinical, human blood (British Columbia); 12/a serotype, CC8	+	Knabel et al. (2012)
01-7210	Liverwurst sausage (British Columbia), 1/2a serotype, CC8	+	Knabel et al. (2012)
02-4056	Clinical, human blood (Ontario); 1/2a serotype, non CC8	-	Knabel et al. (2012)
03-0402	Clinical, human blood (Alberta); 1/2a serotype, CC8	+	Knabel et al. (2012)
06-6956	Clinical, human blood (Quebec); 1/2a serotype, non CC8	-	Knabel et al. (2012)
08-6040	RTE meat (Ontario); 1/2a serotype, CC8	+	Knabel et al. (2012)

Table 5-1. Continued.

Strains/ plasmids used	Description	LGI1[a]	Reference
95-0093	Clinical, human blood (Alberta); 1/2a serotype, CC8	+	Knabel et al. (2012)
95-0151	Clinical, human blood (Ontario); 1/2a serotype, CC8	+	Knabel et al. (2012)
99-3046	Clinical, human blood (Ontario); 1/2a serotype, CC8	-	Knabel et al. (2012)
01-5373	Clinical, human blood (Ontario); 1/2a serotype, CC8	-	Knabel et al. (2012)
03-5833	Clinical, human blood (Alberta); 1/2a serotype, CC8	-	Ziegler (2012)
08-5375	Clinical, human blood (Ontario); 1/2a serotype, CC8	-	Knabel et al. (2012)
LR39-1	RTE fish (British Columbia); 1/2a serotype, CC8	+	This study
Controls			
EGD-SmR	EGD derivative resistant to streptomycin; 1/2a serotype, non CC8	-	Gaillard et al. (1986)
81-0861	Coleslaw (Nova Scotia); 4b serotype	-	Knabel et al. (2012)

[a]*Listeria* genomic island (LGI1) present (+) or absent (-).
[b]Thermosensitive replication origin of plasmid pE194.
[c]CC8, clonal complex 8; based on multilocus sequence typing.

5.2.2 Screening for LGI1

Conventional polymerase chain reactions (PCR) were used to screen for the presence of the 50 kb LGI1. Briefly, four sets of primers were used for screening, targeting loci 1859, 1861, 1862, and 1901 (Table 5-2). DNA was isolated from overnight cultures grown on TSA (Acumedia). A single colony was resuspended in 100 µl of 1 x Tris-EDTA buffer, heated at 90°C for 10 min, cooled on ice for 2 min, and spun at 16,000 x *g* for 5 min. PCR reactions (25 µl), using 5 U of AmpliTaq Gold 360 DNA polymerase (Applied Biosystems, Life Technologies, Carlsbad, California, USA), 0.4 µM of respective primers, 200 µM dNTPs (Invitrogen Canada Inc., Burlington, ON), and template DNA (1 µl) were cycled as follows: 95°C for 5 min; 35 cycles of 94°C for 30s, 52°C for 30 s and 72°C for 20 s; followed by 72°C for 5min. Images of ethidium bromide-stained bands were visualized on 1% agarose gel using Image Master VSD (Amersham Pharmacia Biotech, Uppsala, Sweden). *Listeria*

monocytogenes 08-5578 (Gilmour et al., 2010) strain obtained from the Canadian National Microbiology Laboratory was used as a positive control.

Table 5-2. Oligonucleotide primers used in experiments[a].

Primer (Tm)[b]	PCR conditions and oligonucleotide sequence (5'–3')[c]	Product size (bp)	Digestion enzymes	Primer effic. (%)
LGI1 screening	95°C for 5 min; 35 cycles of 94°C for 30s, 52°C for 30 s and 72°C for 20 s; followed by 72°C for 5 min.			
LGI1-1859-F (57°C)	AAG AGC GCG AAG CTG AAA GAT A	77	N/A[d]	N/A
LGI1-1859-R (55°C)	CCT CAT CTT GGA ATC GTT CCA			
LGI1-1861-F (52°C)	GAT ACT GGC GAA AGC TTC TA	316	N/A	N/A
LGI1-1861-R (50°C)	GGT TTC GGG TTA ATG ATG TA			
LGI1-1862-F (53°C)	GAG CAA CAC CAC CTA AGT TC	299	N/A	N/A
LGI1-1862-R (52°C)	CAG TCG CTA TCG TAC TTG AA			
LGI1-1901-F (55°C)	TGA TCC GCC GTA TTA GCA AAC	69	N/A	N/A
LGI1-1901-R (59°C)	AAG CCG TGC ATG ATC TTC CT			
Mutant construction				
emrE	95°C for 2 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1.7 min, followed by 72°C for 10 min.	1588 (AD)		N/A
SOE-A	CCC CTG CAG AGA CCC TCG GCT TTG CGT CC	881 (AB)	*Pst*I	
SOE-B	GCA GGG GTT GTA GGC CTG AAC			
SOE-C	*GTT CAG GCC TAC AAC CCC TGC* AAG TTC AAG TAC GAT AGC GAC	707 (CD)		
SOE-D	CCC GGT ACC GAT GGC GTG AAA ACG GCG GC		*Kpn*I	
emrE-XF	GCC ACA AAA GGG CAG GTT			
emrE-XR	TAA AGC TCT CCC GCA GTA CC			
lmo1851	95°C for 2 min; 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1.3 min, followed by 72°C for 2 min.	1083 (AD)		N/A
SOE-A	CCC CTG CAG ATC CAT TAG AGC ATC AAT TTG	537 (AB)	*Pst*I	
SOE-B	TTA CTA AAA GAA ATC AGT TCT			
SOE-C	*AGA ACT GAT TTC TTT TAG TAA* ATT AGC CAC TTC ATC TTC TAT	546 (CD)		
SOE-D	CCC GGT ACC CAT TAT AGC AAC TTG ATT GTG		*Kpn*I	

Table 5-2. Continued.

Primer (Tm)[b]	PCR conditions and oligonucleotide sequence (5'–3')[c]	Product size (bp)	Digestion enzymes	Primer effic. (%)
Mutant construction				
sel1	95°C for 2 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min, followed by 72°C for 10 min.	3603 (AD)		N/A
SOE-A	GG TCT AGA GCT GCT TGA TGA GGT ATG C	501 (AB)	*Xba*I	
SOE-B	GCA TTC CAC ATT GAC CGC			
SOE-C	*GCG GTC AAT GTG GAA TGC* CGG TAA CAG TAG CTT GCT ATC ATC	504 (CD)		
SOE-D	GG GGT ACC ACA TGA GCC TAT CAG AAT TAA CCC		*Kpn*I	
sel1-XF	CAT CTA CAC CGA CAA ATA CCG CA			
sel1-XR	GCA ATC TTG TGC GAG TCT TTC			
Quantitative real-time PCR (qRT-PCR) primers				
16S rRNA-F[e]	TTA GCT AGT TGG TAG GGT	318		91.3
16S rRNA-R	AAT CCG GAC AAC GCT TGC			
emrE-JKq-F	GTT GCT ATA GCG GTG ATT GGA GT	102		104.3
emrE-JKq-R	GTT CAG GCC TAC AAC CCC TG			
lde-JKq-F	TCC CAA TGG CTT TCG CAC AA	136		99.4
lde-JKq-R	ATT CGA CCT GCA ACC TCA CG			
lmo1861-JKq-F	GCT TAC AGA AGA AGG AGC GCA	101		99.6
lmo1861-JKq-R	CCC TAC GTT GTT CCT GCG G			
mdrL-JK2q-F	TCG AGC TGG TTG GGG TTT TG	96		97.1
mdrL-JK2q-R	ATC CCA ATT GCA TGG CCT GG			
*sigB*q-F[f]	TGT TGG TGG TAC GGA TG	221		100
*sigB*q-R	CAT TCT GCA ACG CCT C			

[a]All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa, US).
[b]Tm, melting temperature in °C.
[c]Endonuclease restriction sites are underlined; regions complementary to SOE-B primers are italicized.
[d]N/A, not applicable.
[e]Primers validated by Tasara and Stephan (2007).
[f]Primers designed by Arguedas-Villa et al. (2010).

5.2.3 Preparation of *L. monocytogenes* competent cells

A single colony of *Listeria monocytogenes* 08-5578 was used to inoculate 5 ml of BHI (16 x 150 mm tubes), incubated overnight at 37°C with shaking (200 rpm). Fresh BHI (50 ml) in a 250 ml flask was inoculated with the overnight culture (500 µl), and subsequently incubated at 37°C with shaking, until cells reached OD_{600} of 0.2 (BioRad iMark! Microplate Absorbance Reader, Hercules, CA, US). Penicillin G (50 µl of 100 mg/ml stock; Sigma-Aldrich, Oakville, Ontario) was added to the culture, which was incubated for additional 2 h at 37°C with shaking (200 rpm). Following incubation, the culture was chilled on ice for 15 min, transferred to four centrifuge tubes (15 ml; Corning™ Polypropylene, Fisher Scientific, Ottawa, ON) and spun at 5,939 x *g* (Eppendorf 5415 R) for 1 min at 4°C. The supernatant was discarded, and 1.2 ml of HEPES (1 mM; Sigma-Aldrich) with sucrose (0.5 M; Sigma-Aldrich) and glycerol (10% wt/vol; Fisher Scientific) was added to each tube. The contents were gently mixed with a pipet and were spun at 5,939 x *g*. Supernatants were discarded, and the resulting pellets were re-hydrated with 100 µl of HEPES/sucrose/glycerol solution. Competent cells were used immediately or were stored at -80°C until used.

5.2.4 Generation of deletion mutants

Non-polar deletion mutants of putative regulator (*lmo1851*; locus 1851), adhesin (*sel1*; locus 1866), and efflux-pump (*emrE* homolog; locus 1862) genes were generated in *L. monocytogenes* 08-5578, using the allelic exchange protocol described by Camilli et al. (1993). In summary, splicing by overlap extension (SOE) PCR was used to excise the desired sequences. A list of oligonucleiotide primers, thermocycling conditions, and restriction endonucleases used is provided in Table 5-2. The procedure is based upon removing a

portion of sequence between two flanking sequences, followed by splicing of the two flanking regions together. *Pfu*Turbo CX DNA polymerase 2.5 U (Agilent Technologies Inc., Mississauga, ON) was used in all PCR reactions according to the manufacturer's instructions, with 0.4 μM of each oligonucleotide primer and *L. monocytogenes* 08-5578 genomic DNA, isolated with the DNeasy Blood and Tissue kit (Qiagen), used as a template. SOE oligonucleotide primers (A and B) were designed to amplify appropriate DNA fragments (Table 5-2) at the 5' end of the gene of interest. The primer SOE-A included a 5' restriction endonuclease site. SOE-C and SOE-D primers were designed to amplify appropriate DNA fragments at the 3' end of the gene of interest. SOE-C primer included an overhang complementary to the primer SOE-B, and SOE-D primer included a second restriction endonuclease site. PCR fragments (e.g., SOE-AB, and SOE-CD) were purified using the QIAquick PCR Purification kit (Qiagen, Toronto, ON), and subsequently used as templates in a PCR reaction with SOE-A and SOE-D primers. Resulting SOE-AD PCR product was electrophoresed (Bio-Rad Horizontal Electrophoresis System) on a 1% agarose gel (Fisher Scientific), and ethidium bromide-stained bands were visualized using Image Master VSD (Amersham Pharmacia Biotech, Uppsala, Sweden) to confirm the presence of a single band of appropriate size. When more than one band was present, the band of the appropriate size was cut out from the agarose gel and further purified using the QIAquick Gel Extraction kit (Qiagen). SOE-AD PCR product and pKSV7 were purified with QIAquick PCR Purification kit (Qiagen), digested with appropriate endonucleases (FastDigest, Fisher Scientific; Table 5-2), and confirmed by running on a 1% agarose gel stained with ethidium bromide (Image Master VSD). Once confirmed, products were purified once more, and SOE-AD PCR product was ligated (T4 ligase, Thermo Scientific) into a suicide shuttle vector pKSV7

(Cornell University, Ithaca, NY), which can be expressed in both *Escherichia coli* and *L. monocytogenes* (Camilli et al., 1993; Smith and Youngman, 1992). The vector containing the gene of interest was first inserted into *E. coli* and subsequently electroporated (details described below) into *L. monocytogenes*. *Escherichia coli* transformants were selected on LB agar plates containing 100 μg/ml of ampicillin (AMP_{100}; Sigma-Aldrich). Plasmids were obtained from *E. coli* using GeneJET Plasmid Miniprep Kit (Thermo Scientific). They were sequenced at the Nucleic Acid Protein Service Unit (NAPS) at the University of British Columbia with NAPS-prepared primers (-21M13 and M13R) to confirm the absence of nucleotide deletions and polymorphisms, and subsequently electroporated into competent *L. monocytogenes* 08-5578 cells. Following the electroporation procedure (described in section 5.2.5), *L. monocytogenes* were grown on BHI agar plates containing chloramphenicol (10 μg/ml; CHL_{10}), at 30°C. Strains were incubated at this temperature for two days, to promote plasmid replication and integration into the chromosome. Colonies with CHL resistance were used to inoculate BHI broth (5 ml) containing CHL_{10}, and incubated overnight at 37°C, with shaking (100 rpm). Following incubation, 50 μl of culture was used to inoculate 5 ml of fresh BHI broth containing CHL_{10}. Cultures were incubated at 40°C with gentle shaking (100 rpm), and passaged (1:100 dilution) in BHI broth containing CHL_{10} up to 10 times, forcing the insert carried by pKSV7 to homologously recombine with the cell's wild type (WT) gene. A second round of passaging (10 to 20 times) in BHI broth without CHL was performed at 30°C (with shaking, 100 rpm), to promote recombination where cells revert to WT phenotype or become mutants. Screening for vector excision was performed by replica plating on BHI plates, and BHI plates containing CHL_{10}. PCR amplification with SOE-A and SOE-D primers was used to confirm the allelic exchange. Mutants were sequenced at NAPS

(University of British Columbia) using SOE-A and SOE-D (*lmo1851*), or XF and XR primers (*emrE,* and *sel1*).

5.2.5 Electroporation

Competent cells were thawed on ice. Purified plasmid construct (2 µg) was added to 50 µl of One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen), and 100 µl of competent *L. monocytogenes* 08-5578 cells, mixed and transferred to a pre-chilled (ca. -20°C) cuvette (0.1 cm; Bio-Rad, Hercules, CA). Electroporation was performed using a BioRad Gene Pulser (Bio-Rad, Hercules, CA, US) set to 1.1 kV pulse. Immediately following the treatment 250 µl of S.O.C. medium (Invitrogen) was added to the cuvette containing *E. coli,* and 1 ml of BHI containing 0.5 M sucrose (Sigma-Aldrich) was added to the cuvette containing *L. monocytogenes*. Contents were transferred to 1.5 ml sterile tubes (Eppendorf Safe-Lock Tubes™, Fisher Scientific, Ottawa, ON) and incubated at 37°C with shaking (*E. coli*) to allow expression of the antibiotic resistance gene, or tubes were placed on ice for 5 min (*L. monocytogenes*), followed by static incubation at 30°C, for up to two hours. Electroporated cells were spread (10 to 150 µl) onto pre-warmed LB plates containing AMP_{100} (*E.* coli) or BHI plates containing CHL_{10} (*L. monocytogenes*), and incubated at 37°C, overnight. Colonies were confirmed by PCR and appropriate SOE-A and SOE-D primers.

5.2.6 Antimicrobial agents

The list of compounds and concentrations used in experiments is provided in Table 5-3. Antimicrobial agents used in the study included antibiotics: chloramphenicol, ciprofloxacin, erythromycin, gentamicin, and tetracycline; quaternary ammonium compounds (QAC) such

as E-San® 10%, a sanitizer containing 5% N-alkyl dimethyl benzyl ammonium chloride (Epsilon Chemicals Ltd, Edmonton, AB), and a benzalkonium chloride (BAC) with alkyl distribution from C_8H_{17} to $C_{16}H_{33}$ (Acros Organics, New Jersey, US). Other antimicrobial compounds tested included acriflavine (Sigma-Aldrich), a cationic dye used in enrichment media during isolation of *Listeria* spp.; triclosan [Irgasan, 5-Chloro-2-(2,4-dichlorophenoxy) phenol; Sigma-Aldrich], a broad spectrum antimicrobial agent that inhibits enoyl-acyl-carrier protein reductase in fatty acid synthesis (Russell, 2004); and reserpine (Sigma-Aldrich), an efflux pump inhibitor (Godreuil et al., 2003). Antibiotic stock solutions were prepared according to manufacturer's instructions, and stored at -20°C for up to two months. Other antimicrobial agents were stored according to the manufacturer's recommendations (i.e. at 4°C or at room temperature). Working solutions of water-soluble agents (1,000 ppm) were prepared by diluting the concentrated sanitizers in sterile distilled water on the day of the experiment. These were stored at 4°C, and used within 3 h of preparation. CIP was dissolved in dimethyl sulfoxide (DMSO; 1 ml) and sterile deionized water (9 ml), while triclosan was dissolved in 70% ethanol (1 ml of 10,000 ppm transferred to 9 ml of 70% ethanol). A working solution of reserpine (10,000 ppm; Sigma-Aldrich) was prepared in DMSO with 20 µl added to bacterial cultures (total volume 10 ml in TSB). The highest volumes of DMSO used for dissolving CIP and reserpine, and 70% ethanol used for dissolving triclosan, were applied as controls to check for the diluent effect.

