ISOLATION OF *LISTERIA* SPECIES AND CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* FROM A READY-TO-EAT SEAFOOD PROCESSING FACILITY IN BRITISH COLUMBIA: EXAMINATION OF SOURCE, PERSISTENCE, AND RISK

by

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Abstract

Foodborne *Listeria monocytogenes (Lm)* causes listeriosis, a rare but severe disease affecting at-risk populations. Contamination of ready-to-eat (RTE) food has been linked with persistent *Lm* in the food processing environment (FPE), though mechanisms of persistence are not fully understood. Recent surveys in British Columbia (BC) detected *Lm* in seafood processing facilities and in RTE seafood products, yet little is known regarding *Lm* persistence and risk posed by strains from these sources. The objectives of this study were to determine the prevalence of *Listeria* spp., including *Lm*, in a RTE seafood processing facility and assess persistence capabilities and potential risk of *Lm* recovered. Environmental and RTE food samples were collected over 18 months from a BC seafood processor (n=2,959) and assessed for the presence of *Listeria* spp. Isolated *Lm* were subjected to phenotypic and genetic characterization and a subset of isolates (n=28) were characterized for attributes that may facilitate FPE persistence: surface adherence, resistance to quaternary ammonium-based sanitizers, and adaptation to salt and refrigerated temperatures. Ability of cold-smoked salmon from this processor to support *Lm* growth, evaluation of virulence gene (*inlA*) sequence, and antibiotic resistance in the subset were used to assess consumer risk. Non-*Lm Listeria* spp. and *Lm* were found in 2.6% and 1.5% of samples, respectively. Molecular characterization revealed raw materials as the primary contamination source and two recurrent subtypes. *Lm* typically possessed one attribute favorable to persistence in the FPE, though there was no association between these attributes and strain recurrence. Cold-smoked salmon supported the growth of *Lm* and most strains recovered in the FPE (n=14) belonged to serotypes linked to listeriosis (92%) and possessed full-length *inlA* (93%). These strains, however, did not show resistance to antibiotics commonly used to treat listeriosis. The results of this study highlight the importance of processor-level control strategies to minimize *Lm* FPE persistence, product contamination, and risk to consumers. While these results improve understanding of *Lm* in a BC seafood processing environment, more work is needed to determine whether these strains are truly persisting in this FPE and how these findings compare to similar facilities.

Preface

This thesis is original, unpublished, independent work by the author, Keely Johnston.

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List of abbreviations

ANOVA	Analysis of variance
a_w	Water activity
BAC	Benzalkonium chloride
BC	British Columbia
BHI	Brain heart infusion
BRIL	Brilliance agar
Caps	Cold acclimation proteins
CDC	Center for Disease Control
CFU	Colony forming unit
Csps	Cold shock proteins
CV	Crystal violet
DNA	Deoxyribonucleic acid
EC	Epidemic clone
EGR	Exponential growth rate
ELFA	Enzyme-linked fluorescence assay
EPS	Extracellular polymeric substance
FAO	Food and Agriculture Organization of the United Nations
FCS	Food contact surface
FDA	Food and Drug Administration
FPE	Food processing environment
FSIS	Food Safety and Inspection Service
HSD	Honest significant difference
LAB	Lactic acid bacteria
LEB	*Listeria* enrichment broth
LIPI-1	*Listeria* pathogenicity island
LLO	Listeriolysin O
LPD	Lag phase duration
LRRs	Leucine-rich repeats
MFB	Modified Fraser broth
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing

MvLST	Multi-virulence-locus sequence typing
NaCl	Sodium chloride
NFCS	Non-food contact surface
OD_{600}	Optical density at 600 nm
OXA	Oxford agar
PAL	Palcam agar
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMSC	Premature stop codon
PVC	Polyvinyl chloride
QAC	Quaternary ammonium compound
RTE	Ready-to-eat
TSA	Tryptic soy agar
TSB	Tryptic soy broth
US	United States
w/v	Weight/volume
WHO	World Health Organization
WPS	Water phase salt

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For my parents, Bill and Jill, and sister, Alexis

And will you succeed?
Yes! You will, indeed!
(98 and ¾ percent guaranteed).
- Dr. Seuss

Chapter 1: Literature review and research overview

1.1 Foodborne disease and food safety in Canada

The Public Health Agency of Canada estimates that one in eight Canadians, or four million, acquire a foodborne disease annually (Thomas et al., 2013). This value can be attributed to 30 known pathogens tracked by the public health system as well as unspecified agents that cause gastrointestinal symptoms (Thomas et al., 2013). Of those tracked by disease surveillance, Norovirus caused the highest burden of illness followed by *Clostridium perfringens*, *Campylobacter* spp., and *Salmonella*, circa 2006 (Thomas et al., 2013). These findings show that, although Canada was a top-performing country in the 2014 World Ranking: Food Safety Performance Report (Charlebois and MacKay, 2014), there continues to be room for improvement in the area of foodborne illness prevention and control in Canada.

Despite having a low incidence when compared to other foodborne pathogens, the bacterium *Listeria monocytogenes* is a major food safety concern (Farber and Peterkin, 1991; Thomas et al., 2013). As the etiological agent of invasive listeriosis, *L. monocytogenes* is more likely to cause death than other bacteria that cause foodborne illness (Thomas et al., 2013). Although *L. monocytogenes* was identified as a pathogen in 1924, it was not thought of as a serious food safety risk until 1981 when consumption of contaminated coleslaw caused illness in 41 individuals and 17 deaths (Kornacki and Gurtler, 2007). More recently, *L. monocytogenes* became a high-profile foodborne pathogen in Canada after a nationwide outbreak in 2008 associated with delicatessen meats (Health Canada, 2011a). In the United States (US), consumption of foods contaminated with *L. monocytogenes* results in an estimated 94% hospitalization rate, the highest of all pathogens monitored, and a 16% death rate (Scallan et al.,

2011). Although similar data are not available for Canada, recent foodborne illness outbreaks associated with *L. monocytogenes* have resulted in 20-40% mortality despite early antibiotic treatment (Health Canada, 2011a). These data combined with the ability of *L. monocytogenes* to survive food processing hurdles aimed at inhibiting pathogen growth in food (e.g., cold temperatures, acidity, high salt concentrations), makes this microorganism a serious threat to food safety and of concern in the food industry (Vázquez-Boland et al., 2001).

1.1.1 *L. monocytogenes*, listeriosis, and food safety in British Columbia

In British Columbia (BC), the overall incidence of foodborne illness between 2008 and 2013 was similar to reported national estimates and occurrence of listeriosis has remained steadily below the Canadian average (BC Centre for Disease Control, 2014). Despite this lower incidence of listeriosis, *L. monocytogenes* still poses a food safety risk in BC. Numerous recalls of a variety of ready-to-eat (RTE) foods recently demonstrates that *L. monocytogenes* is present in the BC food supply (BC Centre for Disease Control, 2015; Government of Canada, 2015). In addition, a recent study evaluating the occurrence of *L. monocytogenes* in BC dairy, fish and meat food processing environments (FPEs) revealed issues with control of this pathogen in these establishments (Kovacevic et al., 2012a).

1.2 Overview of *L. monocytogenes*

1.2.1 Characteristics and classification of *L. monocytogenes*

L. monocytogenes belongs to the genus *Listeria*, a group of closely related Gram-positive, rod-shaped, facultative anaerobic bacteria (Liu, 2006). Until recently *Listeria* taxonomically consisted of six species. Over the last five years, though, 11 novel species have been discovered

(Table 1.1). *L. monocytogenes* is the only species of *Listeria* that is a major concern to human health (Kathariou, 2002), although *L. ivanovii*, *L. innocua, L. gray,* and *L. seeligeri* have been implicated in mammalian disease (Chen and Nightingale, 2013; Guillet et al., 2010; Kathariou, 2002; Perrin et al., 2003; Rapose et al., 2008)

L. monocytogenes is non-spore forming and has low GC content (Liu, 2006). Additionally, *L. monocytogenes* possesses peritrichous flagella which give the bacterium a characteristic tumbling motility, although this only occurs between 20°C and 30°C (Farber and Peterkin, 1991; Peel et al., 1988). Despite not being able to form spores, *L. monocytogenes* has a remarkable ability to survive and grow in environments considered unfavorable for many other bacteria. Studies have shown that *L. monocytogenes* can grow in pH as low as 4.4, salt concentrations up to 10% w/v, water activity down to 0.92, and through a range of temperatures (-0.4°C to 45°C) (Gandhi and Chikindas, 2007; Valderrama and Cutter, 2013).

L. monocytogenes strains can be separated into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab, and 7) based on their somatic and flagellar antigens (Graves et al., 2007). Serotypes 1/2a, 1/2b, and 4b strains are responsible for the majority of listeriosis cases (McLauchlin, 1990; Swaminathan and Gerner-Smidt, 2007). In addition, these bacteria form a structured population composed of four divergent lineages (I, II, III, and IV), recently substantiated using multilocus sequence typing of 2,000 isolates that spanned different sources, continents, and decades (Haase et al., 2014; Orsi et al., 2011; Wiedmann et al., 1997). Most *L. monocytogenes* strains can be grouped into either Lineage I or II, while Lineage III and IV strains are rarely isolated and are genetically very diverse (Orsi et al., 2011). Lineage I is

comprised of serotypes 1/2b, 3b, 4ab, 4d, and 4e and Lineage II of 1/2a, 1/2c, 3a, and 3c serotype strains. Serotypes 4a, 4b, and 4c span across lineages (Orsi et al., 2011; Valderrama and Cutter, 2013).

Table 1.1 Summary of *Listeria* spp.

Listeria spp.	***Reference***
L. monocytogenes	Reviewed by den Bakker et al., 2014
L. grayi	
L. innocua	
L. welshimeri	
L. seeligeri	
L. ivanovii	
L. marthii	
L. rocourtiae	
L. fleischmannii	
L. weihenstephanensis	
L. aquatic sp. nov.	den Bakker et al., 2014
L. floridensis sp. nov.	
L. cornellensis sp. nov.	
L. grandensis sp. nov.	
L. riparia sp. nov.	
L. booriae sp. nov.	Weller et al., 2014
L. newyorkensis sp. nov.	

Molecular typing methods have been developed to differentiate *L. monocytogenes* strains beyond lineage and serotype. Pulsed-field gel electrophoresis (PFGE) is known as the "gold standard" and has been shown to be a reproducible and highly discriminatory method of subtyping *L. monocytogenes* (Graves et al., 2007; Jadhav et al., 2012). PFGE uses infrequently cutting restriction enzymes, such as *Asc*I and *Apa*I, to digest genomic DNA into large fragments. These fragments are then separated using agarose gel electrophoresis with periodic changes in the orientation of the electric field across the gel (Graves et al., 2007). This method allows for differentiation of isolates based on the banding patterns produced (Gasanov et al., 2005; Graves

et al., 2007). In contrast, ribotyping employs frequent cutting restriction enzymes, such as *Eco*RI, that digest genomic DNA into many small fragments followed by separation via gel electrophoresis. The fragments are then probed for ribosomal DNA (rDNA) sequences and banding patterns are visualized (Graves et al., 2007). To elucidate the population structure among strains, multilocus sequence typing (MLST) is often used. MLST involves partial sequencing of multiple housekeeping genes and then comparison of these sequences to determine the genetic relatedness of isolates (Jadhav et al., 2012). A similar method to MLST, multi-virulence-locus sequence typing (MvLST), has also gained acceptance for population structure analysis. MvLST sequences virulence genes rather than housekeeping genes to determine genetic relatedness of strains (Cantinelli et al., 2013; Chen et al., 2007). While the methods described here represent those most commonly employed currently, there are many other subtyping techniques, including whole-genome sequencing, that are being utilized to study *L. monocytogenes* isolates, as reviewed by Jadhav et al. (2012).

1.2.2 Distribution of *L. monocytogenes*

Numerous studies refer to *L. monocytogenes* as ubiquitous, or widely distributed, in the environment (Chang et al., 2012; Haase et al., 2014; Ribeiro and Destro, 2014). As a saprophytic organism, the natural habitat of *L. monocytogenes* is considered to be decomposing plant materials. Consequently, these bacteria can be isolated from various sources in the natural environment including vegetation, soil, and surface water (Vázquez-Boland et al., 2001). *L. monocytogenes* can also be found in human-made environments such as farms, FPEs, households, and in a wide variety of foods (Farber and Peterkin, 1991; Valderrama and Cutter, 2013; Vázquez-Boland et al., 2001).

Using MLST and MvLST typing methods certain genetic groups of *L. monocytogenes* have been shown to be distributed widely over time and space (Cantinelli et al., 2013). These strains are known as epidemic clones (ECs), as they include genetically similar isolates that have been involved in geographically or temporally distant or large single foodborne illness outbreaks (Cantinelli et al., 2013). A total of seven ECs are currently recognized in the literature (Table 1.2). It has been suggested that ECs are successful clones that have been implicated in many outbreaks due to their wide distribution (Cantinelli et al., 2013). ECV, the clone associated with the 2008 delicatessen meat outbreak in Canada, is serotype 1/2a. A retrospective study of *L. monocytogenes* isolated in Canada over 30 years using MLST and MvLST found this EC was the predominant cause of listeriosis in Canada between the years 1988-2010 (Knabel et al., 2012).

Table 1.2 *Listeria monocytogenes* epidemic clones

EC	***MLST type***	***Serotype***	***Associated outbreaks (Outbreak year, Country, Source)***	***References***
I	CC1	4b	1981, Canada, coleslaw 1983-1987, Switzerland, Vacherin Mont d'Or 1986-1987, USA, Jalisco soft cheese 1992, France, pork tongue in jelly	Cantinelli et al., 2013; Kathariou, 2002
II	CC6	4b	1998-1999, USA, hotdog 2002, USA, delicatessen turkey	
III	CC11	1/2a	1988, USA, turkey franks 2000, USA, sliced turkey deli meat	
IV /Ia	CC2	4b	1970, USA, vegetable 1987-1988, United Kingdom, Ireland, pâté 1997, Italy, gastroenteritis outbreak	Cantinelli et al., 2013; Knabel et al., 2012
V	CC8	1/2a	2008, Canada, ready-to-eat meat outbreak	
VI	CC5	1/2b	1996, Canada, imitation crab meat 2011, USA, cantaloupe	Cantinelli et al., 2013; Lomonaco et al., 2013
VII	CC7	1/2a	2000, Canada, whipping cream 2011 USA, cantaloupe	

EC: epidemic clone; MLST: multi-locus sequence typing

1.3 Foodborne transmission of *L. monocytogenes*

Nearly all sporadic and outbreak cases of listeriosis can be linked to consumption of foods contaminated with *L. monocytogenes* (Health Canada, 2011a; Mead et al., 1999; Schlech, 2000). Numerous foods have been implicated in disease worldwide, and have included items from virtually all food categories (FDA/FSIS/CDC, 2003; Health Canada, 2011a). In fact, the 2003 Risk Assessment on RTE foods indicated that no food category is risk free in regards to *L. monocytogenes* contamination and resultant disease (FDA/FSIS/CDC, 2003). However, RTE foods are known to pose the highest risk. These products have a significant potential for *L. monocytogenes* contamination and are not processed further by the consumer before consumption to inactivate potential contamination (FDA/FSIS/CDC, 2003; Health Canada, 2011a). In the US, Gombas et al. (2003) found the prevalence of *L. monocytogenes* in RTE products ranged from 0.17 to 4.7%. More recently, a large survey of select RTE foods in the European Union recovered *L. monocytogenes* from 0.5 to 10.4% of RTE retail samples tested (European food safety authority, 2013). As a result of this contamination RTE foods have been implicated in the majority of food recalls and listeriosis cases reported in recent years, both in Canada and worldwide (FDA/FSIS/CDC, 2003; Health Canada, 2011a).

1.3.1 Ready-to-eat foods and *L. monocytogenes*

Within the RTE food category certain foods have been deemed to pose higher risk for causing listeriosis (FDA/FSIS/CDC, 2003). Risk ratings have been assigned to 23 categories of RTE food based on (*i*) risk characterization and (*ii*) the probability of exposure to *L. monocytogenes*

from consuming these foods resulting in adverse health outcomes (FDA/FSIS/CDC, 2003). Overall, RTE meats, dairy products, and seafood pose the highest risk to consumers.

Delicatessen meats have the highest predictive risk for causing listeriosis (on both a per serving and per annum basis) (FDA/FSIS/CDC, 2003). While delicatessen meats have a relatively low frequency of contamination in the US (0.8 to 2.1%), these products can support the growth of *L. monocytogenes* under typical storage conditions and have a long shelf life compared to other RTE food categories (FDA/FSIS/CDC, 2003). In addition, retail level cross-contamination of RTE meats with *L. monocytogenes* has the potential to considerably increase the risk of human listeriosis cases (Pradhan et al., 2010).

Similar to RTE meats, smoked seafood is indicated as a high-risk product on a per serving basis (FDA/FSIS/CDC, 2003). RTE seafood products contaminated with *L. monocytogenes* have been linked to sporadic and outbreak cases of listeriosis, can support the growth of this pathogen, and have the highest frequency of contamination compared to other RTE food products (Di Ciccio et al., 2012; FDA/FSIS/CDC, 2003; Jami et al., 2014). A review of *L. monocytogenes* occurrence in smoked fish worldwide reported that on average 16% of products are contaminated with this bacterium, although high variation in prevalence of *L. monocytogenes* is found in these products (from 0 to 80% of samples tested) (Jami et al., 2014). Due to the high frequency of contamination and characteristics of smoked fish products that favor growth of *L. monocytogenes*, these products have been deemed a moderate risk on a per annum basis (FDA/FSIS/CDC, 2003; Jami et al., 2014; Tocmo et al., 2014). However, owing to a short shelf life, typically low contamination levels (<10 CFU/g), and relatively low consumption of smoked

seafood in North America, the risk of contracting listeriosis from these products is reduced compared to other RTE products (FAO/WHO, 2004; FDA/FSIS/CDC, 2003).

Unpasteurized and pasteurized fluid milk, high fat dairy products and soft, unripened cheeses pose a moderate to high risk for causing listeriosis (FDA/FSIS/CDC, 2003). RTE dairy products have been linked to listeriosis cases worldwide (Swaminathan and Gerner-Smidt, 2007). Although the joint FDA/FSIS/CDC risk assessment (2003) assigned less risk to RTE dairy products, a number of listeriosis outbreaks have been associated with both unpasteurized and pasteurized cheeses in Canada (Health Canada, 2011a). In 2002, three different outbreaks associated with cheese resulted in a total of 150 illnesses (Gaulin and Ramsay, 2003; McIntyre et al., 2014). In 2008, an outbreak of listeriosis causing 40 illnesses and 2 deaths was linked to cheeses in Quebec (Gaulin et al., 2012; Health Canada, 2011a). In addition, numerous recalls prompted by *L. monocytogenes* contaminated Canadian cheeses have been conducted both in BC and across Canada in recent years. A joint draft review by the FDA and Health Canada has addressed these outcomes from soft-ripened cheese in both the US and Canada (FDA/Health Canada, 2012).