5.2.7 Minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) of antimicrobials and other compounds listed in Table 5-3 were determined using a slightly modified agar dilution method (e.g., antibiotics,

and QACs) described by Elhanafi et al. (2010), and microbroth dilution protocol (e.g., acriflavine and triclosan). Briefly, for the agar dilution method, strains were applied to Mueller-Hinton agar (MHA-B; 1.2% agar, Difco) supplemented with 5% defibrinated sheep blood (Alere Inc., Ottawa, ON), and incubated at 37°C overnight. Two colonies were transferred into 200 µl of Mueller-Hinton broth (Difco), and 5 µl of the suspension was spotted in duplicate onto MHA-B plates containing appropriate concentrations of antimicrobials/other compounds. Following 48 h incubation at 30°C, MICs were determined as the lowest assessed concentration that prevented confluent growth.

Table 5-3. List of antimicrobial agents used in experiments.

Antimicrobial agent[a] (abbreviation)	**Concentrations tested (ppm)[b]**
Antibiotics	
Chloramphenicol (CHL)	2.5, 5, 10, 15, 20
Ciprofloxacin (CIP))	2.5, 5, 10, 15, 20
Erythromycin (ERY)	1, 2.5, 5, 10, 15
Gentamicin (GEN)	1, 2.5, 5, 10
Tetracycline (TET)	2.5, 5, 10, 15, 30
Quaternary ammonium compounds (QAC)	
Benzalkonium chloride (BAC)	5, 10, 20, 25, 20
E-San	2.5, 5, 10, 15, 20
Other compounds	
Acriflavine	12, 16, 20, 24
Triclosan	1, 2, 4, 8
Reserpine	20

[a]All antibiotics, acriflavine, and triclosan came from Sigma-Aldrich.
[b]ppm, parts per million, equivalent to µg/ml.

For MIC determination using the microbroth dilution method, strains were grown in 5 ml of TSB (16 x 150 mm tubes) at 30°C with shaking (200 rpm) for 16-18 h. Following incubation, strains were diluted 1:100 in TSB (final volume 10 ml) and exposed to different concentrations of each antimicrobial agent (Table 5-3) in 96-well plates (Costar®, Corning®

Inc., Corning, NY, US), in duplicate. Plates were incubated at 30°C in a SpectraMax M2 (SoftMax Pro 6.3 version software; Molecular Devices, Sunnyvale, California) plate reader, with OD_{600} measured at 30 min intervals for 24 h. MICs were defined as the lowest concentration that prevented growth. All MIC experiments were performed at least three times.

5.2.8 Growth in the presence of sub-lethal concentrations of antimicrobials

Growth of *L. monocytogenes* in the presence of sub-lethal concentrations of E-San (0.78 and 1.56 ppm), and BAC (1 and 2 ppm), with (20 ppm) or without reserpine (efflux inhibitor) was assessed in a SpectraMax (Molecular Devices, Sunnyvale, California) plate reader at 30°C. Briefly, single colonies were inoculated into 5 ml of TSB (16 x 150 mm tubes) and incubated at 30°C, with shaking (200 rpm). Following 16 h incubation, cultures were diluted 1:100 in TSB containing appropriate concentrations of antimicrobials/compounds. Aliquots (200 µl) for each strain and treatment were transferred in duplicate into specified wells of a 96-well plate. The OD_{600} levels were monitored at 30 min intervals for 24 h. The OD_{600} data were fitted to growth curves to obtain the lag phase duration (LPD) and maximum growth rates (MGR), using the DMFit 3.0 Excel add-in program (ComBase, Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, UK), based on the models by Baranyi and Roberts (1994). Experiments were performed at least three times. Blank controls containing TSB, or TSB with appropriate concentrations of the tested antimicrobial were included in each run and these OD_{600} values were subtracted from the OD_{600} values for strains containing respective treatments. The relationship between OD_{600} measurements and viable cell numbers of the WT *L. monocytogenes* 08-5578 and deletion

mutant strains was derived from counts obtained by the spread plate method on TSA-YE at seven time points (i.e. 0, 1, 3, 5.5, 8, 10, and 24 h) representing early logarithmic, late logarithmic and late stationary growth phases at 30°C.

5.2.9 RNA isolation and cDNA preparation

Listeria monocytogenes WT strain was grown in 25 ml of TSB (50 ml tubes; Corning) at 30°C, with shaking (200 rpm). After 14 h incubation 5 ml of the culture were transferred to a sterile 10 ml tube and BAC was added to achieve a 10 ppm concentration; 5 ml were retained as a control. Following 1 h incubation at 30°C with shaking (200 rpm) the cultures were used directly for RNA extraction using the RNA PowerSoil® total RNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, California). Recovered RNA samples were treated with RTS DNase™ (MO BIO), and immediately placed at -80°C or converted to cDNA using the QuantiTect® Reverse Transcription kit (Qiagen, Toronto, ON). RNA was quantified and checked for quality with a spectrophotometer (NanoDrop ND1000, Thermo Scientific, Toronto, ON) and by gel electrophoresis.

5.2.10 Gene expression

Quantitative real-time PCR (qRT-PCR) assays examining transcript levels of two known efflux pumps in *Listeria* (e.g., *lde* and *mdrL*), *sigB*, and LGI1 encoded *emrE*, *lmo1851* and *lmo1861* were performed following 1 h exposure of *L. monocytogenes* 08-5578 strain to 10 ppm BAC. The list of primers used and their efficiencies are provided in Table 5-2. Primers were designed using Geneious 5.4 software (Biomatters Ltd., Aukland, New Zealand) and optimized to achieve specific target gene amplification (product with a single melting peak)

and PCR efficiency between 97 and 105% (Table 5-2). cDNA templates derived from *L. monocytogenes* 08-5578 treated with BAC (10 ppm) for 30 min were used in PCR optimization and amplification efficiency evaluation. Reactions were carried out in a final volume of 20 µl containing 1 µl cDNA template, 10 µl SsoAdvancedTM SYBR® Green Supermix (Bio-Rad), and 0.25 µM forward and reverse oligonucleotides (Table 5-2). Thermocycling conditions included initial denaturation at 95°C for 3 min, followed by 39 cycles at 95°C for 10 sec, 56°C for 5 sec, 72°C for 12 sec, melting curve measurement (65 to 95°C by 0.5°C increments, for 5 sec), and cooling (4°C), using CFX96 Touch™ Real-Time PCR Detection System (BioRad). Target gene transcript levels were quantified using CFX Manager™ Software 3.1 (BioRad), and normalized to the levels of *16S rRNA* housekeeping reference gene (Tasara and Stephan, 2007). Cycle threshold (Ct) standard deviation (SD) for all genes was ≤0.3. Gene expression fold change reported represents the means and standard deviations based on three independent assays and each sample run in duplicate.

5.2.11 Adhesion and invasion assays

The adhesion and invasion efficiency of *L. monocytogenes* 08-5578 (WT) and its Δ*sel1* mutant were assessed according to the protocol described in Chapter 3, section 3.2.4, with slight modifications. Briefly, TC-7 subclone of Caco-2 cells (Chantret et al., 1994), kindly provided by Dr. Monique Rousset (Centre de Recherche des Cordeliers, Paris, France) *via* Dr. Catherine Jumarie (Université du Québec à Montréal, Canada), and HeLa (ATCC® CCL-2™) cells ($1x10^5$ cells per well; passages 5 to 20) were cultured in vented cap, tissue culture treated flasks (growth area 75 cm^2; Falcon™, Fisher Scientific, Ottawa, ON) containing 30 ml of Dulbecco's modified Eagle's minimum essential medium (DMEM) (HyClone;

Thermo Scientific, Toronto, ON) supplemented with 10% inactivated fetal calf serum (Gibco, Life Technologies, Burlington, ON), 1% nonessential amino acids (Gibco), and 1% Gluta-MAX (Gibco) for two days (5% CO_2, 37°C) to reach confluence (i.e. non-differentiated cells). Bacterial cultures grown statically overnight in BHI (5 ml; 16 x 150 mm tubes) at 30°C were pelleted by centrifugation (5,939 x *g* at 22°C; Eppendorf 5415 R), washed once, resuspended in 1x Dulbecco's phosphate-buffered saline (DPBS) (HyClone) with magnesium and calcium, and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (Genesys 10UV; Thermo Spectronic, Rochester, NY, US). Prior to infection, bacterial cultures were diluted in DMEM to approximately 5 $x10^6$ CFU/ml, as assessed by counts obtained by the spread plate method on TSA-YE. Bacterial suspensions (0.5 ml) were added to Caco-2 and HeLa cells and incubated at 37°C for 30 min or 1 h to allow bacterial adherence and entry, respectively. Infected cells were then washed three times with DPBS, and lysed with sterile ice-cold water for 10 min at 37°C (adhesion) or overlaid with fresh pre-warmed DMEM containing gentamicin (50 mg/l), and incubated at 37°C for 45 min (invasion). Following gentamicin treatment, the cell monolayers were washed three times with DPBS and lysed with sterile ice-cold water for 10 min at 37°C.

The number of viable bacteria was quantified by spreading direct inoculum and serial dilutions (10^{-1} to 10^{-4}) in DPBS onto TSA-YE plates, incubated at 37°C for 24 to 48 h. Adhesion was reported as average $\log_{10}$ CFU/ml, where starting inoculum and recovered cells for each strain were normalized to those of the WT strain. The invasion efficiency was reported as the percentage of the inoculum recovered by the enumeration of intracellular bacteria, normalized to 08-5578 strain (i.e. set at 100%). *Listeria monocytogenes* BUG5 (Tn1545-induced *inlA* mutant from EGD-SmR) (Gaillard et al., 1986), and 10403S, kindly

provided by Dr. Pascale Cossart (Institut Pasteur) and Dr. Kendra Nightingale (Texas Tech University), respectively, were used as other 1/2a serotype controls. Assays for each isolate were carried out in duplicate and repeated at least three times.

5.2.12 Acid stress survival

Survival of *L. monocytogenes* 08-5578 and its LGI1 mutant derivatives in acidic conditions was assessed according to protocols described by Ells and Truelstrup Hansen (2011) and Oliver et al. (2010). Briefly, BHI (50 ml) was adjusted to pH of 4.5, 3.5, and 2.5 (Pinnacle pH meter, Nova Analytics Corporation) with 6 N HCl, and 9.9 ml were distributed into sterile tubes (i.e. five for each pH). Overnight cultures (10 µl) grown in BHI at 30°C (with shaking, 200 rpm) were inoculated into 5 ml of fresh BHI. Following incubation for 16 h at 30°C, 2 ml of cultures were spun at 6,000 x *g* for 10 min at room temperature, washed twice in 0.1% peptone water (2 ml), re-suspended in 2 ml of 0.1% peptone water, and added to pH-adjusted BHI to get 10^7-10^8 CFU/ml (counts were confirmed by enumeration on TSA-YE). Cultures were mixed with a vortex mixer and incubated at 30°C, with shaking (200 rpm). A 100-µl aliquot was removed immediately (t=0), and 1, 2, 4, 6, 8, 10 and 24 h after acidification, diluted in buffered peptone water (BPW; Acumedia) and spread onto TSA-YE, in duplicate. Plates were incubated at 37°C, and counts recorded after 24 h. Experiments were repeated three times.

5.2.13 Cold adaptation

Cold growth adaptation of *L. monocytogenes* and its LGI1 mutant derivatives was evaluated according to the protocol described by Arguedas-Villa et al. (2010). Briefly, a single colony

of each *L. monocytogenes* isolate was inoculated into 10 ml BHI (16 x 150 mm tube) and grown overnight at 37°C with shaking (200 rpm) (~10^9 CFU/ml). Fresh BHI (10 ml) was inoculated with approximately 10^3 CFU/ml and incubated at 4°C until bacteria reached stationary phase (approximately four to seven weeks). Growth was monitored by spreading 10-fold serial dilutions prepared in peptone-saline on TSA-YE. Plates were incubated at 37°C, and colonies were counted after 24 h. Analysis was carried out at time 0 and weekly until stationary phase was reached. LPD and MGR of each strain were calculated from log-converted growth (CFU/ml) data using the DMfit 3.0 (ComBase) program. LGI1 deletion mutants were compared to the parental strain to determine if phenotypic differences existed in survival or growth following exposure to cold.

5.2.14 Salt stress survival

To assess the growth and survival of the isolates at different salt concentrations, *L. monocytogenes* 08-5578 and its LGI1 mutant derivative strains were exposed to 5, 10, 15 and 20% NaCl (wt/vol; Fisher Scientific), according to the protocol described by Ells and Truelstrup Hansen (2011), with slight modifications. Briefly, 10 µl aliquots of overnight cultures grown in BHI at 30°C, with shaking (200 rpm), were inoculated into 5 ml of fresh BHI (16 x 150 mm tubes). Following incubation for 16 h at 30°C (shaking at 200 rpm), cultures were spun (3,000 x *g*, 10 min) at room temperature, washed twice in 0.1% peptone water and diluted to 10^7-10^8 CFU/ml in BHI containing appropriate concentrations of NaCl (16 x 150 mm tubes). Aliquots (200 µl) for each strain and treatment were transferred in duplicate into specified wells of a 96-well plate. The OD_{600} levels were monitored in a SpectraMax (Molecular Devices) plate reader at 30°C at 30 min intervals for 24 h. In

parallel, cultures exposed to 10 and 20% NaCl concentrations in the tubes were mixed with a vortex mixer and incubated at 30°C, with shaking (200 rpm). A 100 µl aliquot removed immediately (t=0), and after 1, 2, 4, 6, 8, 10, and 24 h, was serially diluted in BPW and spread onto TSA-YE, in duplicate. Plates were incubated at 37°C, and counts were recorded after 24 h. Experiments were repeated three times.

5.2.15 Statistical analysis

Data analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA with Dunnett's multiple comparisons test was used to compare LPD, MGR and maximum OD_{600} values, and adhesion and invasion efficiencies between strains. Differences (LPD, MGR, maximum OD_{600}) between isolates possessing LGI1 and those without LGI1 were assessed using the Mann-Whitney test. For all analyses, differences were considered significant if the p value was < 0.05.

5.3 Results

5.3.1 The presence of LGI1 in *L. monocytogenes* from the food chain

LGI1 was detected in one (LR39-1) of the two 1/2a *L. monocytogenes* isolates belonging to CC8 (ST120). The isolate possessing LGI1 was recovered from a retail RTE fish sample. None of the *L. monocytogenes* or other *Listeria* spp. isolates recovered from food processing facilities harbored genomic island LGI1.

5.3.2 Minimum inhibitory concentrations of antimicrobials against *L. monocytogenes*

No differences were observed between the MICs of the antibiotics against WT parent strain and deletion mutants (Table 5-4). All four strains were sensitive to CHL, ERY, GEN and TET, and exhibited reduced susceptibility to CIP. The *L. monocytogenes* isolate possessing a deletion in the *emrE* gene (lt 1862) had two and three times lower MICs for QACs compared to the parent strain, and other mutants (Table 5-5). No differences were observed in MICs for acriflavine or triclosan.

Table 5-4. Minimum inhibitory concentrations (MIC) of different antibiotics against *L. monocytogenes* possessing deletions in LGI1 genes, and wild type (WT) parent strains.

Strain	**MIC (µg/ml)**				
	Chloramphenicol	Ciprofloxacin	Erythromycin	Gentamicin	Tetracycline
08-5578 (WT)	15	5	1[a]	5	5
08-5578:Δ*emrE*	15	5	1	5	5
08-5578:Δ*lmo1851*	15	5	1	5	5
08-5578:Δ*sel1*	15	5	1	5	5

[a]Lowest concentration tested.

5.3.3 Growth of WT *L. monocytogenes* and LGI1 mutants in the presence of sub-lethal concentrations of sanitizers

When exposed to sub-lethal concentrations of QAC sanitizers, the Δ*emrE* mutant showed impaired growth compared to the parent and other mutant strains (Figures 5-1 to 5-3). This effect was particularly pronounced at 1.56 ppm and 2 ppm for E-San and BAC, respectively (Figure 5-3).

Table 5-5. Minimum inhibitory concentrations (MIC) of different antimicrobials against *L. monocytogenes* possessing deletions in LGI1 genes, and wild type (WT) parent strains.

Strain	**MIC (ppm)**[a]			
	Acriflavine[b]	Benzalkonium chloride[c]	E-San[c]	Triclosan[b]
08-5578 (WT)	18	30	20	8
08-5578:Δ*emrE*	18	10	10	8
08-5578:Δ*lmo1851*	18	30	20	8
08-5578:Δ*sel1*	18	30	20	8

[a]ppm, parts per million or µg/ml or µl/ml.
[b]Assessed using microbroth dilution method.
[c]Assessed using agar dilution method.