1.3.2 L. monocytogenes contamination and the food processing environment

As *L. monocytogenes* is widespread in the environment, it is routinely introduced into the food chain through numerous sources (Vázquez-Boland et al., 2001). *L. monocytogenes* is also well equipped to survive food safety hurdles present in these environments (Tompkin, 2002). Despite many potential sources of contamination, the direct source of food product contamination is most often linked to the food production or retail environment (Ferreira et al., 2014; Kathariou, 2002;

Lappi et al., 2004; Tompkin, 2002). Di Ciccio et al. (2012) investigated sources of finished product contamination in a cold-smoked salmon processing facility by comparing PFGE molecular fingerprints of isolates recovered from raw, semi-processed, and finished product along with processing surfaces over a six month period. Although their results showed that raw materials are likely a primary contamination source, they found that product contamination also derived from *L. monocytogenes* isolated in the processing environment (Di Ciccio et al., 2012). Similar findings have been reported in various RTE food sectors using similar methods (Almeida et al., 2013; Gudmundsdóttir et al., 2005; Kathariou, 2002; Lin et al., 2006; Tompkin, 2002).

Many areas in the FPE are considered *L. monocytogenes* "hot spots", or areas where *L. monocytogenes* is likely to be found (Kovacevic et al., 2012a; Tompkin, 2002). *L. monocytogenes* has routinely been isolated from floors, drains, standing water, and equipment (particularly coolers, freezers, conveyor belts, and slicers) (Barancelli et al., 2014; Kovacevic et al., 2012a; Melo et al., 2014; Tompkin, 2002). Although these "hot spots" can be generalized, it is important to note that each facility has unique processes and practices. For example, in large facilities contamination may come from complex process equipment that include hard to reach areas that pose challenges to sanitation and are therefore potential *L. monocytogenes* harbourage sites (Eklund et al., 1995; Johansson et al., 1999). In small processing facilities food contact surfaces (FCS) and utensils may harbour *L. monocytogenes* and result in food contamination during processing (Barros et al., 2007; Holah et al., 2004; Suslow and Harris., 2000). In addition, studies have correlated the level of hygienic practices in facilities to the incidence of environmental *L. monocytogenes* contamination. It has been demonstrated that increased hygiene standards in facilities typically reduce the incidence of *L. monocytogenes* in the processing

environment (Fox et al., 2009; Kabuki et al., 2004). As sources and pathways of contamination vary between processing facilities, it is important to understand the unique processes and practices at each facility.

1.3.3 L. monocytogenes control policies and regulations

Due to the severity of disease caused by *L. monocytogenes* and the risks associated with RTE food products many countries strictly regulate the level of *L. monocytogenes* contamination in these products. In addition to these regulations many countries, including Canada, have released policies and guidance documents to aid in controlling this pathogen in the FPE as a way to reduce the risk of finished product cross-contamination.

Health Canada released the *Policy on* Listeria monocytogenes *in Ready-to-Eat Foods* in 2004, and amended the policy in 2011. The policy aims to limit the amount of *L. monocytogenes* to under 100 CFU/g in RTE food at the point of consumption, using a combination of measures that include inspection, finished product testing, and monitoring the FPE for the pathogen (Health Canada, 2011a). Typically, foods containing <100 CFU/g pose very little risk of causing listeriosis (Chen et al., 2003). Using a risk-based approach the policy categorizes RTE foods based on their ability to support the growth of the bacterium over the stated shelf life. Highest priority is placed on Category 1 foods, or foods that can support the growth of *L. monocytogenes* over the stated shelf life of the product (Health Canada, 2011a). As these foods pose the highest risk, detection of *L. monocytogenes* at any level is deemed a food safety hazard under the policy (Health Canada, 2011a). Category 2 contains two subgroups: (*i*) Category 2A, which includes RTE foods that can support the growth *of L. monocytogenes* but the levels would not exceed 100

CFU/g over the shelf life, and (*ii*) Category 2B, which includes all RTE foods that do not support the growth of the pathogen (Health Canada, 2011a).

Finished product testing has traditionally been used to evaluate control of *L. monocytogenes* in food processing (Tompkin, 2002). In contrast, the Health Canada policy puts emphasis on environmental verification and control through the use of food safety systems, such as Hazard Analysis and Critical Control Point (HACCP) and Good Manufacturing Practices (GMPs), as a way to limit end product contamination (Health Canada, 2011a). A focus on the environment, rather than finished product, is a proactive approach aimed at preventing contamination (Tompkin, 2004). If the prevalence of contamination in a lot is 0.5%, there would be a 61% probability of accepting the contaminated lot after analyzing 100 samples (Tompkin, 2002). Further, a positive end product test result provides no additional information on contamination routes. On the other hand, monitoring the processing environment for *L. monocytogenes* has been described as a cost-effective control measure as it allows processors to assess trends and gives information on potential sources of contamination (Tompkin, 2002). This information allows resources to be directed to controlling *L. monocytogenes* in a certain area, rather than reacting to end product contamination (Tompkin, 2002).

Adherence to the Health Canada policy is required by all federally registered (i.e., processors that export their product beyond provincial borders) RTE food processors in Canada. These regulations are not required for non-federally registered facilities that produce RTE foods (i.e., provincially licensed and inspected processors). In fact, in BC there are no provincial regulations

or guidelines referring specifically to *L. monocytogenes* in the FPE or products from provincially inspected facilities producing RTE foods (Kovacevic et al., 2012a).

In Europe, standards similar to those used in Canada have been adopted. In 2006, Commission regulation No 2037/2005 was released, and replaced shortly after by No 1441/2007. This regulation outlines the specific criteria for producers of RTE foods in the European Union (Melo et al., 2014). Similar to the Canadian policy, this document categorizes RTE foods using a health risk assessment and requires RTE food manufacturers to sample processing areas and equipment as part of their control scheme. In contrast to Canada and Europe, the US has a zero-tolerance policy for the presence of *L. monocytogenes* in all RTE foods, regardless of whether the food can support growth of this pathogen (Shank et al., 1996). Under this policy the presence of any *L. monocytogenes* in finished product, regardless of the level of contamination, renders the food adulterated (Shank et al., 1996). In addition, this policy considers the presence of *L. monocytogenes* on a FCS as a direct indication of product contamination (Shank et al., 1996). The zero-tolerance policy has been subject to much scrutiny, as the absence of *L. monocytogenes* in foods and the FPE is difficult to achieve (Tompkin, 2002). Being the most common cause of Class I recalls in the US, *L. monocytogenes* contamination costs the US food industry between $1.2 and $2.4 billion each year and reduces sales by 22-27% one to two months following the recall (Ivanek et al., 2005). In response to these concerns, the US FDA released a nonbinding compliance policy in 2008 that acknowledges the challenges the RTE food industry faces in regards to *L. monocytogenes*. This policy provides recommendations for environmental monitoring and formulations to aid in reducing instances of *L. monocytogenes* product contamination (FDA, 2008).

1.4 Persistence of *L. monocytogenes* in the food processing environment

Although great efforts have been taken by both food processors and regulators to limit *L. monocytogenes* in the FPE, certain strains have been found to persist in these environments for several months or years. The persistence phenomenon has been demonstrated using varied methodologies across food processing sectors (Ferreira et al., 2014; Verghese et al., 2011). In one study, ribotyping of *L. monocytogenes* isolated from four different fish plants revealed strains that persisted in three facilities (Lappi et al., 2004). In addition, in one of the facilities a persistent strain was responsible for all finished product contamination (Lappi et al., 2004). Through comparison of whole-genome sequences, Orsi et al. (2008) showed striking genetic similarity between food and clinical strains isolated in 1988 and 2000, and linked to a single meat processing facility. These results suggested presence of a persistent strain for at least 12 years in this processing environment. More recently, Leong et al. (2014) analyzed food and environmental samples collected over a one year period from 48 processing facilities in Ireland and found seven plants with persisting strains based on repeated isolation of specific PFGE types. These researchers also found that the same strain persisted in different processing environments, irrespective of sector (Leong et al., 2014).

L. monocytogenes persistence is loosely defined in the literature, with no standardized criteria to differentiate between persisting and sporadic strains (also known as transient or non-persistent strains) recovered in the FPE (Carpentier and Cerf, 2011; Ferreira et al., 2014). Assignment to the persistent category is usually through repeated isolation of *L. monocytogenes* strains belonging to identical subtypes (Ferreira et al., 2014). PFGE is the most commonly employed subtyping method in persistence investigations due to its high discriminatory power (Ferreira et

al., 2014). However, other subtyping methods such as ribotyping have also been used (Ferreira et al., 2014; Lappi et al., 2004; Leong et al., 2014; Williams et al., 2011). More recently, whole genome sequencing has been adopted in retrospective studies of persistence (Holch et al., 2013b; Orsi et al., 2008). When using qualitative and non-statistical methods for classification of persistent strains it is difficult to differentiate between reintroduction of a subtype and a subtype that is persisting (Ferreira et al., 2014; Malley et al., 2013). Further evaluation of facility set up and design, raw materials, and occurrence of positive results has been recommended to supplement persistence categorization (Ferreira et al., 2014). The lack of a quantitative method for classifying persistent strains is a major problem. Many studies have defined strains as persistent merely from isolating recurring subtypes and have not explored the possibility that these strains were obtained through reintroduction. Moreover, some have used methods of subtyping that are not discriminatory enough to suggest clonality (Ferreira et al., 2014). In addition, even though a strain is classified as sporadic it may still persist in the processing environment but might not be detected due to gaps in environmental monitoring (Borucki et al., 2003). In an attempt to provide a quantitative assessment of persistence, Malley et al. (2013) have developed two statistical tests, a binomial test based on ribotype frequencies and a binomial test based on previous positive results, to identify persistent ribotypes.

Despite the problems seen in classifying persistence, certain strains of *L. monocytogenes* are routinely isolated in the processing environment. Numerous hypotheses have been presented to explain the phenomenon of persistence. After finding that persistent strains could be distinguished from non-persistent strains by two genome deletions, Holch et al. (2013b) suggested that specific traits are being selected for in the FPE and that particular genetic and

physiological factors are responsible for persistence of certain strains. The high prevalence of specific serotypes in the FPE (1/2a, 1/2b, and 1/2c) further supports this idea (Valderrama and Cutter, 2013). Some traits suggested to increase the ability of *L. monocytogenes* to persist include improved ability to adhere to surfaces in the FPE and form biofilms, physical adaptation or enhanced tolerance to adverse environmental conditions, and/or resistance or tolerance to sanitizers commonly used in the FPE (Ferreira et al., 2014; Gandhi and Chikindas, 2007; Ribeiro et al., 2014; Verghese et al., 2011). Evidence both for and against each of these characteristics as factors that affect persistence has led others to suggest that persistent *L. monocytogenes* are not necessarily unique, but instead niches and harborage sites allow strains to avoid control measures and remain in the FPE for extended periods (Carpentier and Cerf, 2011). Needless to say, although certain strains are recurring in the FPE there is currently no consensus as to what causes a strain to persist.

1.4.1 L. monocytogenes surface adherence and biofilm formation

1.4.1.1 Overview of biofilms

Bacteria live predominantly by adhering to surfaces in biofilms (Valderrama and Cutter, 2013). The classical definition of a biofilm includes two parts: cell aggregates that are (*i*) attached to a surface and/or each other and (*ii*) embedded in extracellular polymeric substance (EPS) produced by the microorganisms within the biofilm (Costerton et al., 1995; O'Toole et al., 2000). More recent biofilm definitions suggest that EPS formation may not be an absolute requirement for biofilm formation (Monds and O'Toole, 2009). A biofilm is formed to enhance bacterial survival and spread, thereby ensuring reproductive success in a specific ecological niche (O'Toole et al., 2000; Valderrama and Cutter, 2013). It has been suggested that formation of a biofilm is

determined by the nature of the attachment site, the characteristics of the bacterial cell, and by environmental factors (Van Houdt and Michiels, 2010). Steps in biofilm formation include initial attachment of planktonic cells to a surface via van der Waals forces, electrostatic forces, and hydrophobic interactions, followed by cellular proliferation and, in some cases, production of EPS (Chang et al., 2012; O'Toole et al., 2000). With time, a more complex three-dimensional network is formed where mature biofilms contain channels for flow of nutrients and waste products (Chang et al., 2012; da Silva and De Martinis, 2013; Palmer et al., 2007).

1.4.1.2 Characteristics of *L. monocytogenes* biofilms

It is well established that *L. monocytogenes* can adhere to a diversity of abiotic surfaces and subsequently form biofilms on these surfaces (Blackman and Frank, 1996; da Silva and De Martinis, 2013; Djordjevic et al., 2002; Frank and Koffi, 1990). In general, *L. monocytogenes* cannot form thick, multilayer biofilms as commonly described in other bacteria (da Silva and De Martinis, 2013). The architecture of *L. monocytogenes* biofilms varies between strains and conditions during biofilm formation. Structural dynamics have been described as small micro-colonies, a homogeneous layer of cells, ball-shaped micro-colonies surrounded by a network of knitted chains, or honeycomb structure (Nguyen and Burrows, 2014). Little is known about the composition of *L. monocytogenes* EPS; however, research has suggested that large fragments of extracellular DNA, and other components, such as extracellular polysaccharides, a variety of proteins and enzymes, and poly-N-acetylglucosamine are needed for biofilm production (da Silva and De Martinis, 2013; Harmsen et al., 2010; Nguyen and Burrows, 2014). On the genomic level, a number of genes have been associated with *L. monocytogenes* biofilm formation

including those associated with flagella and motility, quorum sensing, gene regulation, and cell surface and cell wall formation (Chang et al., 2012).

Differences between *L. monocytogenes* strains in their ability to adhere to surfaces and form biofilms have been observed (Valderrama and Cutter, 2013). The high proportion of Lineage II strains in the FPE has led to the hypothesis that there is a relationship between phylogenetic division and biofilm forming ability (Valderrama and Cutter, 2013). While many studies do show that Lineage II strains have increased adherence to a variety of surfaces, when compared to Lineage I strains (Borucki et al., 2003; Folsom et al., 2006; Harvey et al., 2007; Nakamura et al., 2013; Norwood and Gilmour, 1999), others have observed the opposite (Harvey et al., 2007; Kalmokoff et al., 2001; Takahashi et al., 2009) or found no differences (Milanov et al., 2009). These conflicting results may be the result of differing methodologies used in the studies, varying sample sizes, or genetic variability in each sample set (Valderrama and Cutter, 2013). Alternatively, this lack of consensus may suggest that *L. monocytogenes* strains have individual environmental requirements that promote biofilm formation related to lineage (Valderrama and Cutter, 2013).

1.4.1.3 *Listeria monocytogenes* biofilms and persistence

In FPEs, *L. monocytogenes* biofilm formation on inert surfaces may represent a source of product contamination, although a direct link between biofilms and food contamination has not been observed (da Silva and De Martinis, 2013; Valderrama and Cutter, 2013). Biofilms have been associated with increased resistance to a variety of physical and chemical stresses, and for these reasons it has been suggested that the formation of biofilms may be a factor in the survival and

persistence of *L. monocytogenes* in FPEs (da Silva and De Martinis, 2013; Harvey et al., 2007; Pan et al., 2006). Notably, Pan et al. (2006) found *L. monocytogenes* biofilms to have increased resistance to sanitizers, with this increased resistance attributed to protective effects of the EPS surrounding the cells in a biofilm. *L. monocytogenes* in biofilms have also been shown to be protected from ultraviolet rays, toxic metals, acids, desiccation, salinity, and antimicrobials (da Silva and De Martinis, 2013; Nguyen and Burrows, 2014). Many studies support the hypothesis that persistent strains adhere to abiotic surfaces more readily than sporadic strains under specific conditions (Borucki et al., 2003; Ochiai et al., 2014; Wang et al., 2015). Others, however, have not found significant differences between persistent and sporadic strains in regard to biofilm-forming ability (Djordjevic et al., 2002; Ortiz et al., 2014b).

1.4.2 Sanitizer resistance

1.4.2.1 Sanitizers used in the food industry

Sanitation is an action that reduces microbial contamination to levels considered to be safe from a public health perspective (Marriott, 2006). Sanitation may be achieved through physical or chemical means, with chemical sanitizing more commonly used in the FPE (Gaulin et al., 2011). Sanitizers are classified based on their active ingredient. Those that have been approved for use in FPEs include acid anionics, carboxylic acids, chlorine compounds, iodine compounds, peroxide and peroxyacid mixtures, ozone, and quaternary ammonium compounds (QACs) (Gaulin et al., 2011; Marriott, 2006). These sanitizers non-specifically affect proteins, DNA, RNA, and/or cell wall constituents through physicochemical interactions, or chemical reactions resulting in a decrease or elimination of the microbial population (Cerf et al., 2010; Marriott, 2006; Mcdonnell and Russell, 1999).

1.4.2.2 *L. monocytogenes* sanitizer tolerance and resistance

At manufacturer recommended concentrations sanitizers are effective at eliminating *L. monocytogenes* (Cruz and Fletcher, 2012). However, several factors, including dilution below the recommended level and presence of interfering substances (e.g., organic material, biofilm EPS), significantly reduce the efficacy of sanitizers in the FPE (Cerf et al., 2010; Sundheim et al., 1998). In these instances *L. monocytogenes* may be exposed to sub-lethal concentrations of sanitizers leading to a selective pressure for acquisition of resistance genes or adaptation of initially susceptible bacteria (Soumet et al., 2005). Of the sanitizers commonly used in FPEs the relatively stable QACs provide the greatest opportunity for tolerance or resistance to develop as these compounds may be present in the environment for extended periods (Allen et al., 2015; Müller et al., 2013). In fact, resistance of *L. monocytogenes* isolated from foods and the processing plant environment to the QAC benzalkonium chloride (BAC) has been found to range from 10% to as much as 42 to 46% (Mereghetti et al., 2000; Mullapudi et al., 2008; Soumet et al., 2005). However, this resistance is at levels well below the recommended concentrations used for QAC sanitizers.

Mechanisms underlying the BAC resistant phenotype have been associated with acquired tolerance, as well as chromosomal and plasmid-mediated resistance (Elhanafi et al., 2010; Kastbjerg and Gram, 2012; Müller et al., 2013). Kastbjerg and Gram (2012) found that repeated exposure of *L. monocytogenes* to sub-lethal concentrations of QACs resulted in increased tolerance to these compounds, albeit the adapted strains were still sensitive to QACs under conditions used in the food industry. This resistance phenotype, seen in many strains of *L. monocytogenes*, may also be due to genetic elements linked to increased BAC resistance:

bcrABC, *qacH* or *emrE*. *BcrABC* is a plasmid-based BAC resistance gene cassette located on a putative composite transposon (Dutta et al., 2013; Elhanafi et al., 2010). This cassette encodes for two proton-dependent multi-drug efflux systems (*bcrB* and *bcrC*) and a TetR family efflux system transcription regulator (*bcrA*) (Elhanafi et al., 2010). Dutta et al. (2013) screened 116 diverse *L. monocytogenes* isolates and found that of the 71 possessing BAC resistance all but one contained *bcrABC* with the genes either present on a plasmid or incorporated into the bacterial chromosome. *QacH* is another gene encoding an efflux pump responsible for increased resistance to BAC. This gene is found on a transposon (Tn*6188*) that inserts into the chromosomal *radC* gene in *L. monocytogenes* (Müller et al., 2013). Of 91 strains screened by Müller et al. (2013) ten possessed Tn*6188* and had an increased resistance to BAC compared to strains lacking this transposon. *EmrE*, a gene found on *Listeria* genomic island 1, also encodes for a small multidrug resistance protein involved in efflux of toxic compounds, including BAC (Kovacevic, 2014 [unpublished]).

1.4.2.3 Sanitizer resistance and *L. monocytogenes* persistence

Resistance of *L. monocytogenes* to sanitizers used in the FPE has been suggested to improve the persistence of these strains (Ferreira et al., 2014; Gandhi and Chikindas, 2007; Kastbjerg and Gram, 2012). Fox et al. (2011) screened persistent and sporadic *L. monocytogenes* for resistance to various QACs and assessed growth dynamics of the strains in the presence of these compounds. The data from their research, and more recent works, suggest that persistent strains have increased resistance to QACs (Fox et al., 2011; Nakamura et al., 2013; Ortiz et al., 2014a). In contrast, Holah et al. (2002) found no differences in susceptibility to QACs between persistent and sporadic strains isolated from FPEs in the United Kingdom using an exposure assay. Further,

although Lundén et al. (2003) did observe differences in resistance between persistent and sporadic strains after two hours of exposure, no differences in adaptive responses towards QACs were noted.

To explain this lack of agreement, it has been hypothesized that persistence due to sanitizer resistance could be an outcome of biofilm forming abilities of certain strains (Nakamura et al., 2013; Pan et al., 2006). Nakamura et al. (2013) calculated the half maximal effective concentration of BAC for persistent and sporadic *L. monocytogenes* using bioluminescence as an indicator of active biomass in both planktonic cells and cells in biofilm. Although persistent strains showed a 2.2-fold increase in resistance to BAC compared to sporadic strains in the planktonic state, this effect was more pronounced (150-fold increase) when persistent strains were tested in the biofilm state. In addition, this study observed that the total amount of EPS in the persistent strains biofilm was higher compared to biofilms of sporadic strains. These data suggest that components of the biofilm, such as EPS, protect *L. monocytogenes* from the effects of sanitizers in the FPE (Nakamura et al., 2013). Strains that can produce large amounts of EPS may be better able to resist sanitizers and persist in the processing environment. Thus, resistance to sanitizers may be due to attributes of EPS substances rather than the intrinsic attributes of the *L. monocytogenes* strain to resist the chemicals directly (Pan et al., 2006).

1.4.3 L. monocytogenes stress adaptation

Stress refers to any adverse factor or condition that unfavourably affects microbial growth or survival (Yousef and Courtney, 2003). In the FPE *L. monocytogenes* is routinely exposed to environmental stresses such as scarcity of nutrients, low pH, high osmolarity, desiccation,

competing bacteria, and cold temperatures (Ferreira et al., 2014; Gandhi and Chikindas, 2007). These stresses may be lethal to the bacteria or sub-lethal, permitting survival and potential adaptation to the stress conditions (Ferreira et al., 2014). Numerous studies have demonstrated marked interstrain variation of *L. monocytogenes* in response to environmental stresses (Arguedas-Villa et al., 2014; Kovacevic et al., 2013a; Ribeiro and Destro, 2014). Understanding how *L. monocytogenes* responds to stressful conditions provides insights into the ability of some strains to better adapt to stresses encountered in the FPE and foods (Gandhi and Chikindas, 2007).

1.4.3.1 Cold temperature stress

The survival and growth of *L. monocytogenes* at refrigeration temperatures (2–4°C) make the control of this foodborne pathogen difficult in the food industry (Gandhi and Chikindas, 2007; Rocourt and Cossart, 1997). An ability to rapidly multiply in a cold environment could also be of benefit to *L. monocytogenes* competing for nutrients with other microorganisms less adapted to proliferation at low temperature either in the environment or in a food matrix (Durack et al., 2013). Refrigeration is widely used in food processing and distribution due to its capacity to prolong the shelf life and decrease food safety risk in many foods (Yousef and Courtney, 2003). The main principles behind cold temperature as a food safety hurdle are that cellular processes are slowed at lower temperatures, the phospholipid bilayer membranes of all cells decrease in fluidity, and hydrogen bonds stabilize in nucleic acid secondary structures resulting in reduced efficiency of translation, transcription and DNA replication (Yousef and Courtney, 2003).

In *L. monocytogenes,* proteins synthesized in response to cold stress can be classified as cold shock proteins (Csps), with *L. monocytogenes* having three: CspA, CspB, and CspD, or cold acclimation proteins (Caps) (Phadtare et al., 1999; Schmid et al., 2009). Csps are rapidly, but transiently, overexpressed in response to cold whereas Caps are continuously synthesized during growth at cold temperatures (Phadtare et al., 1999). Together these proteins aid in the physiological adaptation to low temperatures and allow *L. monocytogenes* to survive and grow despite the stress imparted by the temperature downshift (Yousef and Courtney, 2003). Specifically, the composition of the cell membrane is modified to include more unsaturated and short-chain fatty acids in an effort to reduce carbon-carbon interactions and maintain membrane fluidity needed for normal cellular functions (Durack et al., 2013; Gandhi and Chikindas, 2007). Additionally, highly soluble compounds that have no net charge at physiological pH (termed cryoprotectants) are accumulated inside the cell via three transporters (Gbu, BetL, and OpuC) (Chan and Wiedmann, 2009; Durack et al., 2013; Tasara and Stephan, 2006). Finally, proteins that aid in DNA replication and transcription and in RNA translation help the bacteria to carry out normal cellular function and continue to grow (Chan and Wiedmann, 2009; Tasara and Stephan, 2006).

1.4.3.2 High osmotic stress

Research on *L. monocytogenes* osmotolerance has shown this bacterium is able to survive in up to 10% w/v sodium chloride (Gandhi and Chikindas, 2007; Valderrama and Cutter, 2013). Salt is present in many processing environments and foods; it can be added to impart flavour, and/or to extend shelf life of foods as an antimicrobial agent (Adrião et al., 2008; Bae et al., 2012; Choi and Yoon, 2013). Salt can cause damage to bacterial cells by disrupting the osmotic balance

between cytoplasmic and intracellular environments (Csonka, 1989). The ability of *L. monocytogenes* to adapt and survive in high concentrations of salt makes it difficult to control the pathogen in foods (Gandhi and Chikindas, 2007).

During osmotic stress the increase in extracellular solute concentration causes water movement out of the cell through osmosis. This dehydrates the cell and inhibits transport of substrates and cofactors into the cell. *L. monocytogenes* response to osmotic stress shares many parallels with the response to cold temperature shock, despite the obvious physicochemical differences each exhibit on the cell (Durack et al., 2013). Accumulation of cryoprotectants inside the cell, similar to those discussed previously, aid in regaining osmotic balance within the cell, reducing flow of fluids to the external environment (Durack et al., 2013).

1.4.3.3 Acid stress

L. monocytogenes encounters a low pH environment in acidic foods, during gastric passage and in the phagosome of the macrophages (Cotter and Hill, 2003; Ribeiro et al., 2014). In order to survive in these environments, bacteria need to maintain a relatively neutral intracellular environment, as essential enzymes are impaired outside this range and low cytoplasmic pH may cause DNA damage (Jay et al., 2005). As a facultative anaerobe, *L. monocytogenes* maintains intracellular pH homeostasis through either the electron transport chain or through the multi-subunit enzyme F_0F_1-ATPase that actively translocates intracellular protons across the cell membrane (Shabala et al., 2002). Another way of reducing protons in the cell cytosol, and thus increasing the internal cell pH during acid stress, is through the glutamate decarboxylase system, wherein glutamate is taken in by the cell, undergoes a decarboxylation reaction consuming one

proton in the process, and is exported from the cell via an antiporter (Gandhi and Chikindas, 2007). Through these mechanisms *L. monocytogenes* is able to withstand and adapt to pH as low as 4.4 and overcome this potential food safety hurdle (Valderrama and Cutter, 2013).

1.4.3.4 Persistence and stress response

Limited data are available to link persistence with an enhanced response to FPE stresses. Ringus et al. (2012) found no clear association between persistent strains and their stress gene transcription levels under salt stress. A previous study, however, did show persistent strains to have an increased tolerance to acid (Lundén et al., 2003). This is an overlooked area of persistence research, as stress response and adaptation may be important factors in explaining why some strains are able to persist over others. In addition, many studies have shown that exposure to one stress common in the FPE may enhance survival and growth to another stress, a phenomenon known as cross-protection, further enhancing strain persistence in the FPE (Bergholz et al., 2010; Faleiro et al., 2003; Schmid et al., 2009).

1.5 Human listeriosis

From a public health perspective the presence of both persistent and sporadic *L. monocytogenes* in the FPE is cause for concern. Contamination of RTE foods with this pathogen, and consequent cases of listeriosis, are most often associated with *L. monocytogenes* in these environments (Lappi et al., 2004). Listeriosis has various clinical manifestations depending on the number of bacteria ingested, the host, and the strain causing infection (FDA/FSIS/CDC, 2003; Vázquez-Boland et al., 2001). In general, listeriosis can be divided into two main categories: non-invasive and invasive (Health Canada, 2011a).

Non-invasive listeriosis usually occurs in immunocompetent individuals, presenting as self-limiting febrile gastroenteritis similar to other food-acquired illnesses (Allerberger and Wagner, 2009; Health Canada, 2011a; Painter and Slutsker, 2007). The exact mechanism causing these symptoms remains unknown (Lecuit, 2007). Non-invasive listeriosis has been observed during a number of outbreaks (Aureli et al., 2000; Dalton et al., 1997; Pichler et al., 2009). Using details from these cases aids in the understanding of this disease. For example, *L. monocytogenes* levels in foods implicated in non-invasive listeriosis ranged between 10^3 and 10^9 CFU/g, with most products having a high level of contamination (Pichler et al., 2009). These data suggest that a high dose of *L. monocytogenes* is required in this disease, a notion further supported by oral challenges in healthy nonhuman primates, where ingestion of $>10^9$ cells was needed to induce a clinical effect (Farber et al., 1991). Additionally, the incubation time of patients who suffered non-invasive listeriosis in these reports is quite short, ranging from 20 to 48 h (Pichler et al., 2009).

Invasive listeriosis occurs in humans who have decreased levels of cell-mediated immunity. This group includes the elderly (55 to 60 years and older), neonates, pregnant women, individuals undergoing immunosuppressive therapy, or debilitated adults with underlying diseases (Health Canada, 2011a; Kathariou, 2002; Vázquez-Boland et al., 2001). As an opportunistic pathogen, *L. monocytogenes* can invade otherwise sterile body sites when presented with a weakened immune response (Painter and Slutsker, 2007). Specifically, *L. monocytogenes* has bacterial tropism towards the pregnant uterus, central nervous system, blood, or a combination (Painter and Slutsker, 2007; Vázquez-Boland et al., 2001). In non-pregnant individuals the infection

usually manifests in the central nervous system resulting in meningoencephalitis, or in the blood causing septicaemia (Health Canada, 2011a). In pregnant women symptoms of the infection are often mild, but localization of the pathogen to the placenta may cause miscarriage, stillbirth, or perinatal sepsis or meningitis in the newborn baby (Health Canada, 2011a).

Although most invasive listeriosis cases are linked to suppressed immune function, outbreaks have occurred in populations who seem to be immunocompetent, potentially due to the high bacterial load or enhanced virulence characteristics of the strains implicated in these outbreaks (Schlech, 1993). The infectious dose for invasive listeriosis, like non-invasive listeriosis, continues to be challenging to determine as factors such as virulence of the strain and host susceptibility play a role in disease outcomes. Analysis of foods linked to cases of invasive listeriosis has found varying levels of contamination (Vázquez-Boland et al., 2001). Overall, though, *L. monocytogenes* seems to have lower pathogenicity compared to other foodborne bacterial pathogens (Vázquez-Boland et al., 2001). In contrast to non-invasive listeriosis the incubation time of invasive listeriosis is much longer at around 20 to over 30 days (Swaminathan and Gerner-Smidt, 2007; Vázquez-Boland et al., 2001).

1.5.1 Diagnosis and treatment of listeriosis

Treatment of invasive listeriosis is often accomplished through administration of antibiotics. The choice and timing of appropriate antibiotic therapy is critical (Allen et al., 2012; Lemoy et al., 2012; Schlech, 2000). *In vitro* and *in vivo* clinical experience suggest a high dose of ampicillin, penicillin, or amoxicillin (6 g/daily) in combination with an aminoglycoside, such as gentamicin, for a three week duration as an effective treatment option for treatment of adult listeriosis

(Lemoy et al., 2012; Painter and Slutsker, 2007; Schlech, 2000). This combination is recommended because ampicillin is bacteriostatic for *L. monocytogenes*, and listeriosis relapse has been reported after administration of ampicillin alone (Lemoy et al., 2012). For patients allergic to antibiotics from the penicillin class, trimethoprem-sulfamethoxazole has also proven effective in treatment of listeriosis (Painter and Slutsker, 2007).

1.5.2 Infection cycle and pathogenesis of *L. monocytogenes*

With listeriosis being primarily a foodborne illness, the gastrointestinal tract is the primary site of *L. monocytogenes* entry into the host (Freitag et al., 2009; Lecuit, 2007; Vázquez-Boland et al., 2001). After passing through the gastric environment the bacteria translocate across the intestinal epithelium (Freitag et al., 2009; Lecuit, 2007; Pizarro-Cerdá et al., 2012). From there *L. monocytogenes* is carried by the lymph or blood to mesenteric lymph nodes, the spleen, and the liver (Freitag et al., 2009; Lecuit, 2007).

L. monocytogenes can target and enter a variety of nonprofessional phagocytes including epithelial cells, fibroblasts, hepatocytes, endothelial cells, and a variety of nerve cells as well as professional phagocytes such as macrophages and dendritic cells, and cause infection (Chen and Nightingale, 2013; Vázquez-Boland et al., 2001). Proteins on the surface of *L. monocytogenes* specific to receptors on the surface of these eukaryotic cells allow a close host cell-bacterium interaction and facilitate entry into the host cell via a zipper-like mechanism (Cossart and Sansonetti, 2004; Mengaud et al., 1996).

Once inside the host cell *L. monocytogenes* is able to replicate and spread to neighbouring cells, causing further infection. During entry, the bacterium becomes engulfed in a phagocytic vacuole. This vacuole is disrupted shortly after entry allowing *L. monocytogenes* to replicate in the cytosol of the host cell (Disson and Lecuit, 2013; Vázquez-Boland et al., 2001). Intracytosolic *L. monocytogenes* is then surrounded by actin filaments, which are polymerized to form actin tails on polar ends of the bacterium (Disson and Lecuit, 2013). The actin tails are used to propel *L. monocytogenes* within the cytosol to the periphery of the cell where it then comes into contact with the membrane and protrudes out to an adjacent cell, allowing the bacterium to spread (Travier and Lecuit, 2014; Vázquez-Boland et al., 2001). Once it successfully enters the neighbouring cell, the now double-membrane vacuole is dissolved and the cycle of infection continues (Freitag et al., 2009; Vázquez-Boland et al., 2001).

1.5.3 Major virulence factors of *L. monocytogenes*

The infection cycle described above involves numerous factors all working in concert to promote virulence, evade the host immune response, and adapt to stresses within the host (Camejo et al., 2009; Lecuit, 2007; Vázquez-Boland et al., 2001). Of the more than 50 genes up-regulated during infection, two genetic loci, the internalin operon and the virulence gene cluster *Listeria* pathogenicity island (LIPI-1), house what are commonly described as the core or key virulence factors in *L. monocytogenes* (Camejo et al., 2009; de las Heras et al., 2011; Freitag et al., 2009; Lemoy et al., 2012; Vázquez-Boland et al., 2001).

1.5.3.1 Internalin operon

The internalin operon encodes InlA and InlB, proteins that bind to specific receptors on the host-cell surface and trigger entry of *L. monocytogenes* into nonprofessional phagocytes (Dramsi et

al., 1995; Gaillard et al., 1991; Kathariou, 2002; Vázquez-Boland et al., 2001). These two proteins play a pivotal role in the pathogenesis of *L. monocytogenes*, as they are required for cell adhesion and internalization of the bacterium (Disson et al., 2008). Although *L. monocytogenes* encodes two internalin proteins, as grouped by the presence of tandemly arranged leucine-rich repeats (LRRs) and a signal peptide in their N-terminal domain, InlA and InlB are the only internalins to date that have been directly associated with invasion of host cells (den Bakker et al., 2010; Pizarro-Cerdá et al., 2012).

InlA, an 800 amino acid protein, consists of two functional regions. The N-terminal includes a signal peptide and 15 LRR units. The C-terminal half contains three longer repeat sequences and a cell wall anchor comprising of (*i*) the sorting motif LPXTG and (*ii*) a hydrophobic membrane spanning region of approximately 20 amino acids (Vázquez-Boland et al., 2001). InlA is covalently linked to *L. monocytogenes* peptidoglycan, with LPXTG responsible for this attachment in a process mediated by the enzyme sortase (Vázquez-Boland et al., 2001). The LRR region of InlA interacts with the first extracellular domain of the transmembrane epithelial cell specific E-cadherin protein (Disson and Lecuit, 2013; Lecuit et al., 2001; Pizarro-Cerdá et al., 2012). E-cadherin molecules are involved in formation of adherens junctions in the intestinal barrier, the blood-brain barrier, and the placenta, which may in part explain this pathogen's tropism for localized infections in these areas of the body (Mengaud et al., 1996). This interaction is species specific where proline at amino acid position 16, found in humans and other animals, has been shown to be essential (Lecuit et al., 1999). The interaction of InlA with E-cadherin is followed by actin cytoskeleton rearrangement mediated by the intracytoplasmic

domain of E-cadherin and internalization of the bacteria into the targeted cell via endocytosis (Lecuit et al., 2000).