Significantly longer LPDs were observed with the Δ*emrE* mutant compared to the parent strain (p<0.0001). The resumption of growth took 2.6 and 2.4 times longer under exposure to E-San and BAC at 0.78 and 1 ppm concentrations, respectively. LPDs ware also four and six times longer than in the parent strain when exposed to E-San and BAC at 1.56 and 2 ppm, respectively (Table 5-6).

Similarly, the Δ*emrE* mutant grew 1.3 times slower than the parent strain in the presence of E-San and BAC at 0.78 and 1 ppm, and 2.8 and 1.6 times slower when concentrations were increased to 1.56 and 2 ppm, respectively, (Table 5-6). The Δ*emrE* mutant also had lower maximum OD_{600} values after 24 h in the presence of sanitizers when compared to the parent strain and other mutants (Table 5-6). Similar LPD, MGR, and maximum OD_{600} values were observed for Δ*lmo1851*, Δ*sel1* and the parent strain (Table 5-6).

Significantly shorter LPD (p<0.05) was seen for strains possessing LGI1 than for strains without LGI1 when exposed to sub-lethal concentrations of E-San and BAC sanitizers (Figure 5-4). Similarly, strains possessing LGI1 grew faster (p<0.001), and reached higher

maximum OD_{600} values ($p<0.05$) in the presence of sub-lethal concentrations of E-San and BAC compared to strains without LGI1 (Figures 5-5 and 5-6).

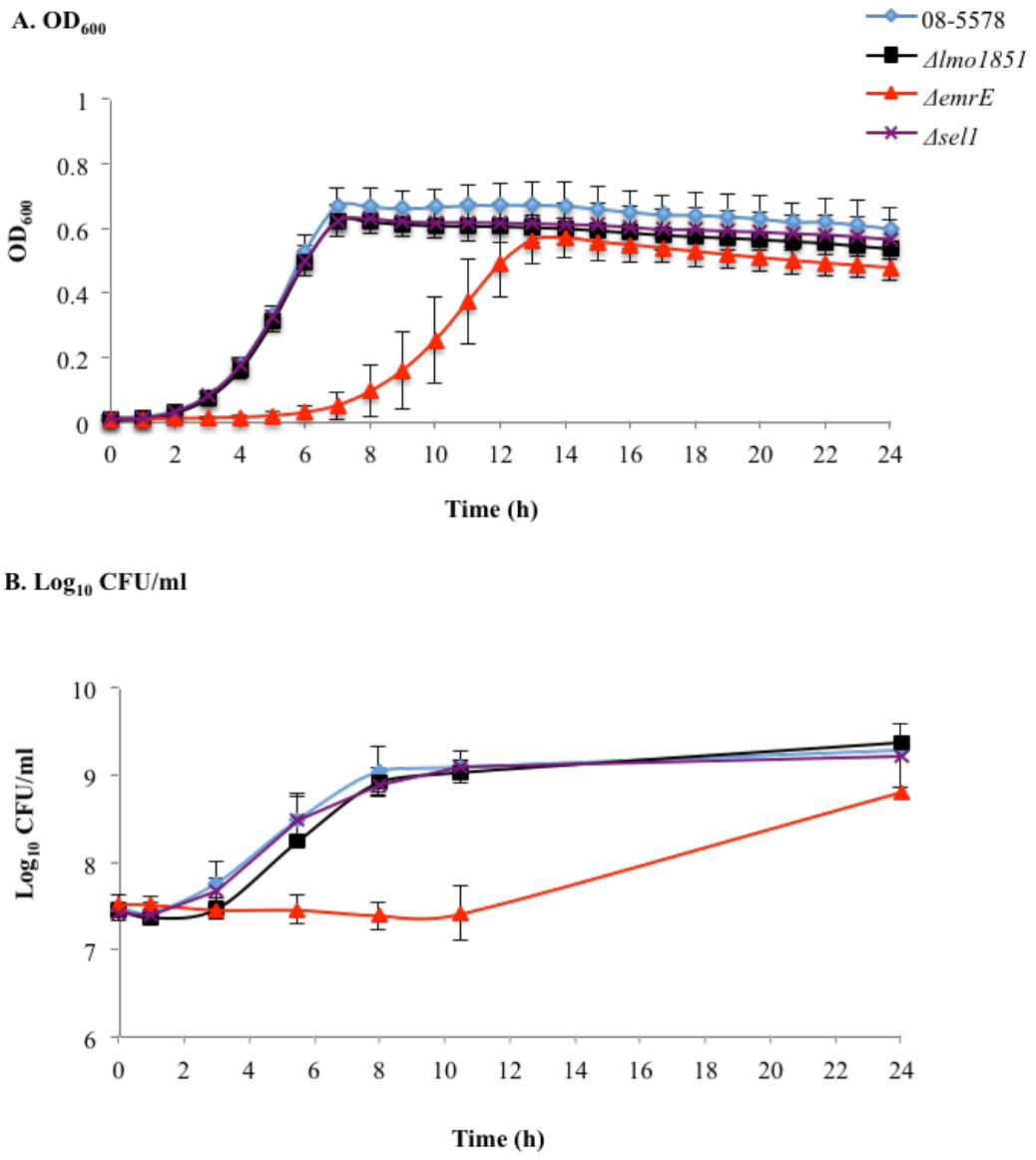


Figure 5-1. Growth of *L. monocytogenes* 08-5578 (WT) parent strain and its isogenic deletion mutants in the presence of E-San (0.78 ppm) sanitizer at 30°C, based on OD_{600} (A) and log_{10} CFU/ml (B) values. The data shown represent the mean OD_{600} and log_{10} CFU/ml values of five and three independent cultures, respectively.

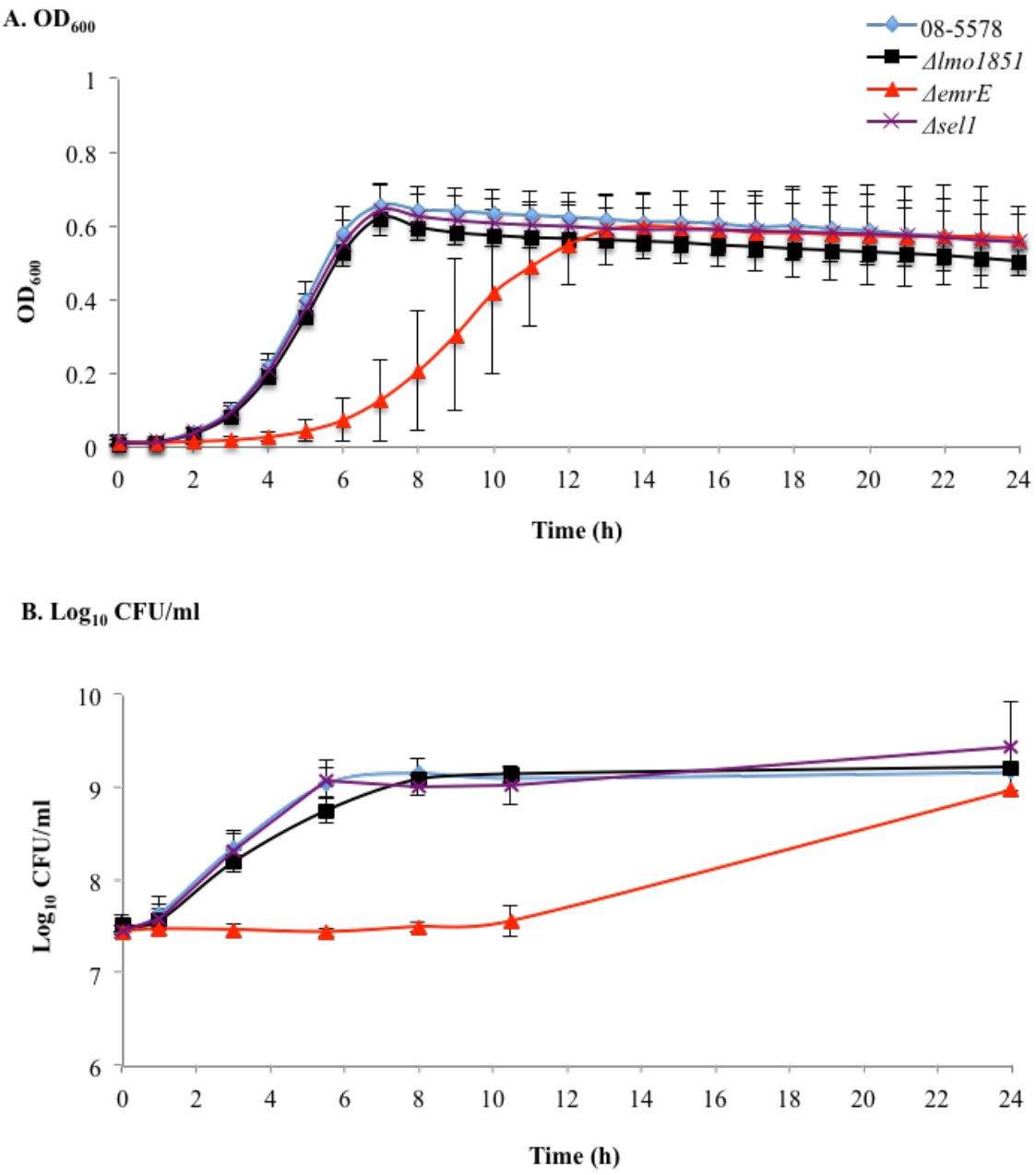


Figure 5-2. Growth of *L. monocytogenes* 08-5578 (WT) parent strain and its isogenic deletion mutants in the presence of benzalkonium chloride (BAC; 1 ppm) sanitizer at 30°C, based on OD_{600} (A) and $\log_{10}$ CFU/ml (B) values. The data shown represent the mean OD_{600} and $\log_{10}$ CFU/ml values of five and three independent cultures, respectively.

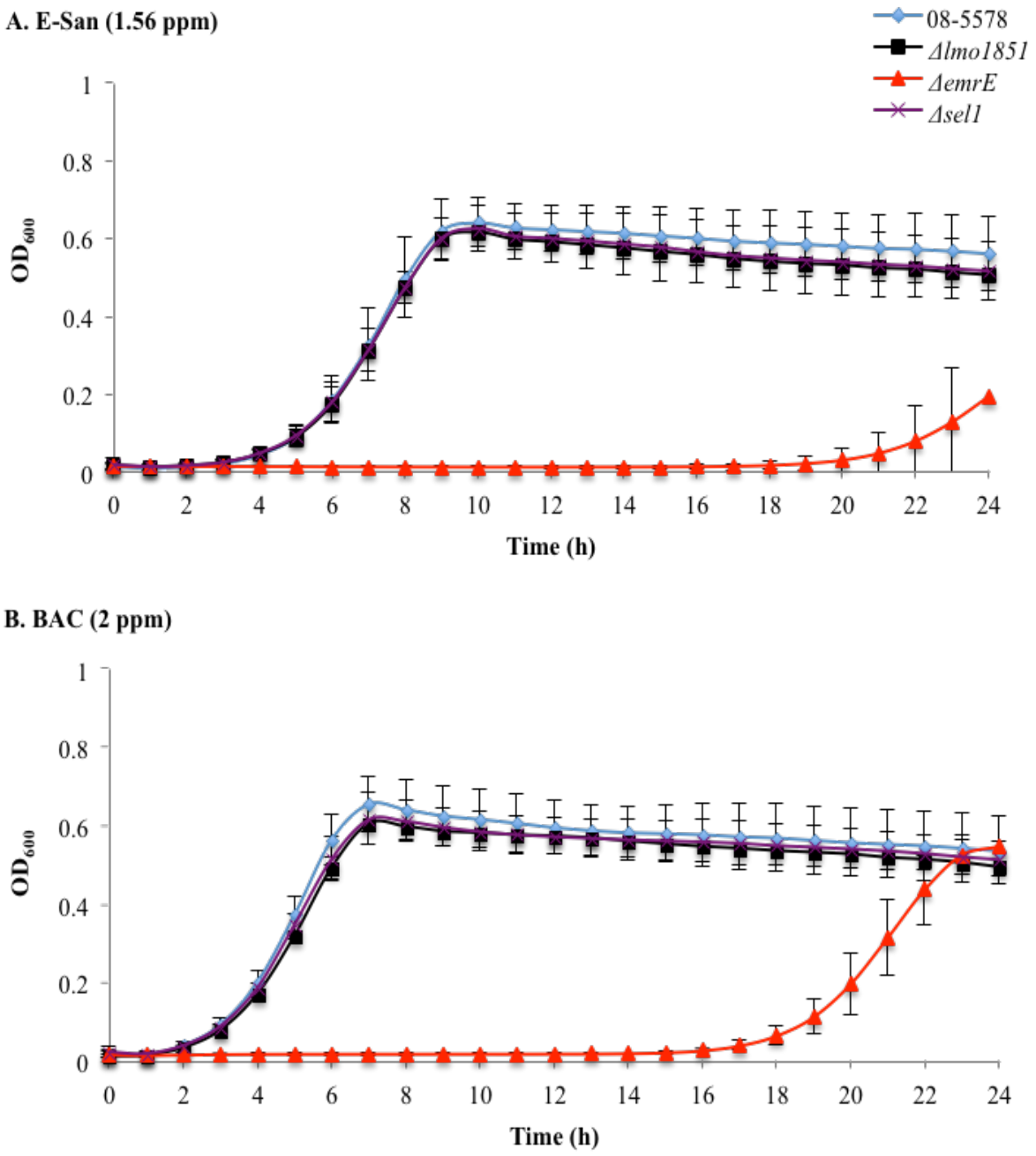


Figure 5-3. Growth of *L. monocytogenes* 08-5578 (WT) strain and its isogenic deletion mutants in the presence of sanitizers E-San at 1.56 ppm (A), and benzalkonium chloride (BAC) at 2 ppm (B) at 30°C. The data shown represent the mean OD_{600} values of five independent cultures.

Table 5-6. Average lag phase duration, maximum growth rate, and maximum optical density of *Listeria monocytogenes* 08-5578 and its LGI1 deletion mutants, Δ*lmo1851*, Δ*emrE*, and Δ*sel1*, when exposed to sub-lethal concentrations of QAC-based sanitizers, E-San and benzalkonium chloride (BAC) for 24 h at 30°C. Values represent mean values from five independent assays, with each sample and treatment measured in duplicate.

Treatment	***L. monocytogenes* strains**			
	08-5578 (WT)	**Δ*emrE***	**Δ*lmo1851***	**Δ*sel1***
Lag phase duration (h)				
E-San				
0.78 ppm	3.31± 0.15	**8.45± 1.34*** **	3.30± 0.30	3.18± 0.29
1.56 ppm	5.17± 0.50	**21.02± 0.79*** **	5.16± 0.49	5.10± 0.55
BAC				
1 ppm	2.87± 0.39	**6.80± 1.40*** **	3.10± 0.23	3.02± 0.20
2 ppm	3.16± 0.19	**18.58± 0.68*** **	3.21± 0.17	3.14± 0.17
TSB	2.68± 0.62	2.79± 0.56	3.04± 0.13	2.96± 0.22
Maximum growth rate (increase in OD_{600} /h)				
E-San				
0.78 ppm	0.19± 0.03	**0.15± 0.02***	0.18± 0.01	0.18± 0.03
1.56 ppm	0.17± 0.02	**0.06± 0.04*** **	0.17± 0.02	0.16± 0.02
BAC				
1 ppm	0.19± 0.01	**0.15± 0.01***	0.18± 0.02	0.19± 0.02
2 ppm	0.20± 0.02	**0.12± 0.01****	0.17± 0.02	0.18± 0.03
TSB	0.19± 0.03	0.21± 0.03	0.19± 0.02	0.21± 0.03
Maximum optical density (OD_{600})				
E-San				
0.78 ppm	0.69± 0.07	0.60± 0.06	0.64± 0.01	0.65± 0.05
1.56 ppm	0.65± 0.06	**0.23± 0.18*** **	0.63± 0.01	0.63± 0.06
BAC				
1 ppm	0.68± 0.07	0.66± 0.11	0.63± 0.01	0.65± 0.06
2 ppm	0.66± 0.07	**0.56± 0.04***	0.62± 0.01	0.63± 0.06
TSB	0.67± 0.06	0.73± 0.07	0.67± 0.06	0.70± 0.06

[a]Statistically significant values are highlighted in bold at $p<0.05$ (*), $p<0.001$ (**), and $p<0.0001$ (***), using one-way ANOVA with Dunnett's multiple comparisons test.