InlB is smaller in comparison to InlA at a length of 630 amino acids. Like InlA, InlB also has two functional regions that include an N-terminal signal peptide and LRR region (made up of 7 units) but this protein lacks the cell wall anchoring motif LPXTG (Vázquez-Boland et al., 2001). Instead, InlB possesses a 232 amino acid region consisting of tandemly arranged repeats starting with the dipeptide GW (the GW motif) which promotes a loose interaction with lipoteichoic acid on the bacterial cell wall (Dramsi et al., 1995; Pizarro-Cerdá et al., 2012). InlB interacts with several host cell receptors (Dramsi et al., 1995; Jonquieres et al., 2001; Khelef et al., 2006; Shen et al., 2000) though Met (the hepatocyte growth factor receptor) plays the most critical role in InlB mediated internalization (Shen et al., 2000). Like InlA, interaction between InlB and the host cell receptor is species specific wherein InlB does not recognize guinea pig and rabbit Met, but does interact with human and mouse Met (Khelef et al., 2006). The interaction between InlB and Met results in actin cytoskeleton rearrangement and endocytosis signalling leading to the internalization of *L. monocytogenes* (Bierne and Cossart, 2002).

Adhesion and invasion into human cells during the *L. monocytogenes* infection cycle involves one or both internalins described above. Separate studies have demonstrated that individually these proteins are sufficient to promote bacterial adhesion to receptors specific to each internalin and internalization into host-cells (Braun et al., 1998; Lecuit et al., 1997). In addition, InlA and InlB can stimulate entry in concert, wherein the two proteins work together to mediate entry into the targeted host cell (Disson et al., 2008; Pentecost et al., 2010; Stavru et al., 2011). InlA is

required for the traversal of the intestinal barrier and is involved in placental invasion (Disson et al., 2008; Khelef et al., 2006; Lecuit et al., 2004). Although InlB is not required for crossing the intestinal barrier, this protein may work with InlA to accelerate bacterial internalization after attachment by locally activating Met and increasing endocytosis in intestinal cells (Pentecost et al., 2010). InlB is also involved in liver and spleen colonization and may play a role in placental invasion (Disson et al., 2008).

1.5.3.2 Virulence gene cluster *Listeria* pathogenicity island 1

L. monocytogenes' lifestyle switch from saprophytic bacteria to intracellular pathogen requires an increase in expression of virulence genes, as these genes are generally expressed at low levels outside the host (Scortti et al., 2007). The major virulence gene locus LIPI-1 encodes factors required for intracellular survival and cell-to-cell spread (Pizarro-Cerdá et al., 2012). The master virulence regulatory protein PrfA is conveniently encoded for by *prfA*, located on LIPI-1 (Freitag et al., 2009). PrfA activates transcription of virulence-associated genes by binding to the PrfA box located upstream from the targeted genes (de las Heras et al., 2011). Numerous triggers to express PrfA, and subsequently the set of virulence genes associated with this regulatory protein, have been described. The promoter region of *prfA* contains a thermoswitch, where at temperatures ≤30°C the RNA transcript forms a secondary hairpin structure preventing ribosome binding and translation of PrfA (Johansson et al., 2002). Available carbon sources have also been linked to virulence gene expression. Sugars common in the natural environment (e.g., glucose, fructose, mannose or cellobiose) down-regulate PrfA-dependent genes while this is not seen in the presence of sugars commonly utilized during intracellular metabolism (e.g., hexose phosphate derivatives) (Freitag et al., 2009; Scortti et al., 2007).

Listeriolysin O (LLO) is a toxin encoded by *hly* (Vázquez-Boland et al., 2001). LLO is required for bacterial vacuolar escape during pathogenesis (Hamon et al., 2012; Vázquez-Boland et al., 2001). In fact, there is a strong correlation between hemolysis and pathogenicity in the genus *Listeria*. Research has demonstrated that the loss of hemolysis in pathogenic strains, *L. monocytogenes* and *L. ivanovii*, results in their reduced virulence (Hamon et al., 2012; Lecuit, 2007; Vázquez-Boland et al., 2001). Aside from its pore-forming ability, LLO has recently been described as the "Swiss army knife of *Listeria*" due to emerging evidence that this toxin plays many roles in the virulence of *L. monocytogenes* (Hamon et al., 2012).

Rapid vacuole escape is facilitated further through two phospholipase C enzymes, PI-PLC and PC-PLC, encoded for by *plcA* and *plcB* on LIPI-1, respectively. These enzymes work with LLO in disruption of the vacuolar membrane (Vázquez-Boland et al., 2001). A metalloprotease, encoded by *mpl* also found on LIPI-1, is required to activate PC-PLC (Domann et al., 1991; Mengaud et al., 1991). Assessing cellular infection of in-frame deletion mutants, Smith et al. (1995) found that PI-PLC is mainly involved in facilitating the escape from the primary vacuole where PC-PLC is important during cell-to-cell spread of the bacteria.

ActA, encoded for by *actA* on LIPI-1, is a 639 amino acid protein solely responsible for actin-mediated intercellular motility during the infection cycle (Tilney and Portnoy, 1989; Vázquez-Boland et al., 2001). ActA recruits actin-related proteins to harness the actin polymerization machinery of the host cell to form a polar actin-based comet tail (Kocks et al., 1992; Stavru et al., 2011; Tilney and Portnoy, 1989). This comet-tail propels *L. monocytogenes* through the

intracystolic space to the periphery of the cell, forming protrusions in the membrane, and allows its spread to adjacent cells (Stavru et al., 2011).

1.6 Research overview

Studies evaluating the presence of *L. monocytogenes* in RTE seafood FPEs and finished products have revealed significant issues with control of this pathogen in this sector (Cruz et al., 2014; Gudmundsdóttir et al., 2005; Kovacevic et al., 2012a, 2012b; Lappi et al., 2004; Miya et al., 2010). In BC, specifically, *L. monocytogenes* was found on both non-food contact surfaces and food contact surfaces in fish facilities (Kovacevic et al., 2012a). Further, various RTE fish finished products in BC were found to be contaminated with *L. monocytogenes* (Kovacevic et al., 2012a, 2012b). One suggestion for the prevalence of *L. monocytogenes* in BC establishments and contamination of finished products is that certain strains may be persisting in these FPEs. However, research on the population diversity of *L. monocytogenes*, as it pertains to persistence in these environments, is lacking. This project aims to assess the population diversity and genetic/phenotypic characteristics of *L. monocytogenes* in a single seafood-processing environment in BC. These data will improve our understanding of the persistence of *L. monocytogenes* and evaluate the risks that these strains may pose to public health. Additionally, this research will be used to provide specific information to a BC seafood processor in relation to the source of contamination in the FPE and the effectiveness of *L. monocytogenes* control strategies currently in place at this establishment.

1.6.1 Hypotheses and research objectives

Hypothesis 1: *Listeria* spp. can be isolated from the FPE and finished products in a BC seafood processing establishment.

Research objectives:

- Conduct environmental sampling and finished product testing at a RTE seafood processing facility over 18 months and analyze samples for the presence of *Listeria* spp.
- Determine the overall prevalence of *Listeria* spp. at this facility and in finished product and assess contamination frequencies and trends.

Hypothesis 2: *L. monocytogenes* strains persisting in the environment of a BC seafood processing facility can be recovered from finished products produced in the facility.

Research objectives:

- Subtype all *L. monocytogenes* isolated during environmental sampling and finished product testing.
- Assess strains for potential persistence in the processing environment using persistence classifications described in previous persistence research.
- Compare persistent strain subtypes to subtypes of isolates recovered from finished products.

Hypothesis 3: *L. monocytogenes* isolated from the FPE of a RTE seafood processor in BC possess characteristics previously associated with persistence of *L. monocytogenes* in the FPE.

Research objectives:

- Determine the capacity of isolates to adhere to abiotic surfaces.
- Assess resistance of isolates to QACs commonly used in food processing and screen for genetic determinants of resistance to these compounds.
- Evaluate isolates' lag phase duration and and exponential growth rate in the presence of two common FPE stresses, high (6%) salt concentration and low temperature (4°C).

Hypothesis 4: *L. monocytogenes* isolated from the FPE of a RTE seafood processor in BC pose a risk to public health.

Research objectives:

- Determine if *L. monocytogenes* isolates from the seafood processing environment have the ability to survive and grow in finished products produced by the facility.
- Evaluate isolates for genetic characteristics associated with increased risk of causing human disease.
- Screen the isolates for resistance to antibiotics commonly used in treatment of listeriosis.

Chapter 2: Evaluation of *Listeria* spp. contamination in a ready-to-eat seafood processing facility in British Columbia reveals reintroduction on raw materials as the primary source of environmental *L. monocytogenes*

2.1 Introduction

Listeria monocytogenes is a bacterial pathogen that can cause a rare but severe human disease, listeriosis, in at-risk populations (Health Canada, 2011a; Kathariou, 2002). Over 90% of listeriosis is caused by consumption of foods contaminated with this pathogen, with ready-to eat (RTE) foods being a primary concern (Health Canada, 2011a; Mead et al., 1999; Scallan et al., 2011). Contamination of RTE foods with *L. monocytogenes* is most often associated with post-lethality treatment contamination of finished product via the food processing environment (FPE) harboring *L. monocytogenes* (Lappi et al., 2004; Tompkin, 2002). The potential for *L. monocytogenes* to recur in the FPE for months to even years, through strain specific traits that aid in persistence or through reintroduction, further challenges control of this pathogen in the FPE (Ferreira et al., 2014; Holch et al., 2013b; Lappi et al., 2004).

In British Columbia (BC), increased prevalence of *L. monocytogenes* in fish processing facilities and RTE products suggests issues with control of this pathogen despite strategies in place to minimize *L. monocytogenes* in these areas. Kovacevic et al. (2012a) found *Listeria* spp., indicators that conditions are favorable to *L. monocytogenes* survival, 3.9 times more often in fish processing facilities when compared to meat and dairy processing facilities located in BC. Further, a survey of RTE products in BC found that 20% of RTE fish samples contained *Listeria* spp. with a quarter of these samples containing *L. monocytogenes* (Kovacevic et al., 2012b). One suggestion for the high occurrence of *L. monocytogenes* in BC seafood and seafood processing plants is that certain strains may be persisting. However, research on the population diversity of

L. monocytogenes in BC RTE seafood processing environments is lacking. Based on the issues and gaps in knowledge noted above, the control of *Listeria* spp. in a single RTE seafood processing facility in BC over 18 months was evaluated and contamination patterns of *Listeria* spp. and persistence of *L. monocytogenes* in this establishment were assessed.

2.2 Materials and methods

2.2.1 Description of seafood processing facility

The seafood processing facility evaluated in this study is a large Canadian Food Inspection Agency inspected operation located near Vancouver, BC. The facility produces a variety of RTE seafood products, with their main output being cold-smoked salmon. The processor sources headed and gutted raw materials from numerous suppliers spanning the globe, including farmed and wild fish. During this study, 15 wild salmon suppliers and 11 farm salmon suppliers were used to process cold-smoked salmon product.

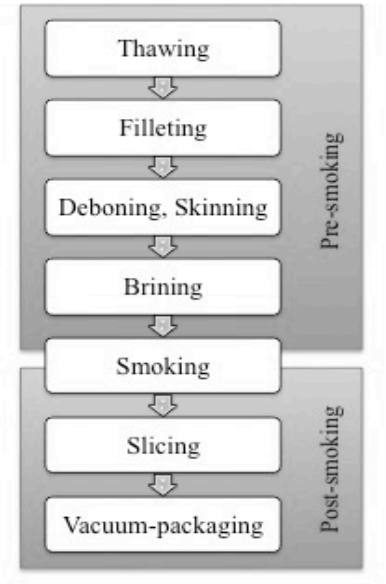


Figure 2.1 Cold-smoked salmon process flow diagram.

2.2.1.1 Control strategies in place at processing facility

A variety of *L. monocytogenes* control measures were in place at the facility through the duration of the study including (*i*) routine environmental and finished product sampling, (*ii*) comprehensive daily cleaning and sanitation, and (*iii*) detailed food safety systems and good manufacturing practices. Positive results from environmental and finished product sampling were immediately addressed through cleaning and sanitation and follow-up sampling. The

cleaning and sanitation regime utilized a variety of commonly used chemical sanitizers and ozonated water. All areas were cleaned and sanitized at the end of each workday. In addition, ozonated water was used to wash raw fish surfaces, and was run through skinning and deboning machines during production. Further, all surfaces were sprayed down with ozonated water at each break throughout the day. Food safety systems such as pre-requisite programs and Hazard Analysis and Critical Control Points (HACCP) and good manufacturing practices were followed and enforced.

2.2.2 Environmental sampling

Environmental sampling at the seafood processing facility described above was conducted over an 18-month period. A total of 120 locations in the facility were pre-selected to (*i*) span the whole process from raw to finished product areas; (*ii*) include both food contact surfaces (FCS) and non-food contact surfaces (NFCS) and (*iii*) include likely sites of *Listeria* spp. contamination, as determined through historical data supplied by the processor and a review of the literature (Health Canada, 2010; Kovacevic et al., 2012a; Tompkin, 2002). A list of sites sampled can be found in Appendix A. At each visit 30 sample sites were randomly selected from the pre-determined list, cycling through all sample sites every four visits. Fresh gull feces were also collected from the facility parking lot during each visit. Sampling between January 2013 and August 2013 occurred on a bi-weekly basis, with monthly sampling from September 2013 to June 2014. Samples were collected at least 5 h after the start of processing and before end-of-day cleaning and sanitation commenced following the Health Canada protocol for environmental sampling (MFLP-41; Health Canada, 2010). Post-operational swabbing was selected as a means of assessing the microbiological environment during production and because pre-operational

swabs were historically negative at this plant. Sterile cellulose sponges (Qualicum Scientific Ltd., Ottawa, ON; 3M Canada, London, ON), composite tissues, or stick swabs (3M Canada, London, ON), all of which contained 10 ml (sponges and composite tissues) or 1 ml (stick swabs) neutralizing buffer (Difco, BD, Sparks, MD), were used for sample collection. Samples were placed in sterile WhirlPak bags, and stored at 4°C until testing, no later than 24 h after collection.

2.2.3 Isolation and confirmation of *Listeria* spp.

Environmental samples were analyzed individually for presence of *Listeria* spp. following Health Canada standard procedures, with slight modifications. From January to August 2013 the culture-based MFHPB-30 protocol for isolation and confirmation of *Listeria* spp. from foods and environmental samples was followed (Health Canada, 2011b). Briefly, WhirlPak bags containing samples were combined with 90 ml (sponges and composite tissues) or 10 ml (swabs) *Listeria* Enrichment Broth UVM formulation (LEB; 3M Canada, London, ON) pre-warmed to 30°C, mixed thoroughly by hand agitation of the bag, and incubated at 30°C for 24 h. Enriched samples were then mixed and streaked onto Oxford (OXA; Oxoid, Fisher Scientific Canada, Ottawa, ON) and Palcam (PAL; Oxoid, Fisher Scientific Canada, Ottawa, ON) agars and incubated at 37°C for a minimum of 24 h. At the same time as plating, 0.1 ml LEB enrichment was transferred to 10 ml Modified Fraser Broth (MFB; Oxoid, Fisher Scientific Canada, Ottawa, ON) and incubated for 24 and 48 h at 37°C with shaking (180 rpm). All positive (darkened) secondary enrichments were plated on OXA and PAL agar and incubated as described above. Plates were checked at 24 h and 48 h for colonies with typical *Listeria* morphology. Hemolysis of presumptive positive colonies was assessed by stabbing 20 typical colonies from selective

agars to horse blood agar (HBA; Dalynn Biologicals, Calgary, AB) and incubating blood plates at 37°C for 24 h. Hemolysis was assessed by observing the presence of cleared zones around stabs. Three colonies representing both hemolytic negative and positive isolates, if available, were streaked onto tryptic soy agar (TSA; Acumedia, Neogen, Lansing, MI) with 5% sheep blood (Dalynn Biologicals, Calgary, AB) and incubated at 37°C for 24 h. After a check for purity, single colonies were confirmed as *Listeria* spp. by Gram staining and microscopy, motility, oxidase, and catalase tests. Species identification was accomplished using the Microgen Listeria ID biochemical test kit (MID-76, Microgen Bioproducts, Alere, Ottawa, ON). Confirmed in-house *L. monocytogenes* and *L. innocua* were used as positive controls.

Beginning in September 2013 the miniVIDAS system (Biomerieux, Saint-Laurant, QC) was used to screen samples following MFLP-77 for detection of *Listeria* spp. in foods and environmental samples using the VIDAS *Listeria* species Xpress (LSX) method (Health Canada, 2012a). The VIDAS assay is an automated enzyme-linked fluorescent immunoassay (ELFA) that uses antibodies specific to *Listeria* for qualitative detection of *Listeria* spp. WhirlPak bags containing samples were combined with 90 ml (sponges and composite tissues) or 10 ml (swabs) LX Broth (Biomerieux, Saint-Laurent, QC) pre-warmed to 30°C, mixed thoroughly by hand agitation of the bag, and incubated at 30°C for 24 h. After mixing again by hand agitation 0.5 ml of the enrichment was transferred to the sample well on the LSX test strip and heated at 97.5°C for 15 minutes using the Heat and Go device (Biomerieux, Saint-Laurent, QC). The strip was allowed to come to room temperature and then loaded into the miniVIDAS machine, along with the Solid Phase Receptacle (SPR), and the assay was run. *Listeria* spp. in samples testing positive were isolated and confirmed following the MFHPB-30 procedure, transferring the LX

broth enrichment to MFB and continuing the steps as described above. Brilliance agar (BRIL; Oxoid, Fisher Scientific Canada, Ottawa, ON) was added to the suite of selective media used previously. All confirmed *Listeria* spp. were frozen at -80°C in 20% glycerol tryptic soy broth (TSB).

In addition to this sampling, when available, *Listeria* spp. isolates recovered from samples taken during routine environmental monitoring (pre-operational and post-operational) and finished product testing (following MFLP-74) were collected from an accredited testing laboratory and frozen as described above.

2.2.4 Subtyping *L. monocytogenes*

One *L. monocytogenes* isolate from each positive sample was characterized by pulsed-field gel electrophoresis (PFGE) and ribotyping by the Canadian Listeriosis Reference Service. *L. monocytogenes* isolates were molecular fingerprinted by PFGE with restriction enzymes *Asc*I and *Apa*I according to the PulseNet standardized protocol and assigned pulsotypes after comparison to the PulseNet Canada database. *Eco*RI Ribotyping was performed using the RiboPrinter microbial characterization system (Qualicon Inc., Wilmington, DL), according to the manufacturer's manual.

2.2.5 Persistence classification

Persistence is often defined by repeated isolation of *L. monocytogenes* in the FPE that are subsequently identified as identical subtypes (as determined by phenotypic or genotypic methods) (Ferreira et al., 2014). For this project PFGE pulsotypes were compared in order to

determine if any isolates were clonal and possibly persisting in the processing environment over time. Although this method is commonly used in persistence research, comparison of PFGE or ribotype patterns over time is a qualitative and non-statistical approach (Ferreira et al., 2014). For these reasons, statistical tools described by Malley et al. (2013) were used to supplement persistence categorization. Specifically, a binomial test was used to compare the plant level frequency of potentially persistent ribotypes over the 18-month sampling period to a reference distribution derived from the publically available Food Microbe Tracker database (www.foodmicrobetracker.com), accessed on January 31, 2015. For comparison, two reference distributions from this database were selected that included all isolates derived from food environments (n=1,351) or isolates derived specifically from seafood processing environments (n=338). If the ribotype was not present in the reference distribution a probability of 1/(total number of isolates in reference distribution) was assigned, as described by Malley et al. (2013). Tests were performed in R (Version 3.1.1; R Foundation for Statistical Computing, http://www.r-project.org/). Using Bonferroni's correction, significant difference between ribotype frequencies was set at $p<0.025$ (0.05/2).

2.3 Results

2.3.1 Prevalence of *Listeria* in the processing area

A total of 2,959 samples were collected and analyzed between January 2, 2013 and June 4, 2014 (Table 2.1). The samples included 47 raw fish surface swabs, 2,345 processing facility swabs, 452 finished smoked salmon samples, and 115 swabs of the environment directly outside the plant (mostly gull feces). In total, 121 samples (4.1%) were positive for *Listeria* spp. In eight of the positive samples two different *Listeria* spp. were isolated. All other positive samples

contained a single *Listeria* spp. Five of the 17 *Listeria* spp. were recovered, although it must be mentioned that the typing method used (Microgen ID *Listeria*) is only capable of identifying six of these species (*L. innocua, L. ivanovii, L. grayi, L. monocytogenes, L. seeligeri,* and *L. welshimeri*); *L. grayi* was the only species absent from this list.

The highest prevalence of *Listeria* spp. was in gull feces collected in the parking lot outside the processing facility (24%; n=115) followed by the surface of raw salmon before entering the processing area (19%; n=47) (Figure 2.2). In the processing environment, 3.5% of samples (n=2,345) were positive for *Listeria* spp., with the majority of these positive samples (96%) recovered from the pre-smoking area. Recovery of *Listeria* spp. was rare in post-smoking areas (0.003%; n=921) and in finished product (0.7%, n=452) over the sampling period (Table 2.1; Figure 2.2).

Of 121 positive samples, *L. monocytogenes* and *L. welshimeri* made up 43 and 50 isolates, respectively. *L. monocytogenes* was found in every sampling category (Table 2.2), though filleting cutting boards (FCS) in the pre-smoking process area resulted in the most *L. monocytogenes* positive samples (56%; n=43). While *L. monocytogenes* was recovered from cold-smoked salmon finished product on three instances, contamination levels were below 25 CFU/g. *L. welshimeri*, on the other hand, predominated NFCS, primarily drains, in the pre-smoking process area (90%; n=50).

Table 2.1 Number and frequency of sampling areas positive for *Listeria* spp.

Category[a]		*Samples analyzed*	*Samples positive for:*					*Total positive samples*[b]
			L. innocua	*L. ivanovii*	*L. monocytogenes*	*L. seeligeri*	*L. welshimeri*	
Raw salmon		47	3 (6.4%)	0 (0.0%)	8 (17%)	0 (0.0%)	0 (0.0%)	9 (19%)
Pre-smoking	FCS	876	4 (0.5%)	0 (0.0%)	23 (2.6%)	0 (0.0%)	2 (0.2%)	29 (3.3%)
environment	NFCS	548	4 (0.7%)	1 (0.2%)	1 (0.2%)	1 (0.2%)	45 (8.2%)	50 (9.1%)
Post-smoking	FCS	553	1 (0.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.2%)	2 (0.4%)
environment	NFCS	368	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.3%)	1 (0.3%)
Finished product		452	0 (0.0%)	0 (0.0%)	3 (0.7%)	0 (0.0%)	0 (0.0%)	3 (0.7%)
Facility exterior		115	17 (14%)	0 (0.0%)	8 (7.0%)	5 (4.3%)	1 (0.9%)	27 (24%)
Total		2,959	29 (1.0%)	1 (0.03%)	43 (1.5%)	6 (0.2%)	50 (1.7%)	121 (4.1%)

[a] Food contact surface (FCS); Non-food contact surface (NFCS). [b] Two different *Listeria* spp. were identified in eight samples, a single species was identified in all other positive samples.

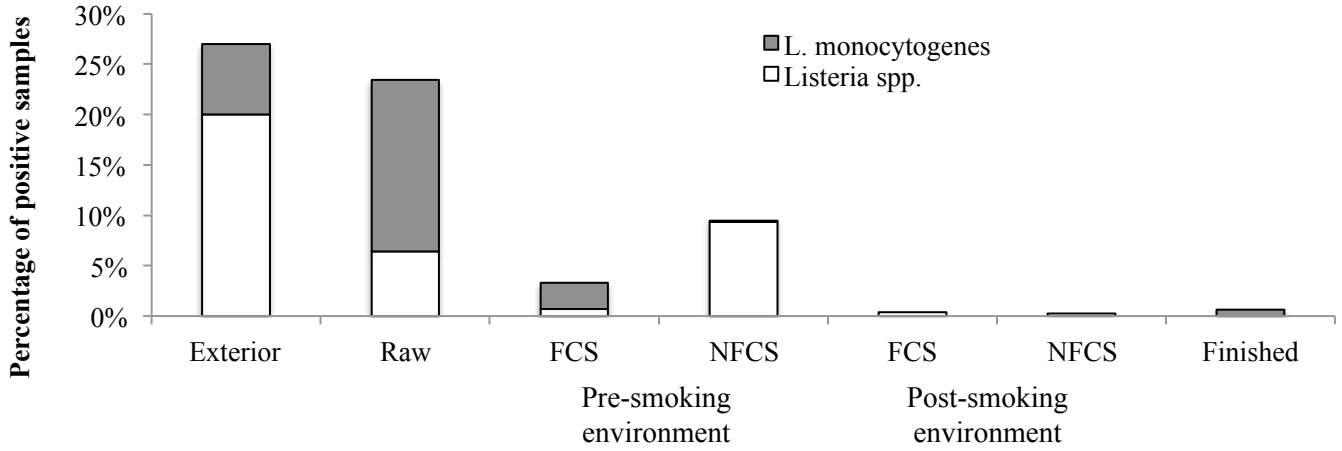


Figure 2.2 Prevalence of *Listeria* spp. contamination in different areas of the processing environment and facility exterior.
(Exterior: facility exterior; Raw: raw product; Finished: finished product; FCS: food contact surface; NFCS: non-food contact surface)

2.3.2 *L. monocytogenes* subtyping

L. monocytogenes from 37 of the 43 positive samples were obtained for PFGE and ribotyping along with two *L. monocytogenes* isolates that had been recovered from the same processing plant in 2012 as part of a previous study. Subtyping of isolates resulted in 15 ribotypes and 22 unique PFGE *Asc*I/*Apa*I pulsotypes (Table 2.2). Fourteen unique pulsotypes were found in the FPE and eight from gull feces. Two predominant ribotypes/pulsotypes were observed over the 18-month sampling period (Table 2.2).

2.3.3 Potential sources and transmission of *L. monocytogenes* in the processing facility

As PFGE subtyping was more discriminatory than ribotyping pulsotypes were used for source tracking. Comparison of pulsotypes showed a link between isolates recovered on the surface of raw salmon from Supplier A and the cutting board after processing fish from this supplier (Table 2.2). *L. monocytogenes* isolated from finished cold-smoked salmon produced with fish from Supplier A could also be linked to one of these pulsotypes (LMACI.0083/LMAAI.0086). This data suggests two possible sources of finished product contamination: raw materials (specifically from Supplier A) or strains introduced by raw materials that are persisting in the processing environment. *Listeria* spp. was rarely found beyond the pre-smoking area (Table 2.1). In fact, *L. monocytogenes* contamination was usually restricted to the cutting board in the pre-smoking area, the first step in the processing line.

Pulsotypes of isolates found in the processing plant (Table 2.2) did not match those recovered from the gull feces directly outside the processing plant (Table 2.3), implying that gull feces were not a source of *L. monocytogenes* contamination in the processing facility.

Table 2.2 Sample locations and instances of recovery for *Listeria monocytogenes* subtypes in the processing facility.

Pulsotype		Ribotype	Sampling area[b]	Date sample taken
***Asc*I pattern**	***Apa*I pattern**	**DUP Similarity[a]**		**(YYYY-MM-DD)**
LMACI.0774	LMAAI.1128	= DUP-20233	Unknown	2012[c]
LMACI.0364	LMAAI.0682	= DUP-19165	Unknown	2012[c]
LMACI.0775	LMAAI.1129	~ DUP-19187	Cutting board	2013-04-03
			Cutting board	2013-04-11
			Supplier A raw salmon surface	2013-04-11
			Supplier A raw salmon surface	2013-05-03
			Supplier A raw salmon surface	2013-05-03
			Cutting board	2013-05-03
			Cutting board	2013-05-09
			Cutting board	2013-05-31
			Supplier A raw salmon surface	2013-11-18
			Cutting board	2014-02-11
			Cutting board	2014-02-17
			Cutting board	2014-05-05
LMACI.0007	LMAAI.0014	= DUP-19186	Shipping area floor	2013-05-06
LMACI.0809	LMAAI.1180	= DUP-18615	Cutting board	2013-09-11
LMACI.0083	LMAAI.0086	~ DUP-1053	Supplier A raw salmon surface	2013-12-02
			Cutting board	2014-02-12
			Cutting board	2014-02-27
			Cutting board	2014-03-27
			Cutting board	2014-03-27
			Cutting board	2014-04-22
			Finished product (Supplier A raw salmon)	2014-04-25
LMACI.0148	LMAAI.1187	= DUP-19165	Supplier A raw salmon surface	2013-12-13
LMACI.0711	LMAAI.0693	= DUP-19169	Cutting board	2013-12-18
LMACI.0015	LMAAI.0024	= DUP-19169	Cutting board	2014-01-11
LMACI.0171/0063	LMAAI.0945	~ DUP-1044	Cutting board	2014-01-18
LMACI.0036	LMAAI.0671	= DUP-19165	Cutting board	2014-02-20
LMACI.0370	LMAAI.0048	= DUP-1038	Cutting board	2014-04-09
LMACI.0060	LMAAI.0857	= DUP-18611	Finished product (Supplier B raw salmon)	2014-04-11
LMACI.0211	LMAAI.1198	= DUP-18645	Cutting board	2014-04-21

[a] Ribotype patterns were identical (=) to ribogroup identified or within 85% similarity (~). [b] The cutting board is the first step in the pre-smoking process line. All positive cutting board samples were collected during cutting of salmon from Supplier A. [c] Isolated during routine sampling at facility before the inception of this project.

Table 2.3 Sample locations and instances of recovery for different *Listeria monocytogenes* subtypes in seagull feces.

Pulsotype		Ribotype	Sampling area	Date sample taken
***Asc*I pattern**	***Apa*I pattern**	**DUP Similarity[a]**		**(YYYY-MM-DD)**
LMACI.0688	LMAAI.0996	= DUP-1044	Gull feces collected in parking lot outside of the processing facility	2013-07-04
LMACI.0107	LMAAI.0915	~ DUP-1052		2013-08-06
LMACI.0041	LMAAI.0033	= DUP-19186		2013-08-06
LMACI.0059	LMAAI.0122/0005	= DUP-1030		2014-01-20
LMACI.0334	LMAAI.0197	= DUP-1035		2014-01-20
LMACI.0163	LMAAI.0140	= DUP-1038		2014-01-20
LMACI.0170	LMAAI.0512	= DUP-1039		2014-03-03
LMACI.0577	LMAAI.1197	= DUP-19165		2014-04-07

[a] Ribotype patterns were identical (=) to ribogroup identified or within 85% similarity (~).

Table 2.4 Binomial test comparison of plant-level ribotype frequencies to two reference distributions derived from Food Microbe Tracker database.

Ribotype[a]	Plant-level frequency[b]	Reference distribution: Food environment ribotypes		Reference distribution: Fish processing environment ribotypes	
		Ribotype frequency [binomial 99.5% CI]	**Significance (*P* value)[c]**	**Ribotype frequency [binomial 99.5% CI]**	**Significance (*P* value)[c]**
~ DUP-19187	0.350 (7/20)	7.40×10^{-4} (0/1351) [1.85×10^{-6}, 6.06×10^{-3}]	$< 2.20 \times 10^{-16}$*	2.96×10^{-3} (0/338) [7.41×10^{-6}, 2.40×10^{-2}]	1.49×10^{-13}*
~ DUP-1053 (DUP-1053E)	0.200 (4/20)	8.14×10^{-3} (11/1351) [2.91×10^{-3}, 1.77×10^{-2}]	1.92×10^{-5}*	2.96×10^{-3} (1/338) [7.41×10^{-6}, 2.40×10^{-2}]	3.57×10^{-7}*

[a] Ribotype patterns were within 85% similarity (~) of ribogroup identified. [b] Plant level frequency represents the number of samples positive for a given *L. monocytogenes* ribotype (excluding the first positive sample) divided by all the *L. monocytogenes*-positive samples isolated from the food processing environment (excluding the first positive sample) for the 18 month sampling period.[c] Statistical significance the frequency of the given ribotype among positive results exceeds the frequency of the ribotype in the given reference distribution. *Values were significant after Bonferroni's correction; $P = 0.025$ (0.05/2).

2.3.4 Persistence of *L. monocytogenes* in the processing environment

Table 2.2 demonstrates that over the 18-month sampling period two strains (as determined by PFGE subtyping) were routinely isolated from the processing environment whereas all other strains were only isolated once. LMACI.0775/LMAAI.1129 isolates were recovered 12 times over 13 months (April 3, 2013 to May 5, 2014) from the surface of raw salmon (four instances) and the cutting board in the pre-smoking process area (eight instances). LMACI.0083/LMAAI.0086 strains were recovered seven times over five months (December 2, 2013 to April 25, 2014) from the surface of raw salmon (one instance), the cutting board in the pre-smoking process area (five instances), and in finished product (one instance). Strain persistence has typically been defined as repeated isolation on different dates of *L. monocytogenes* that are subsequently identified as identical subtypes (Ferreira et al., 2014; Malley et al., 2013). Using this criterion, the data presented here suggests persistence of two strains in this seafood processing environment over the sampling period.

The binomial test described by Malley et al. (2013) compares the in-plant frequency of potentially persistent ribotypes to the frequency of the same ribotype from a reference distribution. In this test persistence is defined as the isolation of a given ribotype at a frequency greater than expected based on the reference distribution (Malley et al., 2013). DUP-19187 was not found in either of the reference distributions used and was assigned a probability of 1/(total isolates in reference distribution) for analysis. Many variants of DUP-1053 were found in each of the reference distributions used (DUP-1053A, DUP-1053B, DUP-1053C, DUP-1053E, and DUP-1053F in the food environment reference distribution; DUP-1053A, DUP-1053B, DUP-1053E in the seafood environment reference distribution). Comparison of the DUP-1053

RiboPrint pattern from this study to the DUP-1053 variants on the Food Microbe Tracker database showed that the pattern best resembled DUP-1053E, and accordingly this ribotype was used for binomial comparison in this study (Appendix B). The results of the binomial test showed that, after Bonferroni correction, both strains had a significantly greater frequency of in-plant isolation compared to reference distributions ($p < 0.025$; Table 2.4).

2.4 Discussion and conclusions

2.4.1 Prevalence of *Listeria* in and around the processing facility

Listeria spp. are abundant in the natural environment, including surface waters and other water bodies close to land and thus these microorganisms can contaminate the external surface of fish in these waters (Embarek, 1994; Jami et al., 2014). Prevalence of *Listeria* spp. on the surface of salmon varies between 0% and 30% depending on geographical area and processing facility (Jami et al., 2014). In a recent study, imported raw fish in the US yielded a prevalence of 4.8% *L. monocytogenes* (Wang et al., 2011). The findings of this study, however, were much higher. Similar to the results from Lappi et al. (2004), 19% of fish tested were positive for *Listeria* spp. Further, *L. monocytogenes* made up 73% of the *Listeria* spp. recovered from these samples, a ratio that is reflected by the findings of Gudmundsdóttir et al. (2005). Two scenarios could explain the slightly higher prevalence in raw fish seen in this study compared to others. Research has shown that higher prevalence of *Listeria* spp. contamination can occur in fish that are caught from bodies of water where land run-off occurs, as rivers and coastal areas have an increase in *Listeria* spp. prevalence (Embarek, 1994; Thomas et al., 2012). This may be the case in this study, as most raw salmon that tested positive for *Listeria* spp. came from a single supplier that catches salmon in fixed trap nets in marine waters along the shoreline, close to the mouth of a

major river. These fish are also headed and gutted before being frozen and shipped, a processing step that may contribute to increased contamination (Thomas et al., 2012). Targeted sampling of raw salmon from suppliers historically positive for *Listeria* spp. may have also elevated the prevalence findings stated in this study.

The recovery of *Listeria* spp. and *L. monocytogenes* in the seafood processing environment is common and continues to be a problem for this industry despite the implementation of control strategies at these facilities (Alali and Schaffner, 2013; Lappi et al., 2004; Malley et al., 2013). The prevalence of *Listeria* spp. varies widely from plant to plant (Corcoran et al., 2006; Di Ciccio et al., 2012; Gudmundsdóttir et al., 2005; Lappi et al., 2004; Malley et al., 2013). Environmental sampling in numerous seafood processing facilities has shown that each plant had a unique contamination profile. Some facilities have virtually no *L. monocytogenes* contamination while others have upwards of 60% environmental samples testing positive for *Listeria* spp. (including *L. monocytogenes*) (Gudmundsdóttir et al., 2005; Lappi et al., 2004). In BC, a survey of provincial fish processors found 32% of food processing environmental swabs (n=72) contained *Listeria* spp., with 56% of these being *L. monocytogenes* (Kovacevic et al., 2012a). Compared to the contamination levels discussed above, this study found a rather low (3.6%) overall prevalence of *Listeria* spp. contamination in the processing environment, with the majority of contamination seen in the pre-smoking processing area by *Listeria* spp. other than *L. monocytogenes*.

Within the processing environment contamination sites can vary (Di Ciccio et al., 2012; Gudmundsdóttir et al., 2005; Tompkin, 2002). The contamination profile in this plant was

distinct in that different species seemed to have environmental niches. *L. monocytogenes* was predominant in raw materials and on FCS whereas *L. welshimeri* was localized to NFCS, particularly drains, in the pre-smoking process areas (Table 2.1). Previous findings have determined that drains and floors usually contain the highest levels of *Listeria* spp. contamination and are particularly hard to maintain *Listeria*-free despite routine cleaning and sanitation (Gudmundsdóttir et al., 2005; Jami et al., 2014). This was the case in the plant sampled for this study as well wherein NFCS in the pre-smoking process areas including drains, floors, and personnel boot soles made up nearly all of the NFCS samples testing positive for *Listeria* spp.