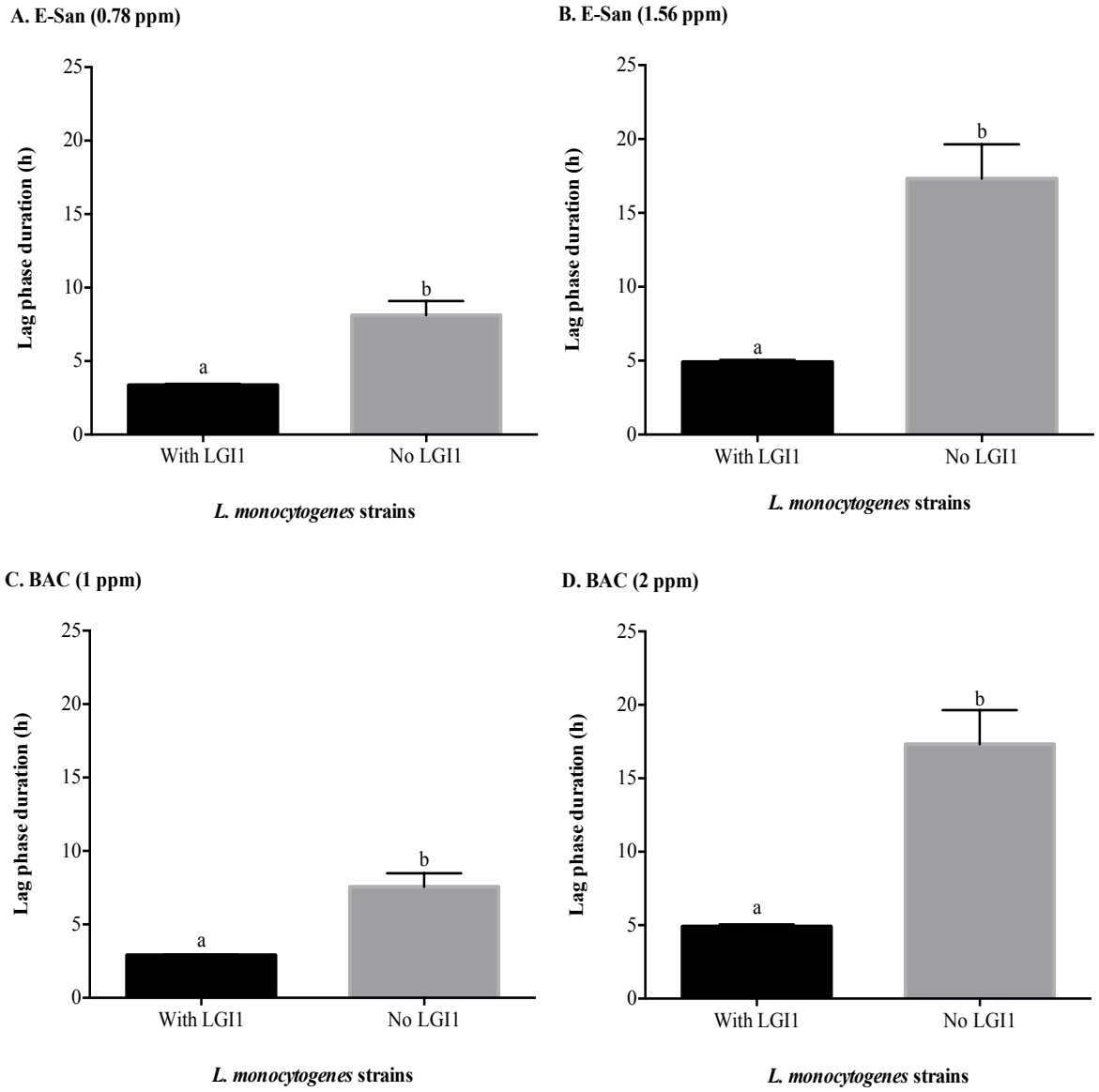


Figure 5-4. Mean lag phase duration (h) of *L. monocytogenes* isolates possessing LGI1 (n=9) and isolates without LGI1 (n=8) when grown in the presence of sub-lethal concentrations of E-San sanitizer at 0.78 (A) and 1.56 ppm (B), and 1 (C) and 2 ppm (D) of benzalkonium chloride (BAC), at 30°C for 24 h. Bars represent mean lag phase duration values and error bars indicate standard errors of the mean. Different letters above the bars represent significant differences (p<0.05) between the groups, determined using the Mann-Whitney test.

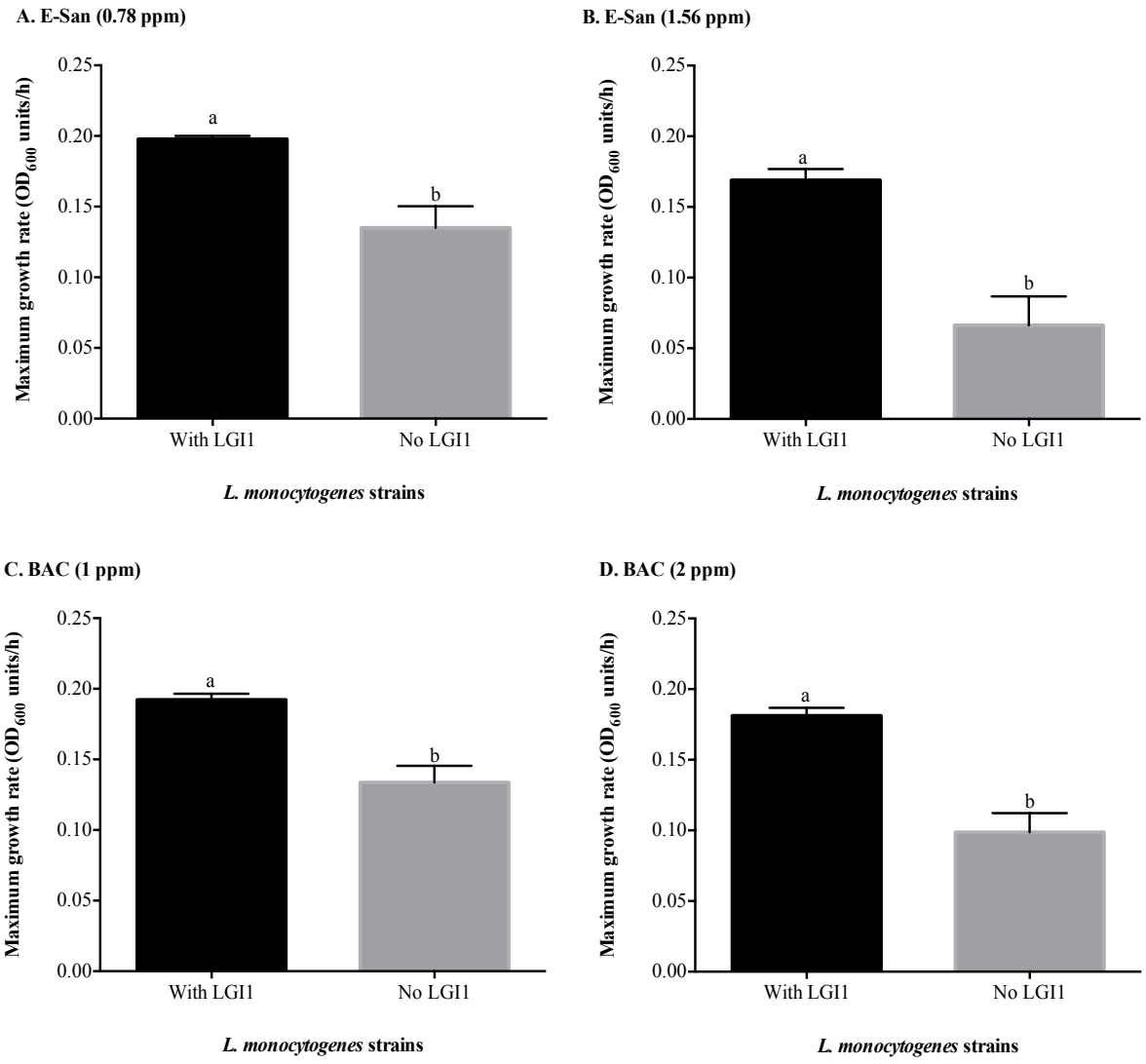


Figure 5-5. Mean maximum growth rate (OD_{600} units/h) of *L. monocytogenes* isolates possessing LGI1 (n=9) and isolates without LGI1 (n=8) when grown in the presence of sub-lethal concentrations of 0.78 (A) and 1.56 ppm (B) of E-San, and 1 (C) and 2 ppm (D) of E-San sanitizer at 0.78 (A) and 1.56 ppm (B), and 1 (C) and 2 ppm (D) of benzalkonium chloride (BAC), at 30°C for 24 h. Bars represent mean growth rate values and error bars indicate standard errors of the mean. Different letters above the bars represent significant differences (p<0.001) between the groups, determined using the Mann-Whitney test.

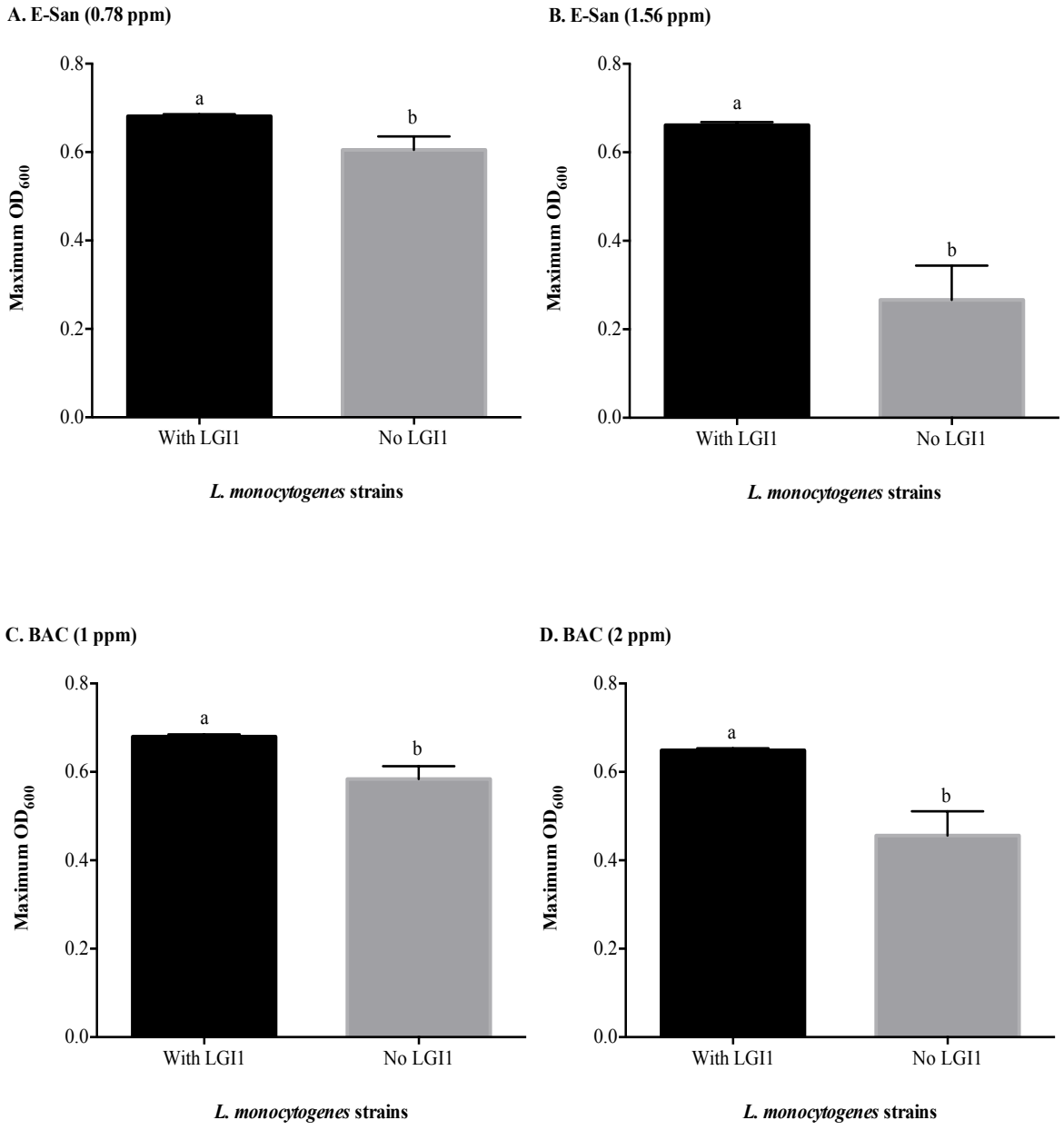


Figure 5-6. Maximum OD_{600} values for *L. monocytogenes* isolates possessing LGI1 (n=9) and isolates without LGI1 (n=8) when grown in the presence of sub-lethal concentrations of E-San sanitizer at 0.78 (A) and 1.56 ppm (B), and 1 (C) and 2 ppm (D) of benzalkonium chloride (BAC), at 30°C for 24 h. Bars represent mean growth rate values and error bars indicate standard errors of the mean. Different letters above the bars represent significant differences (p<0.05) between the groups, determined using the Mann-Whitney test.

The addition of the known efflux inhibitor reserpine to TSB containing lower concentrations of E-San (0.78 ppm) and the two tested BAC concentrations (1 and 2 ppm) only marginally impacted the growth of WT 08-5578 (Figure 5-7). However, at higher concentrations of E-San (1.56 ppm) the effect was more pronounced (Figure 5-7A).

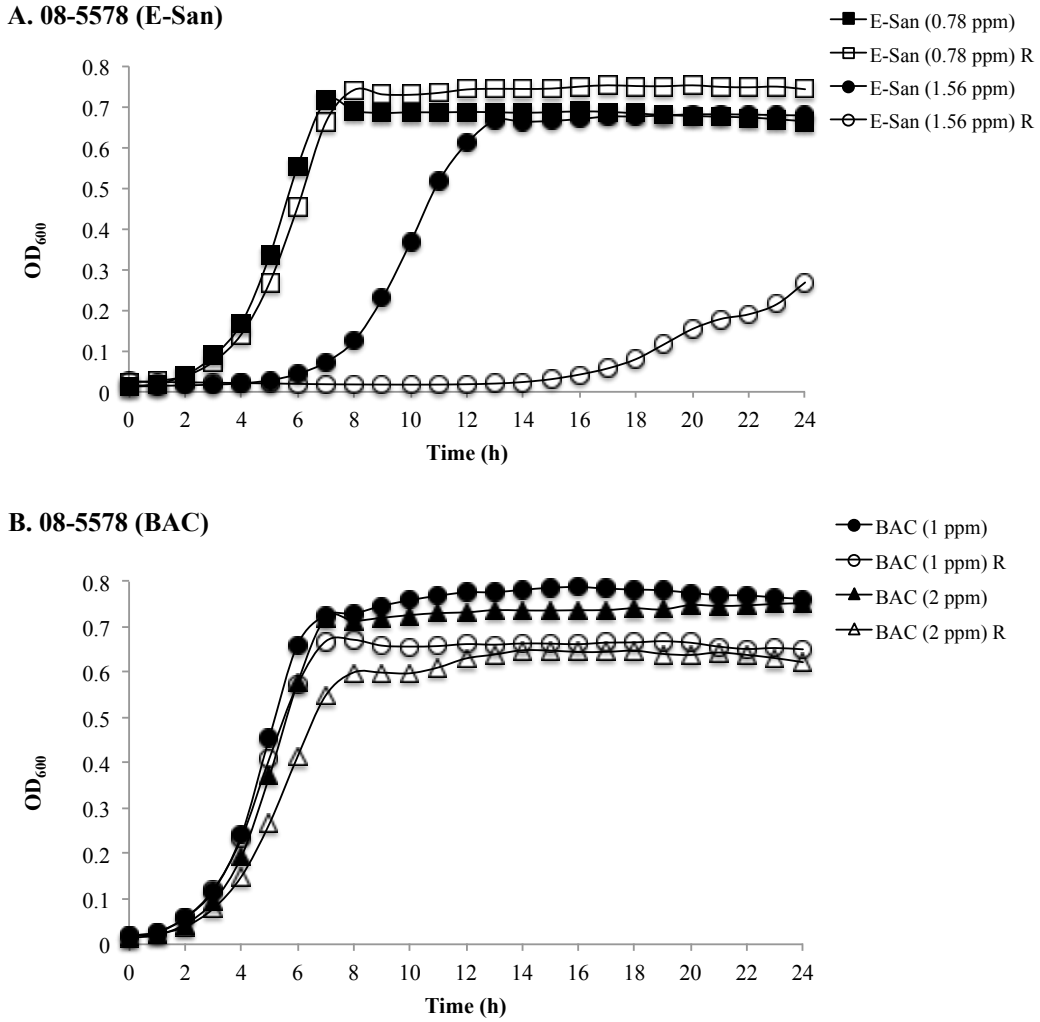


Figure 5-7. Growth of *L. monocytogenes* 08-5578 (WT) strain in the presence of sanitizers E-San at 0.78 and 1.56 ppm (A), and benzalkonium chloride (BAC) at 1 and 2 ppm (B) at 30°C, with (white line markers) and without (black filled line markers) reserpine (R; 20 μg/ml). The data shown represent the mean OD_{600} values of three independent assays. Standard deviation ranged from 0.0010 to 0.24.