L. monocytogenes was recovered on three instances (0.7%, n=115) from cold-smoked salmon finished product by an accredited laboratory during routine testing, all at levels below 100 CFU/g. As a Category 2A product this level of contamination is acceptable by Health Canada standards (Health Canada, 2011a). The frequency of contamination seen in this study is much lower than the average frequency reported by Kovacevic et al. (2012b). The overall frequency of *Listeria* spp. contamination in BC RTE products was previously found to be 10%, with contamination found exclusively in BC RTE fish products (Kovacevic et al., 2012b). Similar to this study, though, contamination levels in the finished products were below 100 CFU/g (Kovacevic et al., 2012b).

When comparing between prevalence data it is important to note that differences in sampling approaches will affect the study outcomes (Jami et al., 2014). Lappi et al. (2004) sampled up to 20 food processing environment sites monthly, with focus on high-risk areas, in four seafood

processing plants over two years. This study found 19.5% of samples contained *Listeria* spp. (including *L. monocytogenes*) (Lappi et al., 2004). In a study by Gudmundsdottir et al. (2005) high-risk areas were sampled, taking 15-20 pre-operational and post-operational swabs over a maximum of eight visits in four different plants. This approach yielded an overall prevalence of 10.4% *Listeria* spp. in the processing environment (73.1% of these samples being *L. monocytogenes*) across the four plants tested. The results presented in the present study were obtained through swabbing of over 120 sites, including high-risk and low-risk areas in a single plant, with samples collected more often and over a shorter period of time when compared to both the above mentioned studies. These differences may in part account for the lower overall prevalence seen in the processing environment at this facility (3.6%). Limitations in comparing prevalence data of *Listeria* spp. in raw fish and finished product samples should also be considered for reasons similar to what has been stated above (Jami et al., 2014; Kovacevic et al., 2012b).

Sampling and isolation methodologies may also affect recovery rates of *Listeria* spp. in the FPE. In this study, prevalence of *Listeria* spp. was determined using Health Canada-approved environmental sampling, isolation, and identification methodologies. Studies have shown that the cotton swab method of sampling is significantly less efficient in recovery of *Listeria* spp. when compared to sterile-sponges and composite tissue methods (Kovacevic et al., 2009; Vorst et al., 2004; Yan et al., 2007). Where possible, sponges and composite tissues were used in this study; cotton swabs were used to collect gull feces and to swab hard to reach areas on processing equipment. Although composite tissue preparation was a bit more labor-intensive compared to sponge swabs, the economic benefit and ease of use in the processing environment of this

method outweigh the additional time needed to prepare these sampling vessels. Neutralizing buffer was used to wet the swabs and was necessary to neutralize sanitizers present in the processing environment during sampling.

This study required a method that would detect and allow for isolation of *Listeria* spp. from environmental swabs and smoked salmon. Two methods were selected for detection from the *Compendium of Analytical Methods* (referred to henceforth as simply *Compendium*), the two-step enrichment culture based method (MFHPB-30) and the VIDAS LSX ELFA method (MFLP-77). Isolation and confirmation of *Listeria* spp. from all samples followed the guidelines outlined in MFHPB-30. Gasanov et al. (2005) recommend that when selecting a method it is important to consider the degree of validation the method has undergone. In the *Compendium*, procedures are categorized by their degree of validation. Laboratory procedures (designated by an MFLP-code) are those that have not been validated by an inter-laboratory study whereas an HPB method (MFHPB-code) has been fully validated through a comparative or collaborative study. The *Compendium* does, however, state that despite not being validated to the same extent as HPB methods, laboratory practices can have greater analytical sensitivity and specificity according to available data from international agencies (Health Canada, 2008).

Both methods selected in this study are widely used in government and industry for detection of *Listeria* spp. in food and environmental samples as they are simple, inexpensive, and allow for high sample throughput (Gasanov et al., 2005). The two-step enrichment method has been called the "gold-standard" for detection and isolation of *Listeria* spp. (Graves and Swaminathan, 2001). The principle of this method is focused on inhibition of the indigenous bacterial flora by taking

advantage of the resistance of *L. monocytogenes* to various selective agents (Donnelly and Nyachuba, 2007; Zunabovic et al., 2011). In this study two selective enrichment broths (LEB and MFB) and either two or three selective agars (OXA, PAL, and BRIL) were used to isolate *Listeria* spp. Acriflavin, lithium chloride, nalidaxic acid, polymyxin B, and/or ceftazidime are added to these media to inhibit competitive microorganisms (Donnelly and Nyachuba, 2007). MFB, OXA, and PAL also contain esculin which can be hydrolyzed by all *Listeria* spp. into 6,7-dihydroxycoumarin, a molecule that complexes with ferric ions present in media resulting in blackening of the broth or colonies and surrounding agar (Fraser and Sperber, 1988). Chromogenic BRIL agar, a modification of *Listeria* agar according to Ottaviani and Agosti (ALOA), was added halfway through the study. This selective and differential agar has been shown to reduce false negatives and allows an enhanced detection of *L. monocytogenes* (Vlaemynck et al., 2000). A proprietary chromogenic substrate X-glucoside is cleaved by *Listeria* spp. enzyme β-glucosidase resulting in blue *Listeria* colonies. The addition of lecithin allows for differentiation between *Listeria* spp. based on the presence of phosphotidylcholine phospholipase C (PC-PLC), an enzyme associated with *Listeria* virulence. The lecithin is cleaved by PC-PLC resulting in an opaque white halo around potentially pathogenic *Listeria* spp.

Although the culture-based method described above is well established and widely accepted, this method is time-consuming, taking between five and seven days to arrive at a result. Additionally, the need for selective agents in the media used for this method may be harmful on stressed or injured cells or selective for certain *Listeria* spp., such as *L. innocua* (Zunabovic et al., 2011). For these reasons the use of these reagents may result in decreased ability to detect stressed cells or decreased ability to detect certain species of *Listeria* in mixed samples (Gasanov et al., 2005;

Zunabovic et al., 2011). Despite using agents to select for *Listeria* spp., other organisms such as *Enterococcus* and *Bacillus* spp. may grow on the agar and can also utilize esculin resulting in colonies that look similar to *Listeria* spp. making it difficult to distinguish between *Listeria* spp. and non-*Listeria* spp. colonies (Gasanov et al., 2005).

As many food processors require positive test results in a more timely fashion, numerous tests have been developed for more rapid detection of *Listeria* spp. (Zunabovic et al., 2011). This study employed the VIDAS LSX ELFA method, which uses an antibody specific to *Listeria* spp. antigens to detect the presence of these bacteria in an enriched sample. This method takes approximately 28 h from sample enrichment to a positive or negative result. However, as this project required isolation and speciation of the *Listeria* spp. in the sample, isolation and identification of the bacteria was still necessary. However, because the VIDAS method was used to screen for positive samples and only positive samples were carried through the isolation steps, this method allowed for decrease in overall number of samples processed using MFHPB-30. Although the addition of fluorescence labels to antibodies increases sensitivity of this assay compared to enzyme-linked immunosorbent assay methods, ELFA has been found to be less sensitive compared to the culture-based method (Gasanov et al., 2005; Zunabovic et al., 2011). For this reason there is a higher likelihood of false negatives with this assay.

2.4.2 Listeria contamination patterns

Some have suggested that *Listeria* spp. can be used as an indicator for *L. monocytogenes* contamination in the FPE (Tompkin, 2002; Williams et al., 2011). Tompkin (2002) has recommended that all samples testing positive for *Listeria* spp., regardless of whether or not they

are confirmed *L. monocytogenes*, should be treated like *L. monocytogenes* in order to be proactive in control of *L. monocytogenes* in the processing environment. While this approach may be a practical and reliable for control in some plants, this may be difficult for plants with a high number of non-*L. monocytogenes* positive samples (Williams et al., 2011). Further, the presence of *L. monocytogenes* only correlates well to presence of *Listeria* spp. in some, but not all, plants (Williams et al., 2011). Alali and Schaffner (2013) performed a systematic literature review to see if the practice of using *Listeria* spp. as an indicator for *L. monocytogenes* contamination holds true in seafood processing environments. Their study found that the presence of *Listeria* spp. was not a good indicator of *L. monocytogenes* on FCS, but were good indicators of contamination by this pathogen on NFCS (Alali and Schaffner, 2013).

The results of this study are in conflict with the findings by Alali and Schaffner (2013) in that *Listeria* spp. may be a good indicator of *L. monocytogenes* on FCS, whereas this may not hold true for NFCS at this establishment. In the pre-smoking processing area, *L. monocytogenes* was found in 79% (23/29) of the positive FCS samples, but was only found in 2% (1/29) of the NFCS positive samples. *L. welshimeri,* on the other hand, was found in 90% (45/50) of the NFCS positive samples in the pre-process area. These results suggest that a sample positive for *Listeria* spp. recovered from the FCS in this area may be a good indicator of *L. monocytogenes* contamination, whereas a positive result from a pre-smoking area drain may indicate *L. welshimeri* contamination. On the surface of raw fish, 89% (8/9) of *Listeria* spp. positive samples were *L. monocytogenes*, with two of these samples having both *L. monocytogenes* and *L. innocua*. These results suggest that the presence of *Listeria* spp. on the surface of raw fish at this establishment would also be a good indicator of raw material *L. monocytogenes* contamination.

Although *L. monocytogenes* may not dominate all areas of the processing environment, the presence of *Listeria* spp. is still an important finding. For example, a finding that *L. welshimeri* is routinely present in the pre-smoking process area is cause for concern as this is an indication that conditions are favorable for the survival and potential growth of *L. monocytogenes* (Little et al., 2009; Ryser, 2007). Therefore, the recommendation by Tompkin (2002) to treat all *Listeria* spp. as a potential threat holds merit regardless of whether *Listeria* spp. can be correlated with *L. monocytogenes* contamination. Presence of *Listeria* spp. other than *L. monocytogenes* is an indication of a sanitation failure which should be addressed to prevent more serious problems in the future (Williams et al., 2011).

2.4.3 L. monocytogenes subtyping

Two molecular subtyping methods, ribotyping and PFGE, were used to analyze the diversity of the *L. monocytogenes* population in and around the seafood processing facility in this study. Both these methods have been demonstrated as effective tools in epidemiological investigations and in studies aimed at tracking the source of contamination and persistence of strains in FPEs (Corcoran et al., 2006; Dauphin et al., 2001; Graves et al., 2007; Gudmundsdóttir et al., 2005; Ho et al., 2007; Jadhav et al., 2012; Kabuki et al., 2004). Although ribotyping is reproducible, and relatively easy due to availability of automated tools, ribotyping is not as discriminatory as PFGE, especially for serotype 4b isolates (Corcoran et al., 2006; Graves et al., 2007). For this reason Corcoran et al. (2006) recommend the combination of PFGE and ribotyping for source tracking. In this study PFGE was more discriminatory than ribotyping in that the 39 subtyped isolates resulted in 22 unique pulsotypes while only 15 ribotypes were identified. That being said, both methods allowed for source tracking and contamination patterns to be observed in and

around the processing facility. However, if taken alone, the ribotype data suggest a link between strains over time in the processing plant and between gull feces and the shipping and receiving area. This link is not evident when looking at the pulsotype data. For this reason, the pulsotype data was used to differentiate between strains in this study.

2.4.4 Potential sources and transmission of *L. monocytogenes*

In seafood processing it is well established that contamination of finished product can come from one of two sources: (*i*) *L. monocytogenes* present in raw materials or (*ii*) cross-contamination from the processing facility, although pinpointing the precise source has proven difficult for many (Alali and Schaffner, 2013; Autio et al., 1999; Little et al., 2009; Thomas et al., 2012; Tompkin, 2002). Gudmudsdottir et al. (2005) found that the primary source of finished product contamination in the plants sampled to be raw materials, whereas others suggest that the processing environment is more often linked to finished product contamination than raw materials (Autio et al., 1999; Di Ciccio et al., 2012; Holch et al., 2013b). Indeed, few studies have been able to link strains from raw materials to finished product contamination, implying that the environment may play a larger role in end-product *L. monocytogenes* contamination (Jami et al., 2014).

Observations from this study pointed to raw materials as the primary source of environmental and end-product contamination. One strain was isolated from Supplier A raw materials, the filleting cutting board, and finished product. A different strain, also isolated from Supplier A raw materials, could be linked to cutting board contamination. In addition, over the study duration, cutting boards were only contaminated after processing of fish from this Supplier A.

While swabs were taken more often after Supplier A fish were processed to assess this recurring problem, cutting board samples were also taken after processing of fish from other suppliers over this time. Taken together, these findings point to raw materials as the primary source of *L. monocytogenes* in this processing environment and in finished products. That being said, not all contamination could be linked to raw materials over the study duration, suggesting that there may be other sources of contamination in this facility.

In terms of the transmission of *Listeria* spp. in the processing environment, *Listeria* spp. was rarely found beyond the pre-smoking process area (Figure 2.2). In fact, *Listeria* spp. contamination was usually contained to the filleting cutting board, the first step in the processing line. This finding suggests that the control strategies in place at this facility (primarily an ozonated water wash of the raw fish before filleting and frequent rinsing of the cutting board with ozonated water) are reasonably effective at eliminating *Listeria* spp. present on raw fish and FCS before it can be transferred further down the processing line. If *L. monocytogenes* is not eliminated from in-process salmon, however, the data from this study suggests that the bacterium may be able to survive the remainder of the process. *L. monocytogenes* was able to contaminate finished cold-smoked salmon in three instances, despite being absent from the post-smoking environment. This indicates that, rather than contamination occurring from *L. monocytogenes* present in the post-smoking environment, *L. monocytogenes* in in-process salmon can survive the cold-smoking process. This is in agreement with previous reports that show the cold-smoked fish process lacks a kill-step capable of eliminating this pathogen from the product (Beaufort et al., 2007).

This study also explored the hypothesis that the exterior may be a potential source of contamination for the processing plant, specifically gulls outside the processing plant that are routinely on the premise. Gulls have been shown to be reservoirs of *Listeria* spp. contamination (Fenlon, 1985). Comparison of the strains recovered from the plant to strains recovered from the gull feces showed that although gull feces outside the processing facility contain *L. monocytogenes* these strains were not the source of contamination in the processing facility.

2.4.5 Persistence of *L. monocytogenes* in the processing environment

Almost every seafood processing facility has an in-house, or persistent, *L. monocytogenes* subtype that can be isolated from the FPE over time (Jami et al., 2014). However, classification of isolates into sporadic and persistent categories has proven challenging (Ferreira et al., 2014). The typical definition of *L. monocytogenes* persistence in the FPE is isolation of identical subtypes over time (Carpentier and Cerf, 2011; Ferreira et al., 2014; Holch et al., 2013b). A wide variety of *L. monocytogenes* strains were recovered from the seafood processing environment sampled over the 18-month study period. Two strains were routinely isolated over this time, with one strain recovered from raw materials, cutting boards and finished product over five months and another strain recovered from raw materials and cutting boards over 13 months (Table 2.2). Using the definition of persistence stated above, both these strains could be classified as persisting in this processing environment.

While the above definition of persistence is widely used, this non-standardized, qualitative approach to persistence classification has been criticized (Carpentier and Cerf, 2011; Ferreira et al., 2014). In response to these concerns, statistical approaches to classify these strains have been

proposed (Malley et al., 2013). The present study used a binomial test described by Malley et al. (2013) to compare the plant-level frequency of the two potentially persistent ribotypes over the 18-month sampling period to two reference distributions derived from the publically available Food Microbe Tracker database. The results of these statistics, where persistence is defined as the isolation of a given ribotype at a frequency greater than expected based on the reference distribution, suggested that both strains had a significantly greater frequency of in-plant isolation compared to reference distributions.

Although this statistical tool is a step in the right direction for persistence classification, the findings of significant persistence must be used with discretion. Selection of a reference distribution is important, as the frequency within these distributions will affect the outcome of the test. The reference distributions used in this study aimed to reflect the strain variation in FPEs specifically, as research has shown that FPE *L. monocytogenes* may differ from *L. monocytogenes* isolated from clinical and animal-derived samples (Orsi et al., 2011). That being said, because the Food Microbe Tracker database is made up primarily of US *L. monocytogenes* isolates this database may not reflect the frequency of ribotypes found in Canada. Findings from Knabel et al. (2012) have demonstrated that although the population of *L. monocytogenes* is diverse, a predominant 1/2a strain unique to Canada has been causing the majority of human illness in Canada for the last two decades, opposite to findings in the US that show 4b strains to be implicated in most listeriosis cases (Orsi et al., 2011; Ragon et al., 2008).

Further, what neither the qualitative or quantitative methods for characterization of persistent strains accounted for is potential reintroduction of strains into the processing facility. In the

present study, both recurring strains were recovered on the surface of raw fish from a specific fish supplier (Supplier A) and only recovered from the processing environment after fish from this supplier was used in production. Further, *L. monocytogenes* from these positive sites seemed to be eliminated after sanitation was performed since pre-operational swab results of the cutting board were always negative. These findings show the importance of raw material testing performed in parallel with environmental monitoring to assess if reintroduction is a potential cause of persisting subtypes.

While subtyping and statistical comparison of ribotype frequency point towards persistence of two strains in this processing facility, the presence of these strains on raw materials and only on FCS after processing these fish suggests reintroduction of *L. monocytogenes* by raw materials. To reconcile this disagreement, the term "recurrent" must be used with these strains to acknowledge that while these strains may be persisting based on the classical definition of persistence reintroduction cannot be ruled out. Nonetheless, the finding of two recurring strains is important, as these strains may have a higher probability of contaminating finished products and causing risk to public health.

2.4.6 Conclusions

Environmental and finished product sampling combined with isolation and identification of *Listeria* spp. allowed evaluation of the *Listeria* population and contamination trends in a single RTE seafood processing facility in BC. The data derived from this study showed (*i*) the overall prevalence of *Listeria* spp. to be 19% on raw salmon surfaces, 3.6% in the processing environment, and 0.7% in finished product with *L. monocytogenes* being present in all

categories; (*ii*) two potentially persistent *L. monocytogenes* strains isolated from processing area FCS over several months; and (*iii*) raw materials as the major source of *L. monocytogenes* contamination in the processing environment. The data further illustrates the challenges that RTE food processors face in regards to *L. monocytogenes* control at the plant-level as these bacteria are continuously introduced into the processing facility via raw materials. Although subtyping allowed for identification of two potentially persistent strains in the processing environment and binomial test statistics supported that these strains are likely persisting in the processing plant, other observations suggest reintroduction of *L. monocytogenes* on raw materials. Collectively, the findings of this study demonstrated that the control strategies in place at this facility appear to be effective at minimizing occurrence, transmission, and persistence of this pathogen.

Chapter 3: Certain *Listeria monocytogenes* from a British Columbia ready-to-eat seafood processing environment possess attributes associated with enhanced survival and growth in the food processing environment and food

3.1 Introduction

A specific subtype of *Listeria monocytogenes* isolated in the processing environment repeatedly over time is often referred to as persisting in that environment. In contrast, subtypes only isolated once over the same time are referred to as sporadically occurring, non-persistent, or transient strains (Carpentier and Cerf, 2011; Ferreira et al., 2014; Holch et al., 2013b). Numerous studies have identified persistent subtypes of *L. monocytogenes* in ready-to-eat (RTE) food processing environments (FPE) despite control measures in place aimed at eliminating this pathogen in these establishments (Holah et al., 2004; Holch et al., 2013b; Lappi et al., 2004; Leong et al., 2014). Separately, research has found that some strains of *L. monocytogenes* possess enhanced ability to adhere to abiotic surfaces and form biofilms (Adrião et al., 2008; Djordjevic et al., 2002; Harvey et al., 2007; Kalmokoff et al., 2001), resist sanitizers commonly used in the FPE (Dutta et al., 2013; Mereghetti et al., 2000; Mullapudi et al., 2008; Müller et al., 2013; Soumet et al., 2005), and adapt to and grow in the presence of environmental stressors associated with these environments (Bergholz et al., 2010; Kovacevic et al., 2013a; Lianou et al., 2006). These differences have lead investigators to hypothesize that strains possessing one or more of these attributes may be better able to persist in the FPE (Ferreira et al., 2014; Holch et al., 2013b).

Several attempts to support the above hypothesis can be found in the literature (reviewed by Ferreira et al., 2014). Studies have linked *L. monocytogenes* persistence to higher adherence phenotypes on abiotic surfaces (Borucki et al., 2003; Ochiai et al., 2014; Wang et al., 2015), decreased susceptibility to quaternary ammonium compound (QAC) sanitizers (Aase et al.,

2000), and enhanced stress response (Lundén et al., 2008). However, others have found no association between persistence and each of these characteristics, (Djordjevic et al., 2002; Heir et al., 2004; Lundén et al., 2003; Ortiz et al., 2014b; Porsby et al., 2008) and therefore scientific consensus regarding this phenomenon is still lacking.

A previous study (outlined in Chapter 2) found *L. monocytogenes* in the FPE of a RTE seafood processor in British Columbia (BC) including strains recovered repeatedly over time, suggesting persistence. In addition, previous work in BC showed that *L. monocytogenes* is more often isolated from fish plants compared to meat and dairy processing facilities (Kovacevic et al., 2012a). Taken together, these data may suggest that certain strains possess characteristics that enhance their survival in seafood processing environments. While many have found persistent strains in seafood processing plants (Dauphin et al., 2001; Holch et al., 2013b; Mędrala et al., 2003), few have characterized these isolates for traits that may be associated with persistence. The objective of this study was to use genetic and phenotypic means to assess isolates from a BC seafood processing facility for their ability to adhere to abiotic surfaces, resist sanitizers commonly used in the FPE, and adapt to and grow under cold temperature and salt stress. Additionally, this research aimed to evaluate if differences in these characteristics could be associated with repeated isolation of a specific subtype in the processing environment.

3.2 Materials and methods

3.2.1 Isolates and subset selection

A subset of strains recovered from a RTE seafood processing facility in BC (described in section 2.2.1) was selected for genetic and phenotypic characterization. The strains were chosen to

represent (*i*) all unique subtypes isolated from the processing environment during an 18-month sampling period and (*ii*) isolates with the same subtype from different sources and/or dates (as available). In total, 28 isolates were selected for characterization (Table 3.2). This subset represents all 20 subtypes, previously determined by pulsed-field gel electrophoresis (PFGE) and ribotyping (see section 2.2.4), recovered over an 18-month sampling period and two subtypes recovered in 2012 from the same facility as part of the plant's routine sampling. These isolates were recovered from raw salmon surfaces (n=4), the process area cutting board (n=11), finished product (n=2), shipping and receiving floor (n=1), and gull feces in the parking lot outside the processing plant (n=8). Specific source data for the two isolates from 2012 was not available, however these strains were isolated inside the processing plant. Isolates were sporadically (n=20) recovered from the interior and exterior of the facility as well as recurring (n=8) in the processing environment. All isolates were stored at -80°C in 20% glycerol tryptic soy broth (TSB).

3.2.1.1 Template DNA preparation

Template DNA used for genetic analysis was obtained for each isolate with the PureLink genomic DNA extraction kit (Invitrogen, Burlington, ON) following the manufacturer's protocol for Gram positive bacterial cells, with slight modifications to increase efficiency of extraction for *L. monocytogenes*. Briefly, isolates were grown in 10 ml brain heart infusion (BHI) broth with shaking (180 rpm) for 16-18 h and 1 ml of the culture was transferred to a centrifuge tube and harvested (10,000 × g, 10 min). For the binding, washing, and elution steps all centrifuge times were doubled. In the elution step, 50 µl elution buffer was added to the spin column and incubated at 37°C for 5 min before being centrifuged at room temperature. DNA template

concentration and purity was assessed with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and extracts were stored at -20°C.

3.2.2 Lineage typing

Lineage typing was completed using the allele specific oligonucleotide multiplex polymerase chain reaction (PCR) described by Ward et al. (2004). Twenty-five microliter reactions contained 1X AmpliTaq buffer, 200 μM of each dNTP, 0.5 μM of each primer (see Table 3.1), 2 mM $MgCl_2$, 0.75 U AmpliTaq 360 polymerase (Applied Biosystems, Foster City, CA), and 100 ng template DNA. Thermocycling parameters were as follows: 94°C for 10 min; 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec; followed by 72°C for 7 min. PCR products were visualized on a 1.5% agarose gel stained with SyberSafe (Invitrogen, Burlington, ON) run at 90 V for 1 h alongside a 100-bp ladder and samples of known lineages as controls. PCR product sizes were compared to the expected results and isolates were assigned to lineage based on these results.

Table 3.1 Lineage typing primers and PCR product sizes.

Lineage	Primer	Primer sequences (5'-3')	PCR product size (bp)
1	*actA*1-f	AATAACAACAGTGAACAAAGC	373
	*actA*1-r	TATCACGTACCCATTTACC	
2	*plcB*2-f	TTGTGATGAATACTTACAAAC	564
	*plcB*2-r	TTTGCTACCATGTCTTCC	
3	*actA*3-r	CGGCGAACCATACAACAT	277
	*plcB*3-r	TGTGGTAATTTGCTGTCG	

Table 3.2 Summary of *Listeria monocytogenes* isolates selected for subset used in characterization.

Isolate	Pulsotype		Ribotype DUP Similarity[a]	Lineage	Recurring (R) Sporadic (S)[b]	Date sample taken (YYYY-MM-DD)	Source[d]
	*Asc*I pattern	*Apa*I pattern					
Lm 362	LMACI.0015	LMAAI.0024	= DUP-19169	II	S	2014-01-11	Cutting board
Lm 371	LMACI.0036	LMAAI.0671	= DUP-19165	II	S	2014-02-20	Cutting board
Lm 372	LMACI.0083	LMAAI.0086	~ DUP-1053	II	R	2014-02-27	Cutting board
Lm 363	LMACI.0171/0063	LMAAI.0945	~ DUP-1044	I	S	2014-01-18	Cutting board
Lm 377	LMACI.0211	LMAAI.1198	= DUP-18645	II	S	2014-04-21	Cutting board
Lm 376	LMACI.0370	LMAAI.0048	= DUP-1038	I	S	2014-04-09	Cutting board
Lm 361	LMACI.0711	LMAAI.0693	= DUP-19169	II	S	2013-12-18	Cutting board
Lm 193	LMACI.0775	LMAAI.1129	~ DUP-19187	I	R	2013-04-03	Cutting board
Lm 368	LMACI.0775	LMAAI.1129	~ DUP-19187	I	R	2014-02-17	Cutting board
Lm 381	LMACI.0775	LMAAI.1129	~ DUP-19187	I	R	2014-05-05	Cutting board
Lm 209	LMACI.0809	LMAAI.1180	= DUP-18615	II	S	2013-09-11	Cutting board
Lm 379	LMACI.0060	LMAAI.0857	= DUP-18611	I	S	2014-04-11	Finished product
Lm 380	LMACI.0083	LMAAI.0086	~ DUP-1053	II	R	2014-04-25	Finished product
Lm 208	LMACI.0041	LMAAI.0033	= DUP-19186	II	S	2013-08-06	Gull feces
Lm 364	LMACI.0059	LMAAI.0122/LMAAI.0005	= DUP-1030	II	S	2014-01-20	Gull feces
Lm 207	LMACI.0107	LMAAI.0915	~ DUP-1052	I	S	2013-08-06	Gull feces
Lm 366	LMACI.0163	LMAAI.0140	= DUP-1038	I	S	2014-01-20	Gull feces
Lm 367	LMACI.0170	LMAAI.0512	= DUP-1039	II	S	2014-03-03	Gull feces
Lm 365	LMACI.0334	LMAAI.0197	= DUP-1035	II	S	2014-01-20	Gull feces
Lm 375	LMACI.0577	LMAAI.1197	= DUP-19165	II	S	2014-04-07	Gull feces
Lm 205	LMACI.0688	LMAAI.0996	= DUP-1044	I	S	2013-07-04	Gull feces
Lm 359	LMACI.0083	LMAAI.0086	~ DUP-1053	II	R	2013-12-02	Raw salmon surface
Lm 360	LMACI.0148	LMAAI.1187	= DUP-19165	II	S	2013-12-13	Raw salmon surface
Lm 200	LMACI.0775	LMAAI.1129	~ DUP-19187	I	R	2013-05-03	Raw salmon surface
Lm 356	LMACI.0775	LMAAI.1129	~ DUP-19187	I	R	2013-11-18	Raw salmon surface
Lm 196	LMACI.0007	LMAAI.0014	= DUP-19186	II	S	2013-05-06	Shipping area floor
Lm 116	LMACI.0364	LMAAI.0682	= DUP-19165	II	S	2012[c]	Unknown
Lm 115	LMACI.0774	LMAAI.1128	= DUP-20233	II	S	2012[c]	Unknown

[a] Ribotype patterns were identical (=) to ribogroup identified or within 85% similarity (~). [b] Recurring isolates belong to subtype repeatedly isolated from processing environment over ≥ 5 months, sporadic strains were recovered once over 18 months. [c] Isolated during routine sampling at facility before the inception of this project. [d] The cutting board is the first step in the pre-smoking process line. All positive cutting board samples were collected during cutting of salmon from Supplier A.

3.2.3 Surface adherence

The ability of each isolate to adhere to abiotic surfaces was assessed using a microtiter plate assay adapted from Harvey et al. (2007). A single *L. monocytogenes* colony grown on tryptic soy agar (TSA) at 37°C for 24 h was inoculated into 5 ml of tryptic soy broth (TSB) and incubated for 18 h at 30°C with shaking (180 rpm). Cultures were transferred (20 µl) to 5 ml of TSB and incubated statically for 18 h at 20°C and then transferred (125 µl) to 5 ml of TSB. Prepared cultures were vortexed for 1 min and 100 µl volumes were transferred into six random wells of a sterile polystyrene (Costar 3370, Fisher Scientific Ltd., Ottawa, ON) or polyvinyl chloride (PVC; Costar 2595, Fisher Scientific Ltd., Ottawa, ON) microtiter plate. Peripheral wells were filled with sterile water to limit edge effects. Each plate included 12 blank wells comprised of 100 µl un-inoculated TSB. Plates were covered with a tightly fitting lid, sealed with parafilm, and incubated statically at 20°C for 48 h. After the incubation period, supernatants were removed from the microtiter plate and optical density was measured at 600 nm (OD_{600}; Spectramax, V6.3; Molecular Devices, Sunnyvale, CA). Following removal of supernatants, microtiter plate wells were washed three times with 150 µl of sterile water using a microplate strip washer (ELx50; BioTek Instruments, Inc., Winooski, VT) to remove loosely associated bacteria, and then dried at 42°C for 45 min. An aqueous 1% crystal violet (CV) solution (150 µl; Fisher Scientific Ltd., Ottawa, ON) was added to each well and incubated at 20°C for 45 min. The CV solution was removed, wells were washed three times with sterile water (150 µl) using the microplate strip washer and air-dried at 42°C for 30 min. Ethanol (95%) was added (100 µl) to each well to solubilize the dye and plates were agitated for 5 min at 150 rpm on a plate shaker; the concentration of CV was determined by measuring OD_{600} (CV-OD_{600}; Spectramax, V6.3; Molecular Devices, Sunnyvale, CA). To correct for background absorbance, the mean CV-OD_{600}

value obtained for the blank wells included in each microtiter plate was subtracted from the mean CV-OD_{600} value obtained for each isolate included in that plate. Three biological replicates were performed for each *L. monocytogenes* isolate on three different days. *L. monocytogenes* HPB399 and HPB642 (kindly provided by F. Pagotto, Health Canada), previously found to be high and low adhering strains, respectively, by Kalmokoff et al. (2001) were used as control strains. Wild-type 10403S and 10403S flagellum-minus deletion mutant (10403SΔ*flaA*; kindly provided by K. Nightingale, Texas Tech University) were also used as controls for each biological replicate as Lemon et al. (2007) previously found that 10403SΔ*flaA* had significantly lower surface adherence compared to the wild-type strain on polystyrene and PVC using the CV assay.

3.2.4 Sanitizer resistance

3.2.4.1 Sanitizer minimum inhibitory concentrations

An agar-based minimum inhibitory concentration (MIC) method adapted from Elhanafi et al. (2010) was used to determine resistance of isolates to the QACs (*i*) benzalkonium chloride, a mixture of N-alkyl (~60% C12, ~40% C14) dimethyl benzyl ammonium chloride compounds, (BAC; Sigma-Aldrich, Oakville, ON) and (*ii*) E-SAN, a quaternary ammonium based sanitizer composed of a 1:1 mixture of N-alkyl dimethyl benzyl ammonium chloride and N-alkyl dimethyl ethyl benzyl ammonium chloride (Epsilon Chemicals Ltd., Edmonton, AB). Briefly, frozen cultures were streaked onto 5% defribrinated sheep blood TSA plates and incubated at 37°C for 24 h. Two or three colonies from the plate were suspended in 200 µl Mueller-Hinton broth, vortexed for 5 sec, and 5 µl of the inoculum was spotted onto Mueller-Hinton agar containing 2% defribinated sheep blood and the desired concentration(s) of sanitizer (0, 2, 5, 10, 15, 20, 25

μg/ml). Plates were incubated at 37°C for 48 h and the presence or absence of confluent bacterial growth was noted. MIC was defined as the lowest concentration of sanitizer that prevented confluent growth on the agar. For each isolate, three biological replicates and two technical replicates were performed. *L. monocytogenes* CDL69 was used as a QAC resistant control (Müller et al., 2013). Resistance was assigned to isolates with MIC values at least two times that of the most susceptible strains.

3.2.4.2 Screening for benzalkonium chloride resistance determinants

BcrABC, a three gene-cassette, *qacH,* found on transposon Tn*6188,* and *emrE*, a gene found on the *Listeria* genomic island 1, all encoding small multidrug resistance proteins involved in efflux of toxic compounds, have all been shown to be associated with resistance to BAC (Elhanafi et al., 2010; Kovacevic, 2014 [unpublished]; Müller et al., 2013). Isolates that were resistant to BAC and/or E-SAN were screened for these genes using conventional PCR and agarose gel electrophoresis, with reaction mixture concentrations and thermocycling parameters summarized below (Table 3.3). All reactions were carried out at 25 μl with AmpliTaq buffer and AmpliTaq 360 DNA polymerase (Applied Biosystems, Foster City, CA). DNA from the following strains were used as controls for their respective screening PCR: CDL69 (*bcrABC*; Müller et al. 2013), Lm 4423 (*qacH*; Müller et al., 2013), and 08-5578 (*emrE*; Gilmour et al., 2010).

Table 3.3 PCR conditions used for screening for benzalkonium chloride resistance determinants.

Parameter		*bcrABC*[a]	*qacH*	*emrE*[b]
Reaction mixture final concentration (25 µl reactions)	Buffer	1X	1X	1X
	dNTP	200 µM each	200 µM each	200 µM each
	Primers[c]	0.2 µM each	0.5 µM each	0.4 µM each
	$MgCl_2$	2 mM	2 mM	1 mM
	Polymerase	0.625 U	0.75 U	2.5 U
	DNA template	100 ng	100 ng	100 ng
Thermocycling conditions	Denaturation	95°C for 5 min	95°C for 7 min	95°C for 5 min
	Cycling	30 cycles:	30 cycles:	35 cycles:
		95°C for 30 sec,	95°C for 30 sec,	94°C for 30 sec
		62°C for 30 sec,	52°C for 30 sec,	52°C for 30 sec
		72°C for 1 min	72°C for 30 min	72°C for 20 sec
	Final extension	72°C for 5 min	72°C for 7 min	72°C for 5 min

Adapted from [a] Elhanafi et al. (2010) and [b] Kovacevic et al. (2012b). [c]Primers and product sizes are summarized in Table 3.4.

Table 3.4 Benzalkonium chloride resistance determinants screening primers.

Gene	Primer	Primer sequence (5'-3')	Product size (bp)
bcrABC[a]	p1	CAT TAG AAG CAG TCG CAA AG CA	1130
	p2	GTT TTC GTG TCA GCA GAT CTT TGA	
qacH[b]	*qacH*-KJ-F	AGC ACT TGC AAT AGT GGG GG	249
	qacH-KJ-R	CCT GCA ATG CTA GCC ATG TT	
emrE[c]	LGI1-1862-F	GAG CAA CAC CAC CTA AGT TC	299
	LGI1-1862-R	CAG TCG CTA TCG TAC TTG AA	

[a]Primers designed by Elhanafi et al. (2010); [b]Custom primers designed in this study using Primer3 (V.0.4.0, http://bioinfo.ut.ee/primer3-0.4.0); [c]Primers designed by Kovacevic et al. (2012b).

3.2.5 Stress response

3.2.5.1 Adaptation and growth under optimal conditions

To assess the ability of isolates to adapt and grow under optimal conditions a spectrophotometric microtiter plate growth assay was performed, following the general protocol by Hall et al. (2014). Specifically, a single colony was picked from TSA into 5 ml BHI and incubated at 30°C for 24 h with shaking (180 rpm). The culture (10 µl) was then transferred to 5 ml fresh BHI and incubated at 30°C for 16 h with shaking (180 rpm). Overnight cultures were washed once with

0.1% peptone water (10,000 $\times g$, 5 min) and diluted to approximately 10^7 CFU/ml in BHI ($OD_{600} \approx 0.01$; confirmed by plating serial dilutions on TSA). The prepared cultures were transferred (200 µl) to a microtiter plate (Costar 3370, Fisher Scientific Ltd., Ottawa, ON) in duplicate, covered and placed into a microplate reader (Spectramax, V6.3; Molecular Devices, Sunnyvale, CA). The plate was incubated at 30°C for 24 h, with OD_{600} measurements taken every 30 min, with shaking for 3 sec before each reading. Four un-inoculated media wells for each plate were used as blanks. Three biological replicates were performed on separate days.