Longer LPDs were observed when reserpine was added to the Δ*emrE* mutant cultures containing sub-lethal concentrations of E-San and BAC (Figure 5-8). At higher concentrations of BAC (2 ppm) growth was visibly suppressed, while with the addition of reserpine at 1.56 ppm E-San completely inhibited growth of the Δ*emrE* mutant (Figure 5-8).

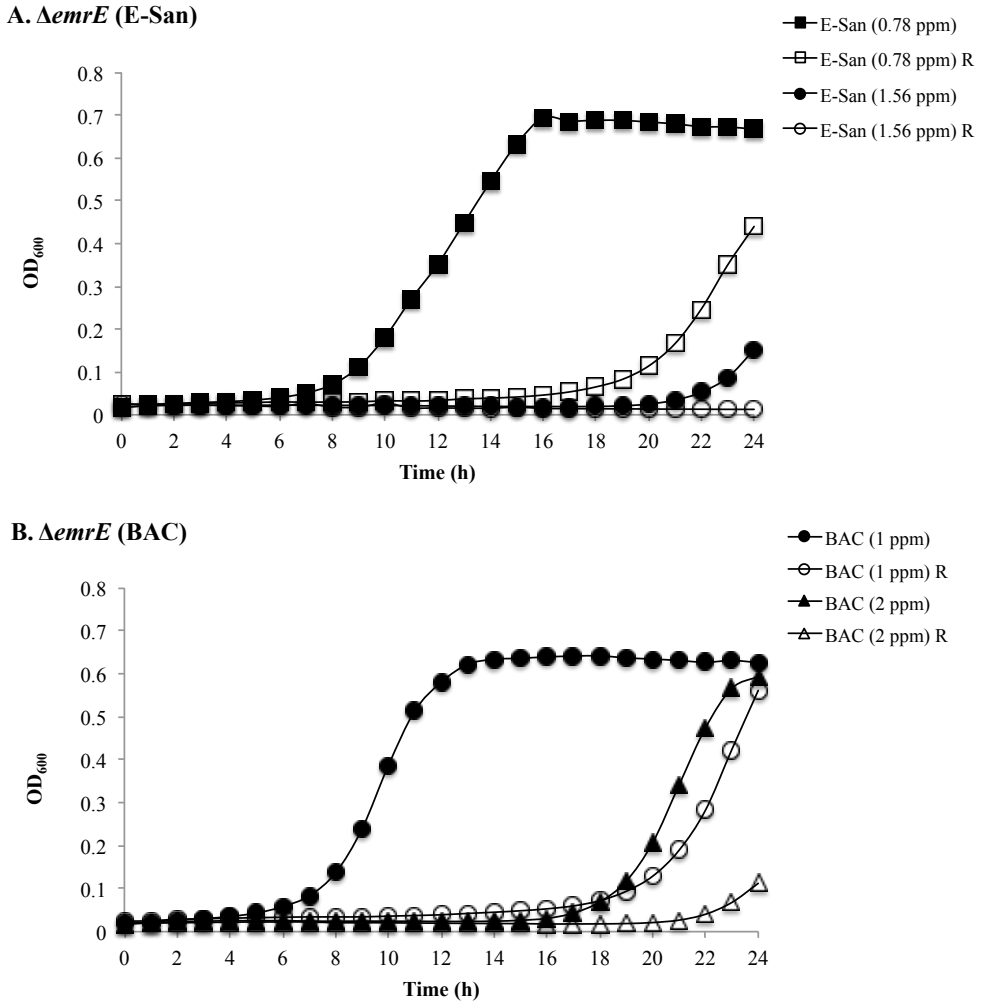


Figure 5-8. Growth of *L. monocytogenes* Δ*emrE* mutant in the presence of sanitizers E-San at 0.78 and 1.56 ppm (A), and benzalkonium chloride (BAC) at 1 and 2 ppm (B) at 30°C, with (white line markers) and without (black filled line markers) reserpine (R; 20 µg/ml). The data shown represent the mean OD_{600} values of three independent assays. Standard deviation ranged from 0.0004 to 0.29.

5.3.4 Gene expression in WT *L. monocytogenes* when exposed to sub-lethal concentration of BAC

When *L. monocytogenes* 08-5578 was exposed to BAC at 10 ppm for 1 h the highest change (82.4-fold) in the expression of LGI1 genes was seen for *lmo1861*, a putative MarR-family transcriptional regulator, followed by *emrE* (49.6), encoding a small multidrug resistance (SMR) efflux pump (Table 5-7). The expression of LGI1 encoded putative response regulator (*lmo1851*) of a two-component transduction system also increased 2.3-fold, while the expression of *sigB*, a major stress response regulator in *L. monocytogenes*, increased 4.1-fold. There was no change in the expression of *lde* and *mdrL*, genes encoding for proteins that belong to the major facilitator superfamily (MFS) of multidrug resistance efflux pumps.

Table 5-7. Gene expression of *L. monocytogenes* 08-5578 strain when treated with benzalkonium chloride (10 ppm) sanitizer for 1 h, relative to the control (TSB).

Functional category and gene name	Physiological function	Fold change[a]
LGI1 encoded		
emrE	Putative efflux pump	**49.6**
lmo1851	Putative response regulator of a two-component signal transduction system	**2.3**
lmo1861	Putative MarR-family transcriptional regulator	**82.4**
Stress response		
lde	Major facilitator superfamily efflux pump	1.7
mdrL	Major facilitator superfamily efflux pump	0.6
sigB	Alternate sigma factor	**4.1**

[a]Relative changes in the expression levels for genes of interest were normalized against a housekeeping gene *16S rRNA*, encoding the RNA component of the smaller subunit of the bacterial ribosome, and compared to the control grown in TSB without the sanitizer treatment. Results that are >2- fold up-regulated are in bold; Ct standard deviation for all genes was ≤0.3.

5.3.5 Adhesion and invasion of Δ*sel1* mutant to TC-7 and HeLa cells

There were no differences in the adhesion or the invasion efficiencies of the Δ*sel1* and WT strains measured with TC-7 and HeLa cell lines (Figure 5-9). The number of adhered cells for both WT and the Δ*sel1* deletion mutant strain were similar to those observed for the control strain 10403S (6.4 $\log_{10}$ CFU/ml). Adhesion efficiencies to TC-7 cells was higher for all strains tested, except the *inlA* deficient mutant BUG5 (Figure 5-9A and B). No differences were observed between the 10403S control strain, and WT and Δ*sel1* mutant when invasion of TC-7 cells was examined (Figure 5-9C). However, significantly fewer WT (08-5578) and Δ*sel1 L. monocytogenes* could invade HeLa cells than the 10403S control strain (Figure 5-9D).

5.3.6 Acid tolerance

No differences were observed between the WT parent (08-5578) strain and LGI1 deletion mutants when strains were grown in BHI that was adjusted to pH 2.5, 3.5, and 4.5 (Figure 5-10). At pH 4.5 bacterial counts remained constant for all four strains, at approximately 7.6 log CFU/ml. When pH was reduced to pH 3.5, bacterial counts started to decline after 10 h, with an overall decrease of approximately 1.5 log CFU/ml within 24 h. Exposure of cells to pH 2.5 resulted in the decrease in bacterial counts for all four strains after approximately 5 h, and no viable bacteria were recovered at 24 h.

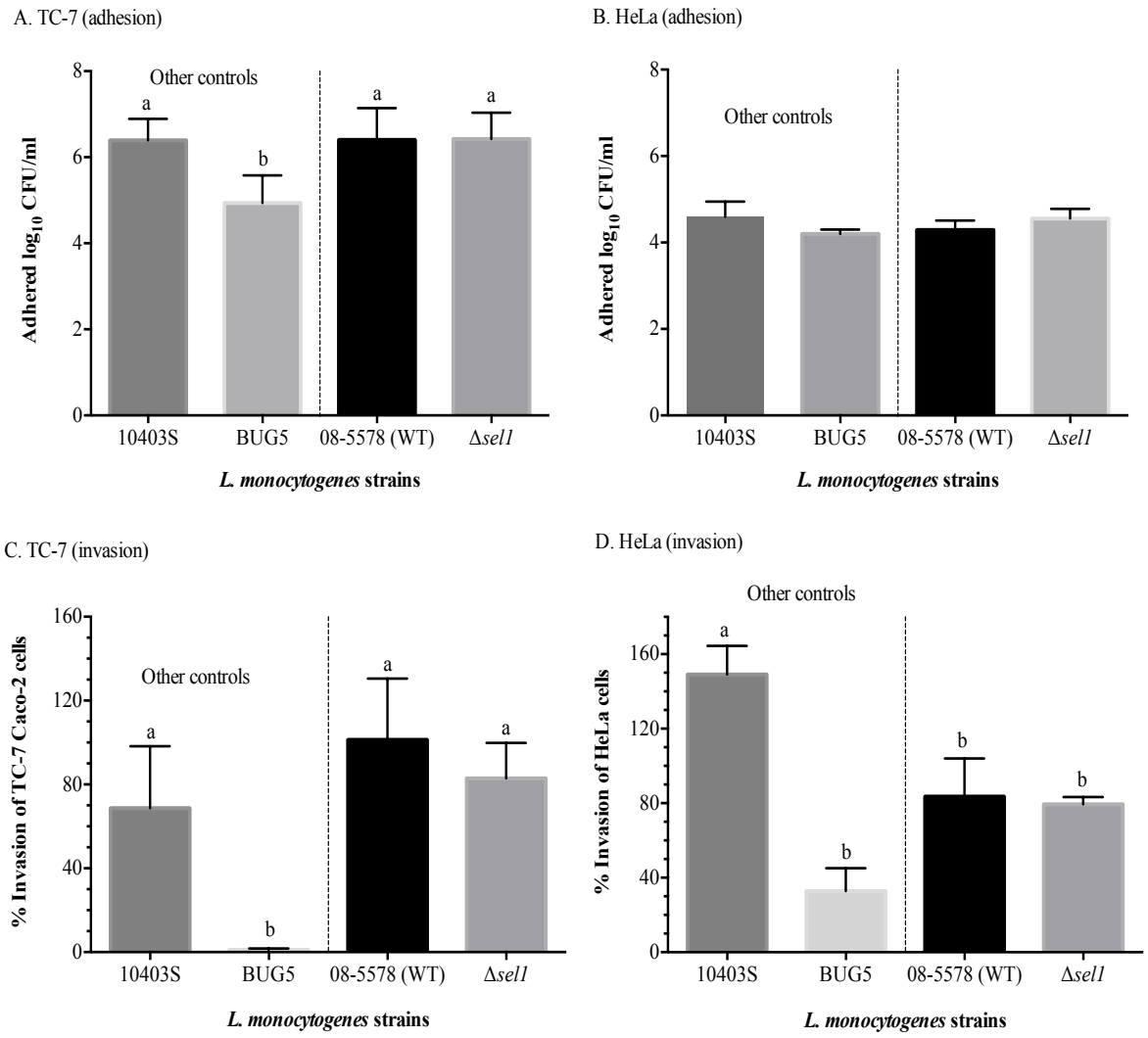


Figure 5-9. Adhesion and invasion efficiencies (% of bacteria recovered relative to the initial inoculum, normalized to 08-5578 strain) of *L. monocytogenes* WT (08-5578) strain and its isogenic mutant possessing deletion in *sel1* gene located on LGI1, compared to a clinical isolate 10403S and a Tn*1545*-induced noninvasive *inlA* mutant of EGD-SmR (BUG5), using TC-7 (A and C) and HeLa (B and D) cells. Assays for each isolate were carried out in duplicate and repeated four times. Bars show mean adhesion (log_{10} CFU/ml) and invasion efficiencies (normalized to 08-5578 strain), and error bars indicate standard errors of the mean. Different symbols above the bars indicate significantly higher adhesion or invasion efficiencies ($p<0.05$; one-way ANOVA with Dunnett's multiple comparisons test).

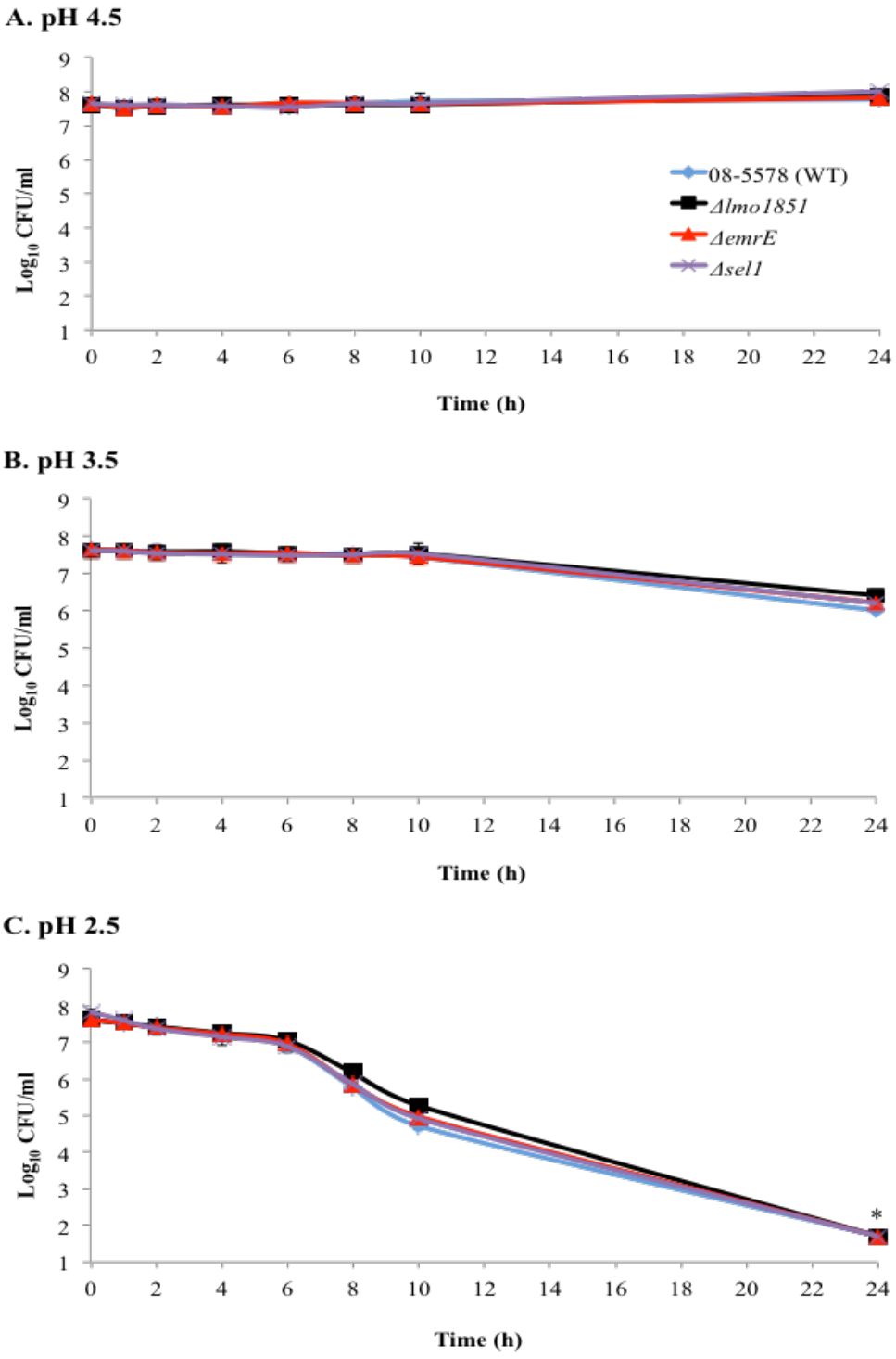


Figure 5-10. Growth of *L. monocytogenes* 08-5578 (WT) strain and its LGI1 mutants in BHI broth adjusted to pH 4.5 (A), pH 3.5 (B) and pH 2.5 (C) with 6 N HCl, at 30°C. The data shown represent the mean $\log_{10}$ CFU/ml values from three different experiments. At pH 2.5 after 24 h, counts were below the limit of detection (i.e. 10 CFU/ml) and they were assigned a value of 5 CFU/ml; correspondingly a $\log_{10}$ CFU/ml value of 1.7 represents a sample in which viable cells were not detected.

5.3.7 Adaptation and growth in cold environment

No differences in the adaptation to cold environment were observed between WT and LGI1 mutants when LPD, MGR, and maximum CFU/ml were measured at 4°C following a downshift from 37°C (Table 5-8). All four strains adapted to cold temperature rapidly, and resumed growth after approximately 2 to 2.5 h following cold exposure (Table 5-8). All four strains reached the stationary phase within approximately three weeks.

Table 5-8. Cold growth adaptation of WT *L. monocytogenes* 08-5578 and its LGI1 deletion mutants based on the lag phase duration (h), growth rate ($\Delta\log_{10}$ CFU/h), and maximum density ($\log_{10}$ CFU/ml) reached during incubation at 4°C following a downshift from 37°C.

***L. monocytogenes* strains**	**LPD (h) (±SD)**	**Growth rate ($\Delta\log_{10}$ CFU/h)**	**Maximum ($\log_{10}$ CFU/ml)**
08-5578 (WT)	2.48 ± 0.74	0.47 ± 0.036	9.22 ± 0.13
Δ*emrE*	1.55 ± 0.56	0.40 ± 0.0065	9.23 ± 0.19
Δ*lmo1851*	2.68 ± 0.59	0.49 ± 0.073	8.99 ± 0.10
Δ*sel1*	2.37 ± 0.17	0.43 ± 0.056	9.18 ± 0.010

5.3.8 Salt tolerance

L. monocytogenes Δ*lmo1851* mutant strain reached slightly higher maximum OD_{600} values compared to the parent and other mutant strains when it was grown in BHI containing 5 and 10% NaCl (Figure 5-11). Differences were, however, not statistically significant (p>0.05). No growth was observed in the presence of 15 and 20% NaCl for the WT and mutant strains based on OD_{600} measurements. Similarly, there was no increase or decrease in viable counts over 24 h in the presence of 15 and 20% NaCl.

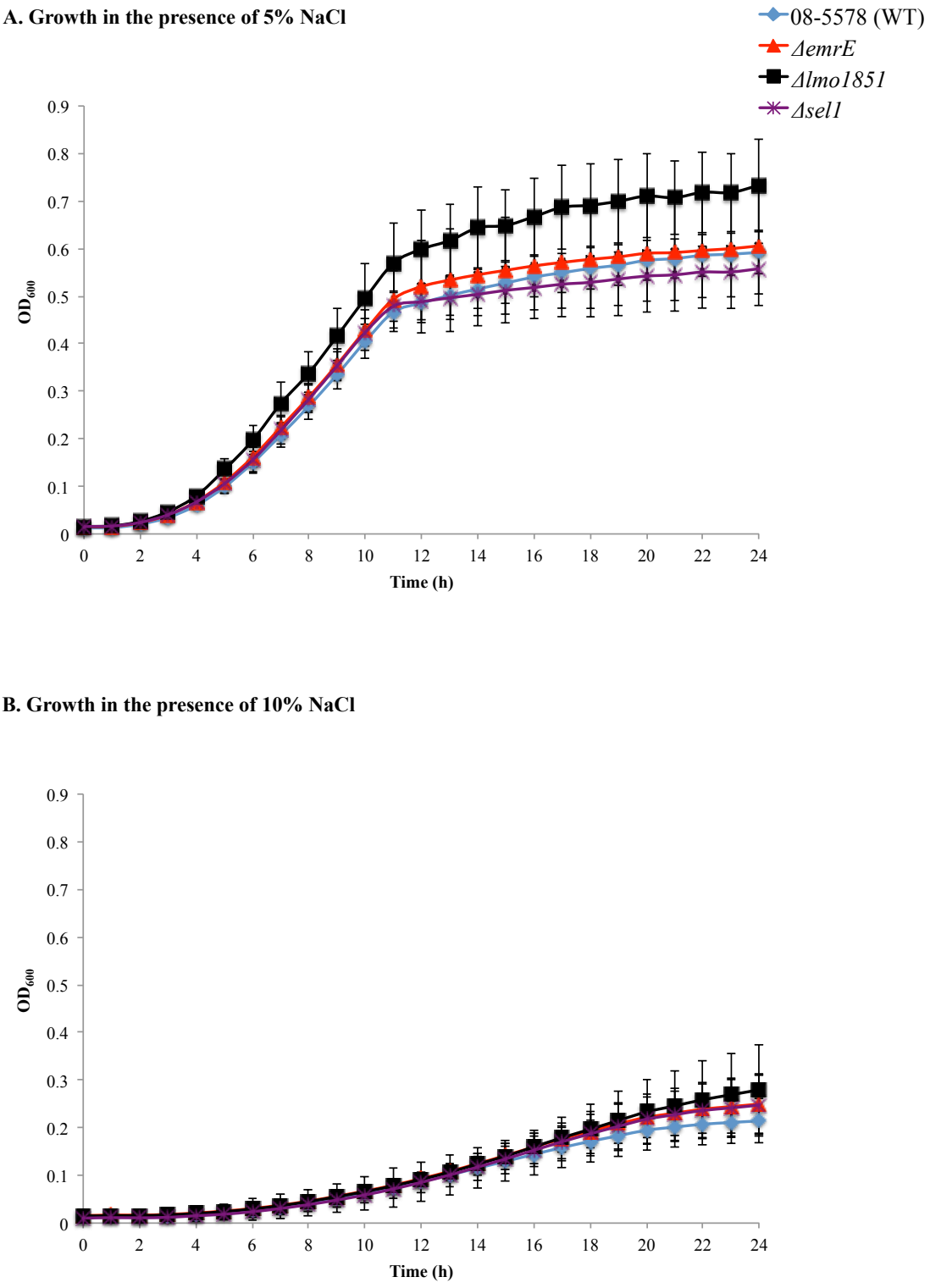


Figure 5-11. Growth of *L. monocytogenes* 08-5578 (WT) strain and its isogenic LGI1 deletion mutants in BHI containing 5 and 10% of NaCl at 30°C. The data shown represent the mean OD_{600} values of three independent cultures.

5.4 Discussion

Screening of *L. monocytogenes* isolates derived from the B.C. food chain for the presence of LGI1 revealed that the genomic island was not widespread in the population. In particular, LGI1 was found in only one isolate recovered from a RTE fish sample obtained at retail. This isolate belonged to serotype 1/2a, ST120 and CC8, which is in agreement with the findings by Knabel et al. (2012) and Ziegler (2012), who observed the presence of the island in a predominant clone belonging to 1/2a serotype and CC8, with a specific PFGE profile LMACI.0001/ LMAAI.0001. In fact this clone has been reported responsible for 40% of Canadian listeriosis outbreaks, and 20% of sporadic clinical cases in the last two decades (Ziegler, 2012). While the presence of only two ST120 isolates in the *L. monocytogenes* collection examined here suggests this sequence type is not prevalent in the B.C. food chain, the presence of LGI1 in one of them is a disconcerting find.

The presence of a number of putative antimicrobial resistance, stress response, and virulence genes on LGI1 could play a significant role in the ability of *L. monocytogenes* to survive in the food chain and cause human listeriosis (Gilmour et al., 2010; Ziegler, 2012). To investigate this hypothesis, LGI1 deletion mutants were created in *L. monocytogenes* 08-5578, an isolate that caused a nationwide listeriosis outbreak in Canada in 2008 in which the whole genome sequencing revealed the presence of the genomic island (Gilmour et al., 2010). Three genes located on LGI1 with putative stress response (i.e. *ΔemrE*, *Δlmo1851*) and virulence (i.e. *Δsel1*) functions were deleted. *Listeria monocytogenes* 08-5578 possessed high tolerance to acidic conditions, refrigeration, and high salt concentrations. However, the putative role of the response regulator of a two-component signal transduction system gene *lmo1851* in increased tolerance to the stressors tested could not be confirmed, leading to the

rejection of the hypothesis that *lmo1851* contributes to enhanced survival of *L. monocytogenes* in acidic, cold, and high salt conditions. Similarly, deletion of the *sel1* gene, encoding for a putative adhesin, did not impact *L. monocytogenes* adhesion and invasion of TC-7 and HeLa cell lines. These results suggest that *sel1* does not affect the virulence potential of *L. monocytogenes* under the conditions tested, and are cause to reject the hypothesis that this gene contributes to the increased adherence and invasion of *L. monocytogenes in vitro*.

In contrast, deletion of the *emrE* gene encoding for a small multidrug resistance (SMR) efflux pump resulted susceptibility to QAC-based sanitizers. MICs of two different QAC-based sanitizers against the Δ*emrE* mutant were up to three times lower than the WT parent strain. When the Δ*emrE* mutant was grown in the presence of sub-lethal concentrations of BAC and E-San sanitizers longer lag phase, slower growth rate, and overall lower maximum cell densities (OD_{600}) were observed compared to the WT strain. Furthermore, addition of reserpine, a known efflux pump inhibitor (Godreuil et al., 2003), affected the growth of both the WT and Δ*emrE* mutant strains in the presence of different concentrations of QAC sanitizers, thus, confirming that increased QAC-tolerance is indeed to due to efflux activity. Collectively, these data demonstrate that LGI1 encoded *emrE* confers increased tolerance to QAC in *L. monocytogenes*. This is in agreement with results reported by Ziegler (2012), who observed that MIC values for QACs were higher for isolates possessing the LGI1 genomic island than genetically similar isolates without LGI1.

The role of *emrE* in QAC tolerance was further confirmed by gene expression analyses. In particular, a significant up-regulation of the *emrE* expression occurred in the presence of BAC sanitizer, along with the increased expression of the *lmo1861* gene, a

putative MarR-family regulator. The expression of *mdrL*, a gene responsible for the production of MdrL chromosomal efflux pump belonging to MFS transporters in *L. monocytogenes,* was not up-regulated in the current study. This result is not surprising, as *mdrL* has previously been shown to be over-expressed only in *L. monocytogenes* that were experimentally adapted to BAC, but not in naturally resistant WT strains (Romanova et al., 2006), such as those tested in the current study. The findings described here also confirm that *lde*, an additional efflux pump belonging to the MFS efflux transporters, does not have a role in *L. monocytogenes* resistance to QAC-based sanitizers. This conclusion was expected, as the Lde efflux pump has been linked primarily to quinolone resistance, and is to some degree believed to contribute to *L. monocytogenes* tolerance to dyes, such as ethidium bromide and acridine orange (Godreuil et al., 2003; Lismond et al., 2008).

When it comes to sanitizer resistance in *L. monocytogenes*, at least two mechanisms have been described for resistance to QACs in WT strains. The presence of a plasmid-borne or chromosomally encoded *bcrABC* cassette and transposon (Tn*6188*)-based QacH efflux pump increases tolerance to QAC-based sanitizers in outbreak, clinical, and food chain *L. monocytogenes* isolates (Dutta et al., 2013; Elhanafi et al., 2010; Müller et al., 2013). However, QAC-sanitizer resistance due to the presence of an *emrE* homolog in *L. monocytogenes* has not been characterized before.

Efflux pumps belonging to the SMR family of proteins are typically 100 to 140 amino acids long, and often confer resistance to aminoglycosides, fluoroquinolones, dyes, and QACs (Bay et al., 2008; Piddock, 2006b; Poole, 2005). The first *emrE* efflux pump was described in *Escherichia coli* (i.e. *E. coli* multidrug resistance E) by Yerushalmi et al. (1995). They established that EmrE contributes to the resistance of *E. coli* to tetracycline,

erythromycin and sulfadiazine. Similar SMR pumps have also been described in *Mycobacterium smegmatis* (e.g., Mmr), *Pseudomonas aeruginosa* (e.g., $EmrE_{Pae}$), and *Staphylococcus* spp. (e.g., QacC/D, QacH); however, substrates for each efflux pump vary depending on the pump and the bacterial species (Piddock, 2006a; Piddock, 2006b; Poole, 2005). Li et al. (2003) demonstrated that an *emrE* homolog in *Pseudomonas aeruginosa* contributed to resistance toward ethidium bromide, acriflavine and aminoglycosides (i.e. amikacin, gentamicin, kanamycin, neomycin, and tobramycin), albeit resistance to aminoglycosides was observed only when tested in low-ionic-strength medium. In *Mycobacterium smegmatis*, an in-frame deletion in the *emrE* resulted in decreased MICs to ethidium bromide, acriflavine, ciprofloxacin and norfloxacin but had no effect on MICs of chloramphenicol, erythromycin, gentamicin, and tetracycline (Li et al., 2004). In *L. monocytogenes*, *emrE* did not appear to contribute to resistance to aminoglycosides, chloramphenicol, ciprofloxacin, tetracycline, and triclosan antimicrobials. It also did not seem to play a role in *L. monocytogenes* tolerance of acriflavine. This result is not surprising, since *L. monocytogenes emrE* did not possess any similarity to other well-characterized SMR efflux pumps from Gram negative and Gram positive bacteria. Comparison of the *L. monocytogenes emrE* region against the genomes present in the National Center for Biotechnology Information database produced a 74% and 72% similarity with a cationic/cationic drug transporter seen in *Desulfitobacterium dehalogenans*, and a small multidrug resistance protein in *Desulfitobacterium hafniense* strain, respectively. *Desulfitobacterium* spp. are anaerobic, motile, Gram positive, rod-shaped bacteria that often reside in environments contaminated by halogenated organic compounds (Villemur et al., 2006). Some homology (66 to 68%) between *L. monocytogenes emrE* and a predicted

multidrug resistance protein in *Clostridium ljungdahlii,* and QAC-resistance proteins observed in *Bacillus thuringiensis* serovar *kurstaki* and *B. cereus* strains was also observed. The presence of these bacteria in soil and effluents, which are also natural environments for *L. monocytogenes,* may result in sharing of the genetic material that confers survival under harsh conditions, though more research is needed to explore this hypothesis.

Listeria monocytogenes isolates possessing increased tolerance to QAC-based sanitizers, which are often used in food processing and handling facilities due to their non-corrosive properties, are of special concern to food processors and health authorities. The risk of selecting for sanitizer-resistant microorganisms when sanitizers are used at concentrations recommended by manufacturers is low. However, it should not be overlooked that inadequate cleaning and sanitation practices can result in exposure of *L. monocytogenes* to sub-lethal concentrations of sanitizers, which in turn will lead to selection pressure for progeny possessing increasing sanitizer tolerance (Rakic-Martinez et al., 2011). Such isolates have an increased chance of survival that may lead to persistence in food processing environments (Lundén et al., 2002; Lundén et al., 2003). This is especially likely to occur if equipment and niches that are difficult to clean and sanitize are encountered (Lundén et al., 2002). Persistent *L. monocytogenes* strains with enhanced sanitizer tolerance may also be more likely to contaminate foods and result in listeriosis cases, as evidenced by a number of outbreaks implicating *L. monocytogenes* isolates that do in fact show an increased tolerance to sanitizers (Elhanafi et al., 2010; Lundén et al., 2002). The *L. monocytogenes* 08-5578 isolate characterized in the current study was implicated in one of the largest listeriosis outbreaks in Canada, with the source of contamination suspected to be a large commercial slicer harboring the bacteria (Weatherill, 2009). Environmental sampling records from this facility showed

the intermittent presence of *L. monocytogenes* on two processing lines for almost a year prior to the outbreak (Weatherill, 2009). Similar scenarios have been reported in other listeriosis outbreaks where *L. monocytogenes* in the processing environment led to contamination of RTE products (CDC, 2002; Mead et al., 2006; Olsen et al., 2005). In fact, it is well established that food product contamination is associated with food processing environments harboring *L. monocytogenes* and subsequent post-processing transfer to finished products (Lappi et al., 2004; Lundén et al., 2002; Olsen et al., 2005; Tompkin, 2002). Following proper protocols for cleaning and sanitation of equipment, such as large slicers and conveyor belts that are difficult to disassemble or possess small openings and crevices, is particularly challenging for operators.

An additional concern with isolates possessing efflux pumps that enhance *L. monocytogenes* tolerance to QAC-based sanitizers is the potential for these isolates to develop enhanced tolerance to antibiotics due to similar mechanisms of action (Poole, 2005). Rakic-Martinez et al. (2011) demonstrated that *L. monocytogenes* strains selected on sub-lethal concentrations of ciprofloxacin (2 μg/ml) or BAC (10 μg/ml) exhibited higher MICs not only to these agents, but also to several other toxic compounds, including gentamicin, the dye ethidium bromide, and the chemotherapeutic drug tetraphenylphosphonium chloride. While the research performed in this study did not show that *emrE* increased tolerance to antibiotics relevant to listeriosis treatment (e.g., aminoglycosides), it is important to note that the adaptation to high concentrations of QAC sanitizers and the antibiotic co-selection phenomenon was not investigated. In future research, it would be of interest to investigate whether the co-selection phenomenon can indeed occur in isolates possessing *emrE*. Additionally, only a small number of antibiotics were tested in the experiments described

here. Future studies including additional antimicrobials comprising different classes of antibiotics and sanitizer compounds would be of great interest. Presently, the co-selection phenomenon occurring in *L. monocytogenes* is not well understood. However, a growing body of evidence suggests that pressures occurring at food processing facilities may contribute to the selection of isolates with enhanced tolerance to different antimicrobials, which is a concern to all food safety stakeholders.

5.5 Conclusions

Data from this research provide evidence that LGI1 encoded *emrE* promotes the survival of *L. monocytogenes* in the environments where this microorganism may come into contact with cationic compounds, such as QAC-based sanitizers. As QACs are commonly used in the food industry, the presence of efflux pumps, such as BcrB and BcrC, QacH, and EmrE that increase *L. monocytogenes* tolerance to QACs, is a concern to food safety. While there is presently no evidence that proper use of sanitizers in food processing will lead to development of resistant microorganisms, the exposure of microorganisms to residual QACs and concentrations below those recommended and regarded as adequate to eradicate *L. monocytogenes* is not an unlikely scenario. This is particularly true if bacteria become embedded in equipment niches that are hard to reach and thus are not properly cleaned and sanitized. Exposure to sub-lethal concentrations of sanitizers may not only lead to persistence, but also select for isolates with increased tolerance to QACs and other antimicrobials with similar modes of action. Presently, the co-selection phenomenon between sanitizers commonly used in food processing and antibiotics relevant in human clinical settings are not well understood. However, sufficient evidence does exist that demonstrates

shared mechanisms of resistance between biocides and antibiotics in *L. monocytogenes* (Christensen et al., 2011; Rakic-Martinez et al., 2011).

Considering the ubiquitous nature of *L. monocytogenes* and the severity of disease it can cause in high-risk individuals, there is a need for improved knowledge of the survival mechanisms that promote *L. monocytogenes* persistence in food processing. In particular, a better understanding of mechanisms that confer resistance to injury due to antimicrobials, and the co-selection phenomenon is needed before we can develop more effective targets for future mitigation strategies. Advances in this area would also enhance the successful development of sensitive and specific detection methods for high-risk *L. monocytogenes* strains.

Chapter 6: Conclusions

Federally registered food processing facilities in Canada are subject to environmental and end-product testing for *Listeria* spp. and/or *L. monocytogenes*. However, this level of inspection is not required or practiced in most non-federally registered food processing facilities, or at the retail level. The presence of *Listeria* spp. and *L. monocytogenes* was investigated in RTE food processing and retail facilities under provincial inspection authority in British Columbia (B.C.) to investigate the risks that are posed to consumers. *Listeria monocytogenes* was recovered from the processing environments in dairy, fish and meat facilities. However, the pathogen was recovered from food contact surfaces and RTE food products only in fish processing facilities. Similarly, *L. monocytogenes* was recovered from retail RTE fish samples, but not from retail RTE meat products. Thus, while control measures for *L. monocytogenes* in dairy and meat facilities appear to be effective in limiting contamination, the results indicate that there is a need for facilities and health inspectors to initiate improved monitoring and management of contamination by *L. monocytogenes* in RTE fish processing and retail sectors.

Genotypic and phenotypic characteristics of the recovered isolates were examined to further assess the risk posed to consumers from the consumption of foods contaminated with *L. monocytogenes*. More specifically, characteristics associated with virulence (e.g., *inlA* genotypes), enhanced stress resistance and survival along the food chain (e.g., cold adaptation, adaptive mutability and antimicrobial resistance, the presence of *Listeria* genomic island 1) were investigated.

Genotypic characterization of the *L. monocytogenes* strains revealed considerable diversity. Multilocus sequence typing found 14 sequence types (ST) in the examined

population. Thirteen of STs have been reported worldwide, and one (ST662) was unique to B.C. isolates. STs associated with clinical cases of listeriosis, namely those belonging to clonal complex 8 (CC8) previously linked to a number of sporadic and outbreak listeriosis cases in Canada, were found in two B.C. isolates. Interestingly, a large proportion (37/56) of *L. monocytogenes* isolates also possessed the full-length *inlA* gene. This gene encodes for a protein necessary for invasion of the human gastrointestinal tract, thereby indicating that a large proportion of isolates from the B.C. food chain have the potential to cause human illness.

Phenotypic properties of isolates, based on the serotype profiles, abilities to acquire point mutations that allow *L. monocytogenes* to survive in the presence of rifampicin antibiotic, and the capacity of strains to adapt to refrigeration, were significantly different. In particular, the majority of isolates recovered from the B.C. food chain belonged to listeriosis causing serotypes, 1/2a and 4b. Interestingly, isolates belonging to 1/2a serotype dominated in food processing environments, while serotype 4b was most commonly recovered from RTE foods. Surprisingly, serotype 4b more readily acquired point mutations that led to rifampicin resistance compared to other serotypes. Reasons for this observation are not clear. While positive selection resulting from the acquisition of advantageous mutations has been reported to contribute to the evolution of numerous genes in 1/2a strains, it is less often seen in 4b serotype strains. In general, 4b strains are typically more conserved in genetic content, exhibiting lower recombination rates, and are less likely to possess plasmids and extra-chromosomal elements. While further work is needed to explore this phenomenon, results obtained in this thesis suggest serotype 4b strains may acquire point mutations more readily than 1/2a strains. Considering that point mutations resulting in rifampicin resistance were

more readily observed in 4b serotypes, and that *inlA* mutations are typically very rare among isolates of this serotype, the results reported here suggest that a selection pressure for the maintenance of *inlA* genes encoding a full-length InlA exists for serotype 4b.

Of concern is also the observation made that isolates possessing rapid cold adaption (i.e. less than 70 h) were more likely to encode a full-length *inlA*, a causally linked virulence determinant. These results substantiate the assertion that isolates lacking *inlA* truncations are a significant concern. In light of these findings, which also showed that isolates with full-length *inlA* were more commonly recovered from RTE food, there is sufficient evidence to indicate that these isolates represent a significant food safety concern for food processors and public health authorities.

Although no resistance to antibiotics used in listeriosis treatments was observed, the results obtained in the current research show that a large proportion of *Listeria* isolates recovered from the B.C. food chain possess resistance, or increased tolerance, to antimicrobials typically effective against listeriae. In addition to resistance to antibiotics for which innate resistance of *Listeria* has been reported previously high levels of ciprofloxacin and clindamycin tolerance were noted. This observation was especially pronounced in 1/2a serotype isolates recovered from food. With the widespread usage of antimicrobials in clinical and animal production settings, the recovery of listeriae originating from the food chain that possess increased levels of antimicrobial tolerance may not be surprising. It has been hypothesized that enterococci and streptococci may transmit mobile plasmids that encode antimicrobial resistance determinants to *Listeria* spp. in farming environments. Furthermore, when a co-selection phenomenon was explored in isolates that were experimentally adapted to high concentrations of ciprofloxacin, a reduced sensitivity to

disparate antimicrobials occurred. Of particular interest was the finding that adaptation to ciprofloxacin also resulted in the increased resistance to benzalkonium chloride, a quaternary ammonium compound (QAC), and in some strains also led to increased resistance to gentamicin, an antibiotic used in listeriosis treatment. Considering that QACs are commonly used as sanitizers in food processing environments, and that repeated exposure of *L. monocytogenes* to sanitizers is a plausible and realistic concern in the food processing industry, there is a need for improved understanding of potential pressures contributing to co-selection of antibiotic and sanitizer resistance mechanisms.

The increased tolerance to QAC-based sanitizers in wild type (WT) *L. monocytogenes* can occur due to plasmid-borne and chromosomally encoded efflux pump genes, such as *bcrABC*, *qacH*, and to a lesser degree *mdrL*. The findings of this thesis provide evidence that a novel efflux pump, encoded by the *emrE* gene, and located on *Listeria* genomic island 1 (LGI1) also contributes to increased *L. monocytogenes* tolerance to QACs. This particular island was discovered during an investigation into the 2008 deli meat outbreak when the genomes of the two *L. monocytogenes* strains linked to the outbreak were sequenced. Along with *emrE*, a number of other genes with putative stress response and virulence functions were found on the island, suggesting it may be a factor for the enhanced ability of *L. monocytogenes* to survive in the food chain. In fact, when one of the outbreak strains, *L. monocytogenes* 08-5578, was exposed to a variety of stresses, high tolerance to acidic, cold, and high salt conditions, and two QAC sanitizers was observed. Deletion of genes *lmo1851*, and *sel1*, with putative regulatory, and adhesion functions, respectively, did not affect the tolerance of the bacterium to acid, cold and high salt conditions, or its adhesion and invasion of human cells (e.g., TC-7 and HeLa). However, an in-frame deletion of the *emrE*

gene resulted in the impaired growth of the strain in the presence of sub-lethal concentrations of two QAC sanitizers, and two to three times lower minimum inhibitory concentration (MIC) values. There was no change in MIC values when Δ*emrE* mutant was exposed to aminoglycosides and other antibiotics, acriflavine, and triclosan. These data suggest that the primary role of EmrE in *L. monocytogenes* is to increase its tolerance of QAC sanitizers. Since these sanitizers are commonly used in the food industry, *L. monocytogenes* strains possessing *emrE* will have an increased ability to survive in food processing environments. Future research should focus on elucidating the role of other genes located on the island. In particular, the role of a putative MarR regulator (*lmo1861*) flanking the *emrE* gene, which was over-expressed during exposure to BAC, a putative *rpoE* unit that may play a role in stress regulation, as well as the role of a number of *vir* genes that could enhance virulence should be investigated.

In summary, the research presented in this thesis provides strong evidence that differences in stress survival and virulence potential exist among food chain-derived *L. monocytogenes*. The research findings also highlight the need for a better understanding of the mechanisms that confer resistance and stress survival in the food chain, in order to control this pathogen in the food industry. Currently, in Canada 100 CFU of *L. monocytogenes* are permitted per ml or gram of food that does not support the growth of *Listeria*, or in foods that have a short shelf life (Health Canada, 2011). However, testing of foods and food processing environments is not practiced or required in all the facilities that produce RTE foods for Canadians. Even in facilities that are inspected by the Canadian Food Inspection Agency (CFIA), and undergo more rigorous testing for *Listeria* spp., *L. monocytogenes* continues to be frequently found. In 2013, 21 recall notifications due to

L. monocytogenes contamination of RTE foods were issued by the CFIA (CFIA, 2014). Of these, 10 were due to separate incidents, where foods from different producers across the country were recalled. Similarly, in 2014 (January to July), 16 separate recalls occurred as a result of various foods being contaminated by *L. monocytogenes*. These recalls are costly and place economic burden on both the government and food producers. In addition, the current food safety system does not differentiate between low and high-risk *L. monocytogenes* isolates. Some of these recalls may be removing foods from the shelves that contain low virulence *L. monocytogenes* strains that do not possess genetic determinants required for human illness. In other instances, we may be allowing a small number of *L. monocytogenes* strains with high virulence potential in our foods.

Research from this thesis provides strong evidence that some *L. monocytogenes* strains possess genetic and phenotypic properties that pose higher risk of foodborne illness to consumers than others. Furthermore, findings from the research provide important new information on how *L. monocytogenes* survive in the food chain. These data highlight the need for better monitoring and detection systems that can capture differences in *L. monocytogenes* isolates. We should aim to replace the current presence or absence testing with a more risk-based system. However, in order to do this, more sophisticated, yet affordable methods that can differentiate between the low and high-risk *L. monocytogenes* strains are needed. In addition, greater focus should be placed on understanding the environmental pressures occurring at the food processing level that result in selection of resistant strains and lead to persistence in the food chain. Such knowledge is needed to develop more effective targets for future mitigation strategies, and prevent the establishment of potentially high-risk strains in food processing environments.

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Appendices

Appendix A – Chapter 2 supplementary figure

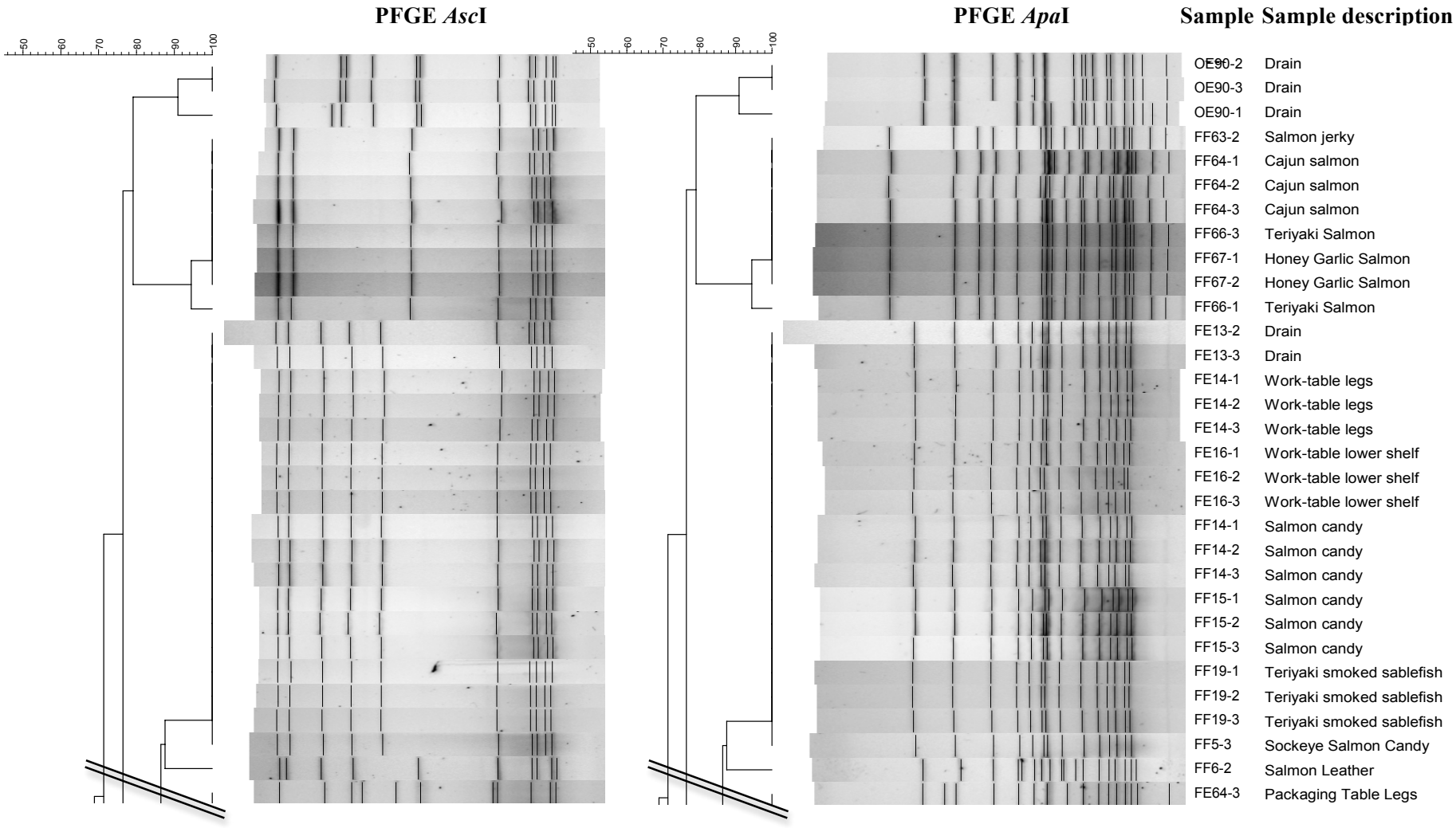

Figure A-1. PFGE patterns for *L. monocytogenes* isolates from food processing facilities, recovered from August to October 2009; with the exception of 160687 isolate, which was recovered from facility d5, during a follow-up inspection in March 2010. Sample designations: DE, dairy environment; FF, fish food; FE, fish environment; MF and OF, meat food; ME and OE, meat environment. Dendrograms represents continuous trees but are split onto three pages for visual clarity; the splitting point is indicated with \\ lines.

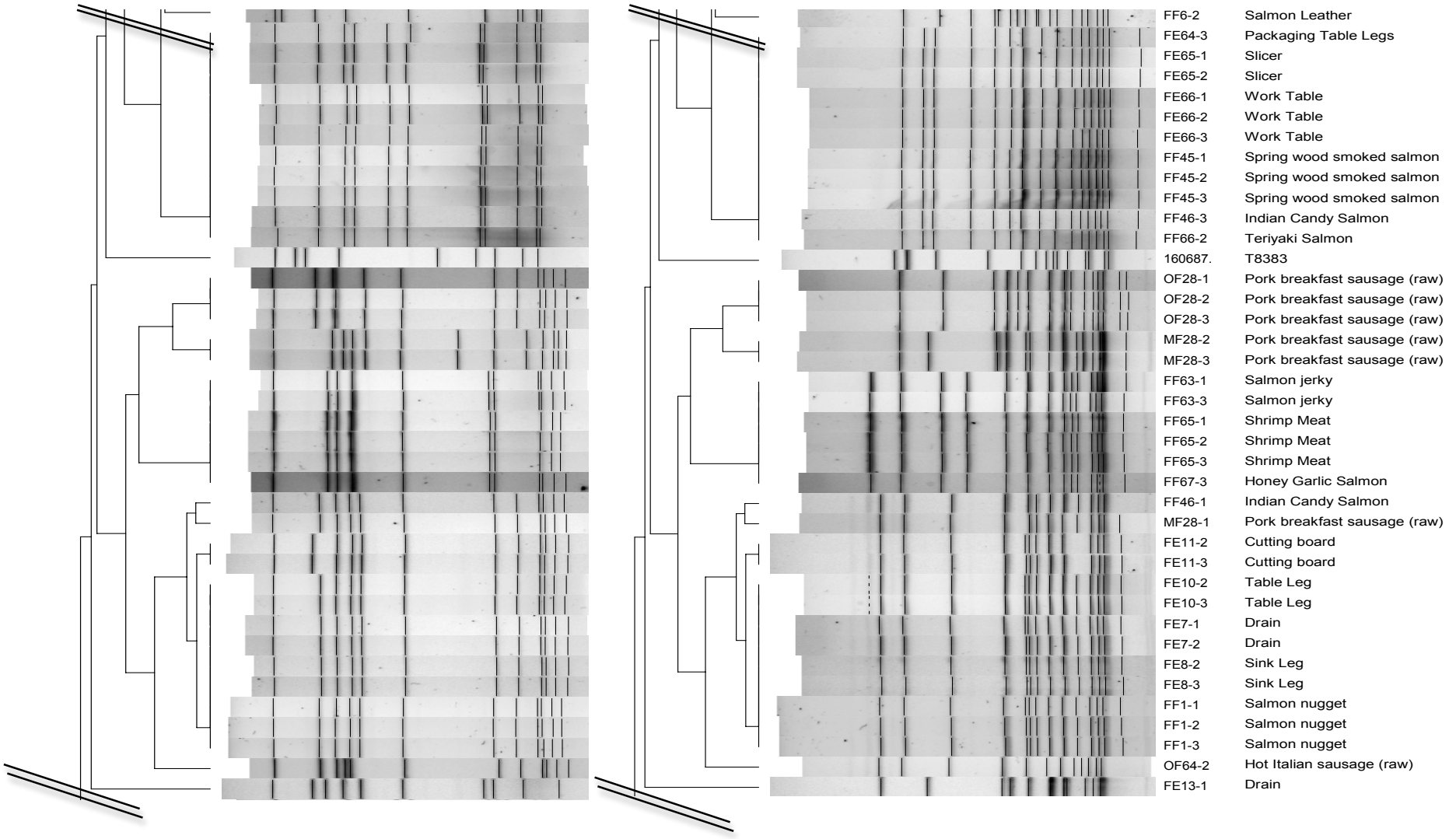

Figure A-1. Continued.

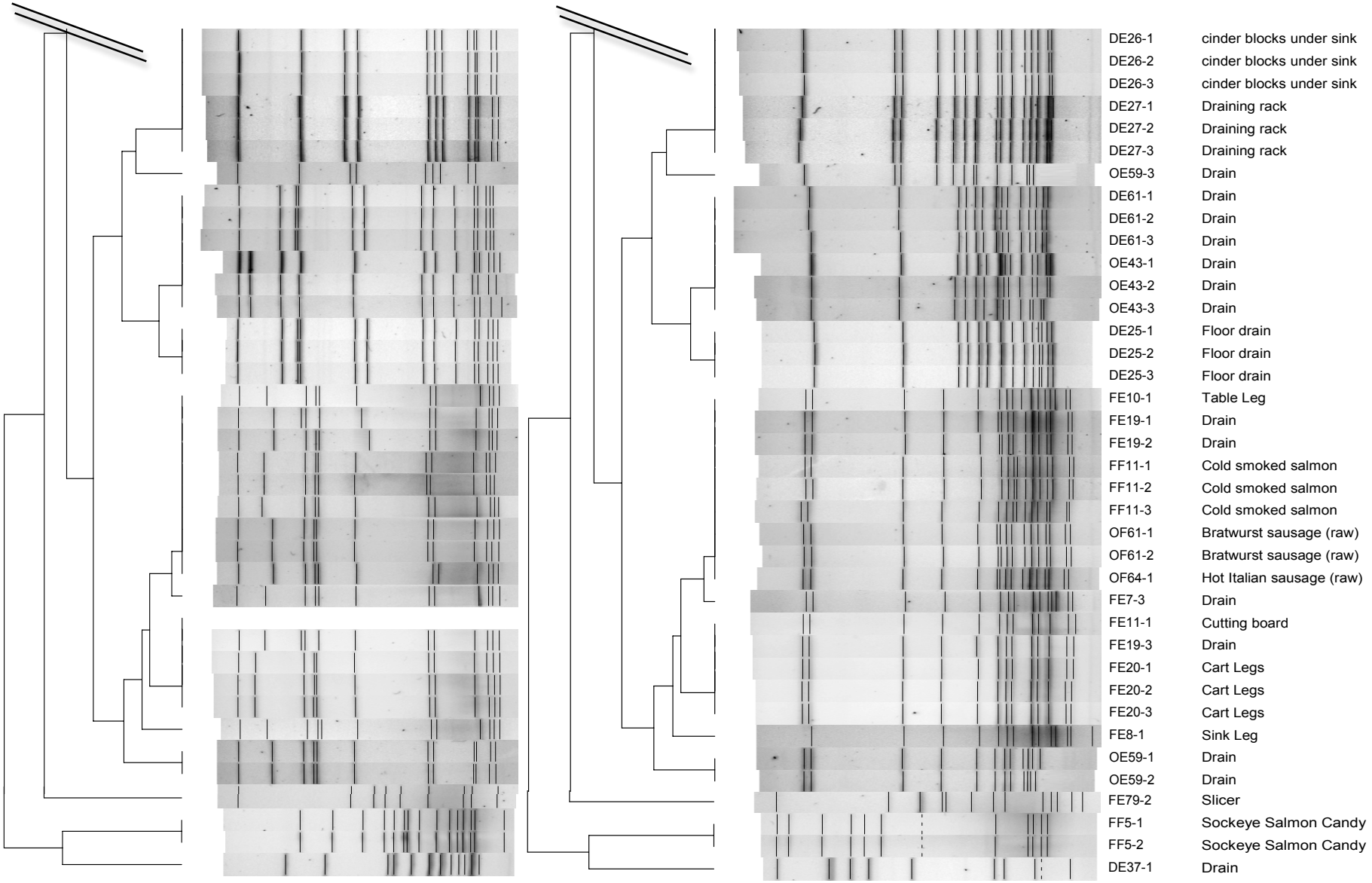

Figure A-1. Continued.

Appendix B – Chapter 3 supplementary table

Table B-1. Summary of mutability, cold growth adaptation, and invasion efficiency of different *inlA* genotypes amongst 56 *L. monocytogenes* isolates recovered from foods and food processing environments of dairy, meat and fish processing facilities.

					Cold growth adaptation[c]			*inlA* Genotype[d]				
Isolate ID	**Fac. type**	**Source[a]**	**Sero-type**	**No. of RIF^R colonies[b] (SEM)**	**Cold growth phenotype**	**Lag phase duration (h)**	**Growth rate (Δlog_{10}CFU/h)**	**Protein length (a.a.)**	**PMSC/ *inlA* mutation type**	**MLST**	**PFGE**	**Mean % invasion efficiency (SEM)**
Food processing facilities												
DE 25-1	Dairy	FPE	1/2a	6.5 ± 1.0	Slow	248.9 ± 44.2	0.0050 ± 0.0006	800	N/A	7	G2	
DE 26-1	Dairy	FPE	1/2a	9.3 ± 2.1	N/A	N/A	N/A	800	N/A	11	F1	
DE 27-1	Dairy	FPE	1/2a	7.5 ±3.0	Intermediate	141.2 ± 6.0	0.011 ± 0.002	800	N/A	11	F1	
DE 37-3	Dairy	FPE	1/2a	1.8 ± 1.0	Intermediate	187.2 ± 68.9	0.0020 ± 0.001	800	N/A	155	N1	
DE 61-1	Dairy	FPE	1/2a	7.3 ± 1.1	Slow	201.2 ± 65.7	0.0035 ± 0.001	800	N/A	7	G1	
MF 28-1	Meat	RUF	1/2c	6.2 ± 3.2	N/A	N/A	N/A	685	11	9	H2	0.027 ± 0.003
MF 28-2	Meat	RUF	1/2a	6.0 ± 4.8	Intermediate	141.3 ± 6.0	0.0018 ± 0.0005	800	N/A	662	J1	
FF 1-1	Fish	RTE	1/2c	4.2 ± 0.7	Intermediate	167.9 ± 54.1	0.0025 ± 0.0006	8	4	9	H1	
FE 7-1	Fish	FPE	1/2c	10.3 ± 5.9	N/A	N/A	N/A	8	4	9	H1	0.034 ± 0.007
FE7-3	Fish	FPE	3a	5.3 ± 1.8	Intermediate	168.6 ± 89.2	0.0025 ± 0.001	699	3	321	K5	
FE8-1	Fish	FPE	3a	4.3 ± 1.4	Intermediate	153.7 ± 22.4	0.0013 ± 0.0009	699	3	321	K3	
FE8-2	Fish	FPE	1/2c	3.7 ± 1.7	N/A	N/A	N/A	8	4	9	H1	
FE10-1	Fish	FPE	3a	10.7 ± 0.9	N/A	N/A	N/A	699	3	321	K4	
FE10-2	Fish	FPE	1/2c	14.0 ± 4.4	Intermediate	149.8 ± 15.5	0.0015 ± 0.0006	8	4	9	H1	
FE11-1	Fish	FPE	3a	6.8 ± 1.6	N/A	N/A	N/A	699	3	321	K2**	
FE11-2	Fish	FPE	1/2c	2.7 ± 1.0	N/A	N/A	N/A	8	4	9	H4	
FE11-3	Fish	FPE	1/2c	2.8 ± 0.9	Intermediate	175.9±17.5	0.0020 ± 0.003	8	4	9	H4	
FE13-1	Fish	FPE	1/2a	4.8 ± 0.7	N/A	N/A	N/A	797	Δ738-740	155	L1	7.19 ± 0.6
FE13-2	Fish	FPE	4b	31.7 ± 4.6	N/A	N/A	N/A	797	Δ738-740	194	A1	
FE14-1	Fish	FPE	4b	43.7 ± 13.4	N/A	N/A	N/A	800	N/A	194	A1	
FE16-1	Fish	FPE	4b	26.5 ± 2.9	N/A	N/A	N/A	797	Δ738-740	194	A1	

Isolate ID	Fac. type	Source[a]	Sero-type	No. of RIF^R colonies[b] (SEM)	Cold growth adaptation[c]			*inlA* Genotype[d]		MLST	PFGE	Mean % invasion efficiency (SEM)
					Cold growth phenotype	Lag phase duration (h)	Growth rate ($\Delta log_{10}CFU/h$)	Protein length (a.a.)	PMSC/ *inlA* mutation type			
Food processing facilities												
FF5-1	Fish	RTE	4b	51.2 ± 19.3	Slow	293.2 ± 22.9	0.027 ± 0.006	797	Δ738-740	194	M1	
FE19-1	Fish	FPE	1/2a	23.8 ± 9.2	Slow	245.1 ± 26.7	0.0027 ± 0.001	699	3	321	K9	
FE20-1	Fish	FPE	1/2a	45.2 ± 17.9	N/A	N/A	N/A	699	3	321	K1	
FF6-2	Fish	RTE	4b	19.7 ± 2.0	Slow	222.2 ± 76.5	0.030 ± 0.0007	800	N/A	2	A2	
FF11-1	Fish	RTE	1/2a	4.0 ± 0.3	Intermediate	156.2 ± 17.3	0.0013 ± 0.0009	699	3	321	K6	
FF14-1	Fish	RTE	4b	30.0 ± 6.2	N/A	N/A	N/A	797	Δ738-740	194	A1	
FF15-1	Fish	RTE	4b	48.0 ± 6.5	Fast	7.1 ± 3.6	0.0018 ± 0.0009	797	Δ738-740	194	A1	
FF19-1	Fish	RTE	4b	29.3 ± 7.7	Fast	39.2 ± 40.0	0.0018 ± 0.002	797	Δ738-740	194	A1	4.76 ± 0.5
FE64-3	Fish	FPE	4b	16.7 ± 3.4	N/A	N/A	N/A	800	N/A	1	B1	
FE65-1	Fish	FPE	4b	47.8 ± 37.1	N/A	N/A	N/A	800	N/A	1	B1	
FE66-1	Fish	FPE	4b	92.3 ± 69.7	N/A	N/A	N/A	800	N/A	1	B1	
FE66-3	Fish	FPE	4b	14.3 ± 2.8	Fast	25.7 ± 12.5	0.0025 ± 0.001	800	N/A	1	B1	
FE79-1	Fish	FPE	4b	33.8 ± 7.2	N/A	N/A	N/A	797	Δ738-740	6	E1	
FF45-1	Fish	RTE	4b	15.3 ± 1.4	Fast	42.4 ± 9.5	0.0018 ± 0.002	800	N/A	1	B1	
FF46-1	Fish	RTE	1/2a	2.2 ± 1.5	Fast	50.7 ± 8.6	0.0013 ± 0.001	800	N/A	9	H3	
FF46-3	Fish	RTE	4b	35.5 ± 26.1	Intermediate	136.8 ± 4.7	0.057 ± 0.008	800	N/A	1	B1	10.91 ± 1.1
FF63-1	Fish	RTE	1/2a	39.2 ± 11.2	Fast	18.8 ± 2.5	0.0015 ± 0.001	800	N/A	91	I2	
FF63-2	Fish	RTE	4b	8.7 ± 2.8	Fast	46.3 ± 19.1	0.0008 ± 0.0009	797	Δ738-740	6	C1	
FF64-1	Fish	RTE	4b	17.8 ± 4.4	Fast	42.7 ± 79.8	0.0015 ± 0.0006	797	Δ738-740	6	C1	
FF65-1	Fish	RTE	1/2a	11.2 ± 5.3	Fast	56.7 ± 19.9	0.0018 ± 0.0005	800	N/A	91	I1	
FF66-1	Fish	RTE	4b	20.7 ± 4.5	Fast	64.0 ± 27.2	0.0008 ± 0.0005	797	Δ738-740	6	C2	
FF66-2	Fish	RTE	4b	13.2 ± 1.6	N/A	N/A	N/A	800	N/A	1	B1	
FF67-1	Fish	RTE	4b	10.3 ± 2.7	N/A	N/A	N/A	797	Δ738-740	6	C1	
FF67-2	Fish	RTE	4b	10.0 ± 1.6	Fast	63.6 ± 6.5	0.0020 ± 0.0008	797	Δ738-740	6	C1	
FF67-3	Fish	RTE	1/2a	18.3 ± 5.7	Intermediate	164.6 ± 5.3	0.0015 ± 0.0006	800	N/A	91	I2	
OF28-1	Meat	RUF	1/2a	52.8 ± 12.2	Fast	68.6 ± 7.1	0.0035 ± 0.002	800	N/A	120	H6	

Isolate ID	Fac. type	Source[a]	Sero-type	No. of RIF^R colonies[b] (SEM)	Cold growth adaptation[c]			*inlA* Genotype[d]		MLST	PFGE	Mean % invasion efficiency (SEM)
					Cold growth phenotype	Lag phase duration (h)	Growth rate (Δlog_{10}CFU/h)	Protein length (a.a.)	PMSC/ *inlA* mutation type			
Food processing facilities												
OE43-1	Meat	FPE	1/2a	50.2 ± 25.2	Intermediate	133.4 ± 18.8	0.0035 ± 0.0017	800	N/A	7	G4	
OE59-1	Meat	FPE	1/2a	7.2 ± 1.6	Fast	67.6 ± 8.8	0.0023 ± 0.0009	699	3	321	K10	
OE59-3	Meat	FPE	1/2a	1.3 ± 0.4	Fast	48.5 ± 33.8	0.0033 ± 0.001	800	N/A	11	F2	
OF61-1	Meat	RUF	1/2a	18.8 ± 7.8	N/A	N/A	N/A	699	3	321	K7	0.011 ± 0.001
OF64-1	Meat	RUF	1/2a	7.7 ± 2.3	N/A	N/A	N/A	699	3	321	K7	
OF64-2	Meat	RUF	1/2c	4.0 ± 1.5	Fast	55.9 ± 21.3	0.0028 ± 0.003	684	11	9	H5	
OE90-1	Meat	FPE	1/2b	4.5 ± 1.0	Intermediate	93.5 ± 14.5	0.0025 ± 0.001	605	1	5	D2	0.006 ± 0.001
Retail facilities												
LR39-1	Retail (fish)	RTE	1/2a	13.7 ± 2.2	N/A	N/A	N/A	800	N/A	120	LMAC I.0001/ LMAA I.0001	
LR59-1	Retail (fish)	RTE	1/2b	6.8 ± 0.7	N/A	N/A	N/A	800	N/A	296	LMAC I.0470/ LMAA I.0584	

[a]FPE, food processing environment; RUF, raw unprocessed food; RTE, ready-to-eat food.

[b]Number of colonies resistant to rifampicin (100 μg/ml) after 24 hr growth at 37°C. SEM, standard error of the mean.

[c]Cold adaptation of isolates when grown at 4°C following a downshift from 37°C in BHI. N/A, not assessed.

[d]*inlA* genotypes include mutations resulting in premature stop codons (PMSCs) or codon deletions (Δ).

[e]% of bacteria recovered from Caco-2 cells relative to initial inoculum; measured in triplicate and repeated two times. Invasion efficiency was compared to wild type clinical isolates 08-5578 (5.04±0.32%) and EGD-SmR (1.01±0.09%), and a Tn*1545*-induced non-invasive *inlA* mutant of EGD-SmR, BUG5 (0.009±0.0008%).