3.2.5.2 Cold shock adaptation and growth

Isolates were assessed for their ability to adapt to cold shock and grow at cold temperatures using a standard culture-based growth assay. Briefly, 10 ml BHI was inoculated with a single colony and grown for 16 h at 37°C with shaking. Cultures were adjusted to OD_{600} of ~0.63 (predetermined to correspond with ~10^9 CFU/ml) and serially diluted to achieve a final concentration of 10^3 CFU/ml in 10 ml pre-chilled BHI and incubated at 4°C until bacteria reached stationary phase. Growth was monitored on days 0, 1, 2, 3, 4, 7, 14, 17, and 21 by plating appropriate 10-fold dilutions prepared in peptone saline on TSA with 0.6% yeast extract and incubating these plates at 37°C for 24 h. Two biological replicates were performed for all isolates, with each biological replicate consisting of two technical plating replicates. The *L. monocytogenes* reference strain 10403S was included for all biological replicates.

3.2.5.3 Salt adaptation and growth

To differentiate between isolate abilities to adapt and grow in the presence of salt a microtiter plate growth curve was employed by scaling down the methods of Bergholz et al. (2010)

following the general protocol for use of microtiter plate readers for growth rate analysis described by Hall et al. (2014). Specifically, a single colony was picked from TSA into 5 ml BHI and incubated at 30°C for 24 h with shaking (180 rpm). The culture (10 µl) was then transferred to 5 ml fresh BHI and incubated at 30°C for 16 h with shaking (180 rpm). Overnight cultures were washed once with 0.1% peptone water (10,000 $\times g$, 5 min) and diluted to approximately 10^7 CFU/ml ($OD_{600} \approx 0.01$; confirmed by plating serial dilutions on TSA) in BHI with 6% NaCl w/v, accounting for the 0.5% NaCl present in BHI. The prepared cultures were transferred (200 µl) to a microtiter plate (Costar 3370, Fisher Scientific Ltd., Ottawa, ON) in duplicate, covered and placed into a microplate reader (Spectramax, V6.3; Molecular Devices, Sunnyvale, CA). The plate was incubated at 30°C for 24 h, with OD_{600} measurements taken every 30 min, with shaking for 3 sec before each reading. Four un-inoculated media wells for each plate were used as blanks. Three biological replicates were performed on separate days. *L. monocytogenes* 08-5578 (Gilmour et al., 2010) was included with each plate; Kovacevic (2014) recently observed that *L. monocytogenes* 08-5578 had enhanced salt adaptation and tolerance.

3.2.5.4 Modeling and evaluation of isolate growth kinetics

Microsoft Excel add-in program DMFit (version 2.1; http://www.ifr.ac.uk/safety/dmfit) was used to determine lag phase duration (LPD) and exponential growth rate (EGR) by fitting OD_{600} and $\log_{10}$CFU/ml growth curves to the model described by Baranyi and Roberts (1994). Results for isolates with the highest and lowest values for each parameter (LPD and EGR) in the absorbance-based assay were verified using a standard culture-based growth assay.

3.2.6 Statistical analysis

Statistical analysis was performed using Minitab version 17. All data was tested for normality using the Ryan-Joiner test. Parametric tests (e.g., ANOVA, Student's t-test) were used to assess differences between source, lineage, and recurrence for normally distributed data. Data that were not normally distributed were log transformed to achieve normal distribution where appropriate. Non-parametric tests (e.g., Kruskal-Wallis, Mann-Whitney U) were used to assess the same differences for non-normal distributions. Relationships between datasets were assessed with Pearson's correlation. Differences were considered significant at a p-value of < 0.05.

Comparisons of isolates attributes within the subset were made using Tukey's HSD test with a 95% confidence interval. Isolates that had significantly higher surface adherence values on polystyrene and PVC compared to isolates with the lowest adherence were classified as high adherers. Isolates were classified as fast adapters if LPD values were significantly shorter compared to the longest LPD duration isolates. Isolates with EGR values significantly higher than the slowest growing isolates were considered fast growers.

3.3 Results

3.3.1 Lineage typing

The subset consisting of 28 *L. monocytogenes* recovered from inside the processing plant and from gull feces in the parking lot included both Lineage I (n=11) and II (n=17) isolates (Table 3.2). The majority of subtypes recovered from inside the processing plant were Lineage II (71%, n=14); this same trend was seen in isolates recovered from gull feces (63%, n=8).

3.3.2 Surface adherence

Assessment of surface adherence revealed that, while all isolates were able to adhere to polystyrene and PVC microtiter plates, adherence was varied (Figures 3.1 and 3.2). Surface adherence was significantly higher on polystyrene compared to PVC ($p=0.0026$; Mann-Whitney U). Using Tukey's HSD test on non-adjusted PVC surface adherence values and on $\log_{10}$ transformed polystyrene surface adherence values, significant differences in surface adherence could be seen between lowest and highest adhering strains, however most isolates were not significantly different in their surface adherence capabilities (Figures 3.1 and 3.2; see Appendix C for complete results). Lineage II isolates had greater adherence than Lineage I isolates on both polystyrene ($p<0.0001$; Mann-Whitney U) and PVC ($p<0.0001$; Student's t-test). One isolate, Lm 363 from Lineage I, had enhanced surface adherence compared to all other Lineage I isolates on both polystyrene and PVC. No differences in adherence were seen between recurring and sporadic isolates on PVC ($p=0.261$; Student's t-test) and polystyrene ($p=0.214$; Mann-Whitney U) or between sources on PVC ($p=0.535$; one-way ANOVA) and polystyrene ($p=0.191$; Kruskal-Wallis). Additionally, surface adherence was not significantly different for isolates from the same subtype on both PVC ($p>0.25$; one-way ANOVA) and polystyrene ($p>0.60$; Kruskal-Wallis).

A strong positive correlation was observed between surface adherence on polystyrene and PVC microtiter plates for the isolates tested in this study (Figure 3.3; $r_p=0.827$, $p<0.0001$). One isolate had high adherence on both polystyrene and PVC (Lm 363), while others only adhered strongly on polystyrene (Lm 208, Lm 209, Lm 116, Lm 377, Lm 365) or PVC (Lm 360). That being said, aside from Lm 209, the high adherers on one surface could be found in the upper 50% of

adherence values on the other surface (Appendix C). On both surfaces, the highest adherers were isolated from inside the processing environment (raw materials and cutting board), and two (Lm 208 and Lm 365) from gull feces.

In order to assure consistency between experiments, two pairs of high and low adhering strains were included for each biological replicate in this study. Figures 3.1 and 3.2 show that 10403S and 10403SΔ*flaA* surface adherence was the same on polystyrene ($p=0.3843$; Mann-Whitney U), and that 10403S had decreased adherence to PVC compared to 10403SΔ*flaA* ($p=0.001$; Student's t-test). *L. monocytogenes* HPB399 and HPB642 were also used as controls in this study. On both surfaces the high adherer (HPB399) had significantly higher adherence than the low adherer (HPB642) ($p<0.001$; Student's t-test [polystyrene] and Mann-Whitney U [PVC]).

To rule out differences in growth as a factor in surface adherence values obtained, final cell density of the supernant was compared to surface adherence values, as others have done (Harvey et al., 2007; Djordjevic et al., 2002). Final cell density did not correlate with surface adherence on polystyrene after 48 h at 20°C. However, there was a significant negative correlation ($r_p=-0.417$, $p=0.018$) between final cell density and surface adherence on PVC (Figure 3.4).

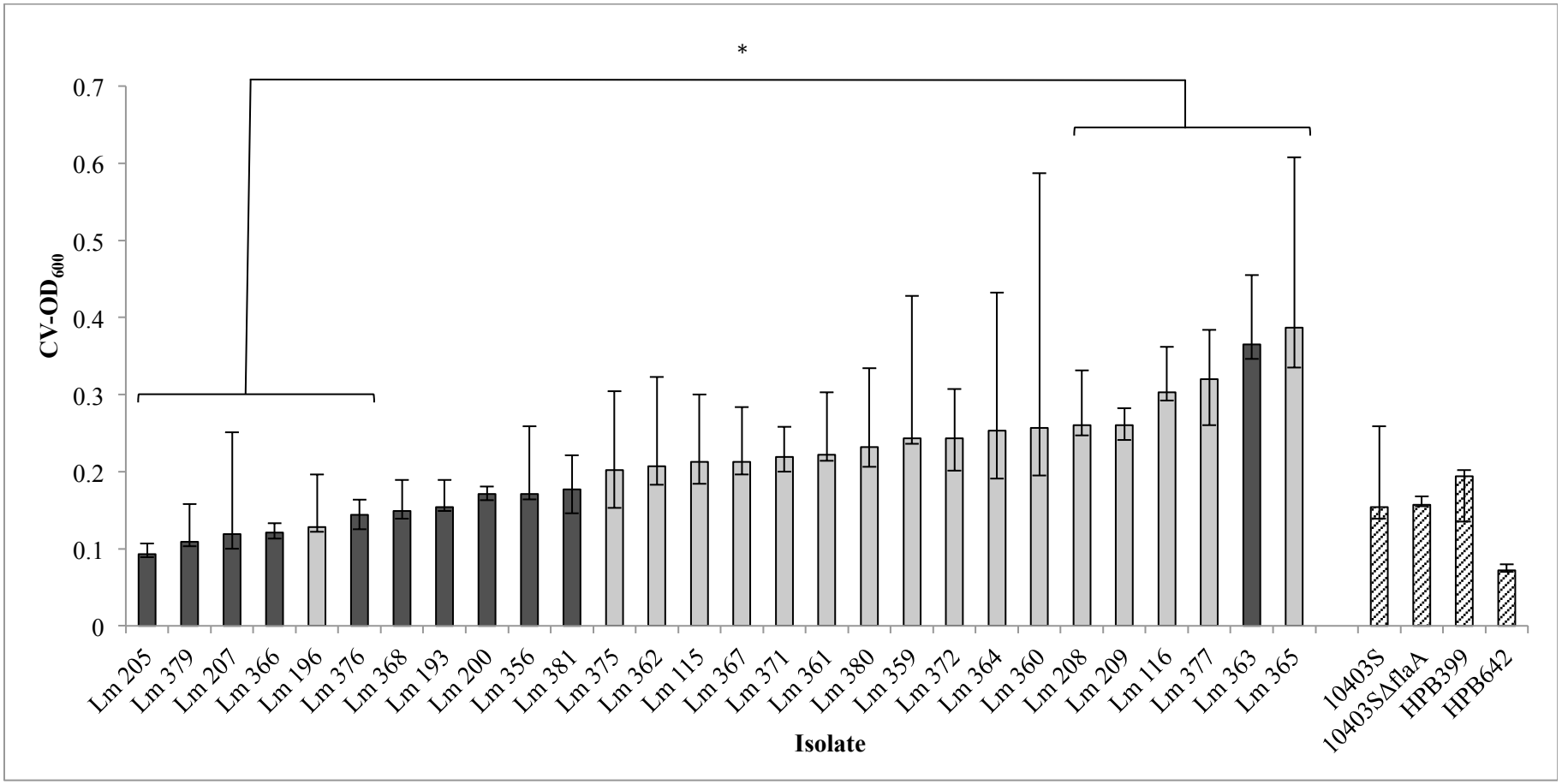


Figure 3.1 Surface adherence values ($CV\text{-}OD_{600}$) for *Listeria monocytogenes* isolates on polystyrene microtiter plates. Bars represent median ± range of three biological replicates. Dark gray bars indicate Lineage I isolates, light gray bars are Lineage II. Bars connected with a star (*) are significantly different ($p<0.05$; Tukey's HSD test on log transformed data). Control strains are included in graph on the far right, but were not included in statistical analysis.

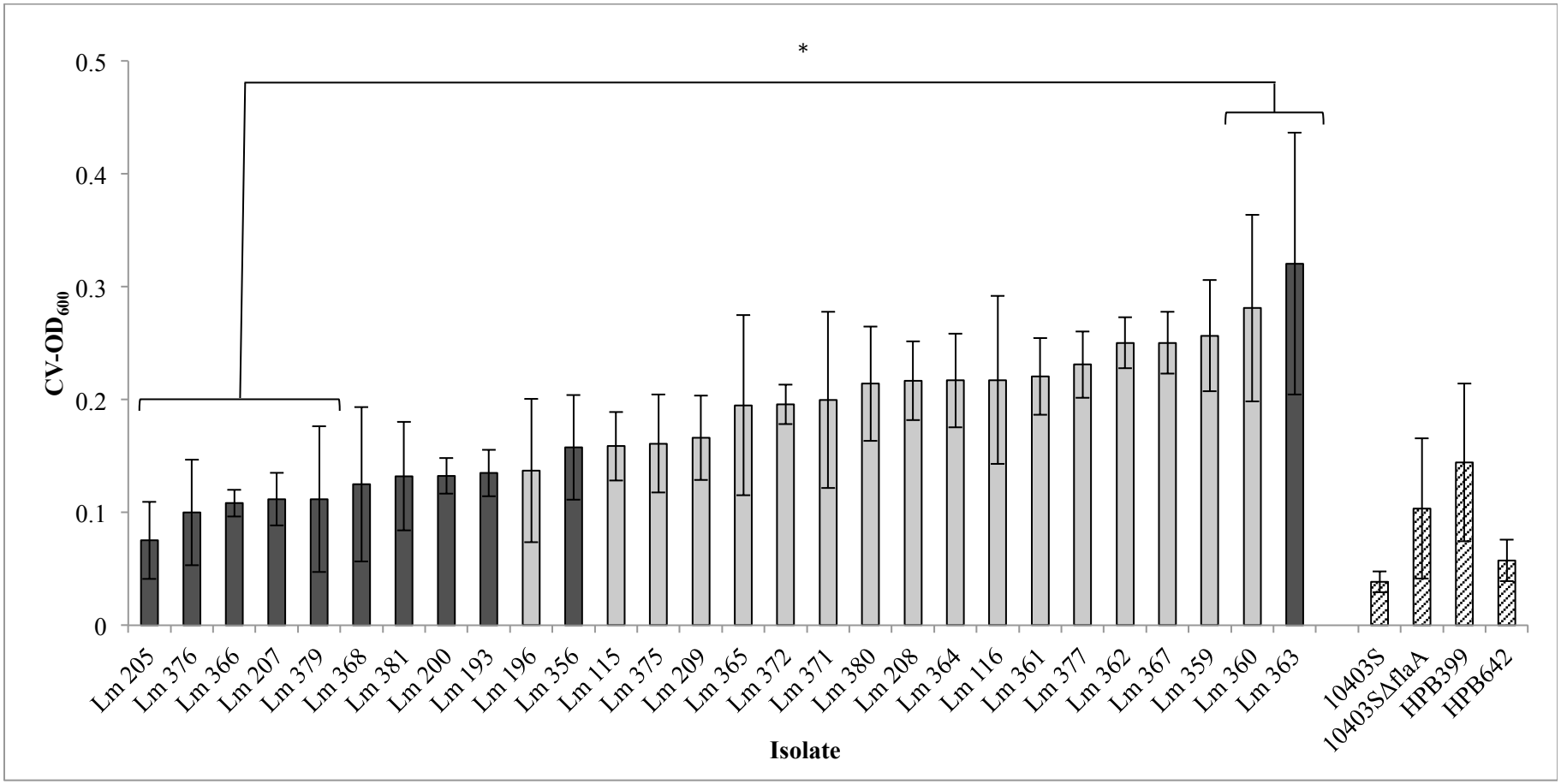


Figure 3.2 Surface adherence values ($CV\text{-}OD_{600}$) for *Listeria monocytogenes* isolates on polyvinyl chloride microtiter plates. Bars represent mean ± standard deviation of three biological replicates. Dark gray bars indicate Lineage I isolates, light gray bars are Lineage II. Bars connected with a star (*) are significantly different ($p<0.05$; Tukey's HSD test). Control strains are included in graph on the far right, but were not included in statistical analysis.

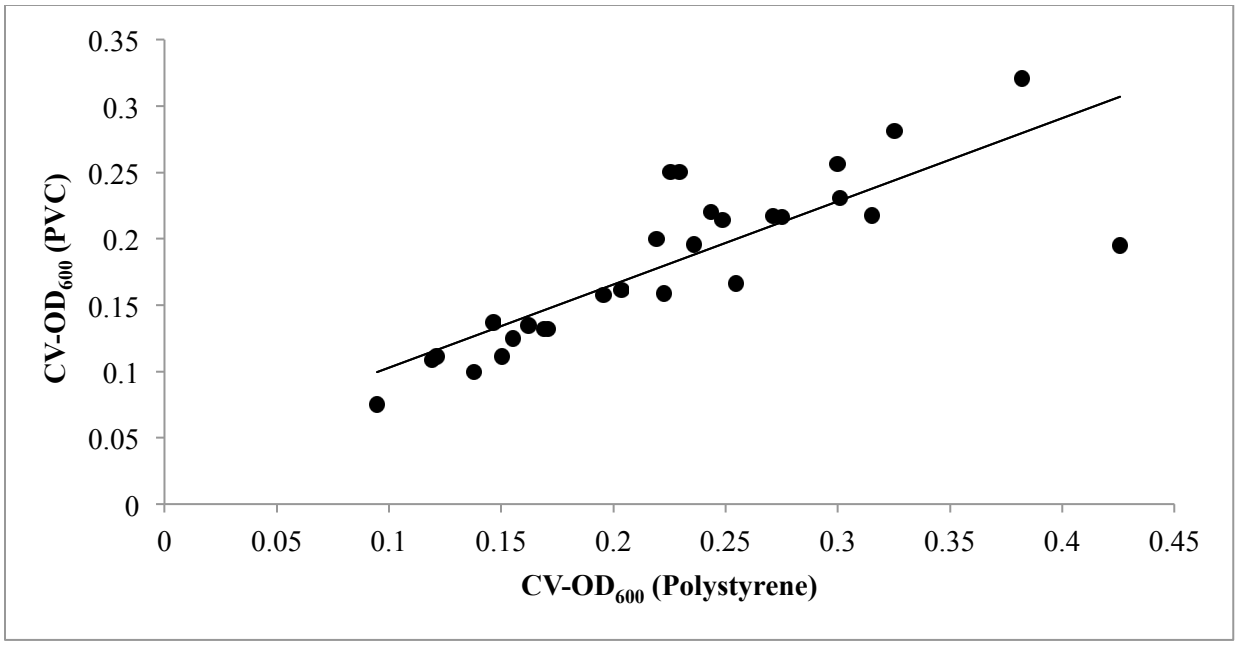


Figure 3.3 Surface adherence on polystyrene versus surface adherence on polyvinyl chloride (PVC) microtiter plates (r=0.827, p<0.0001; Pearson's correlation). Each data point represents the overall mean adherence for each isolate on each surface.

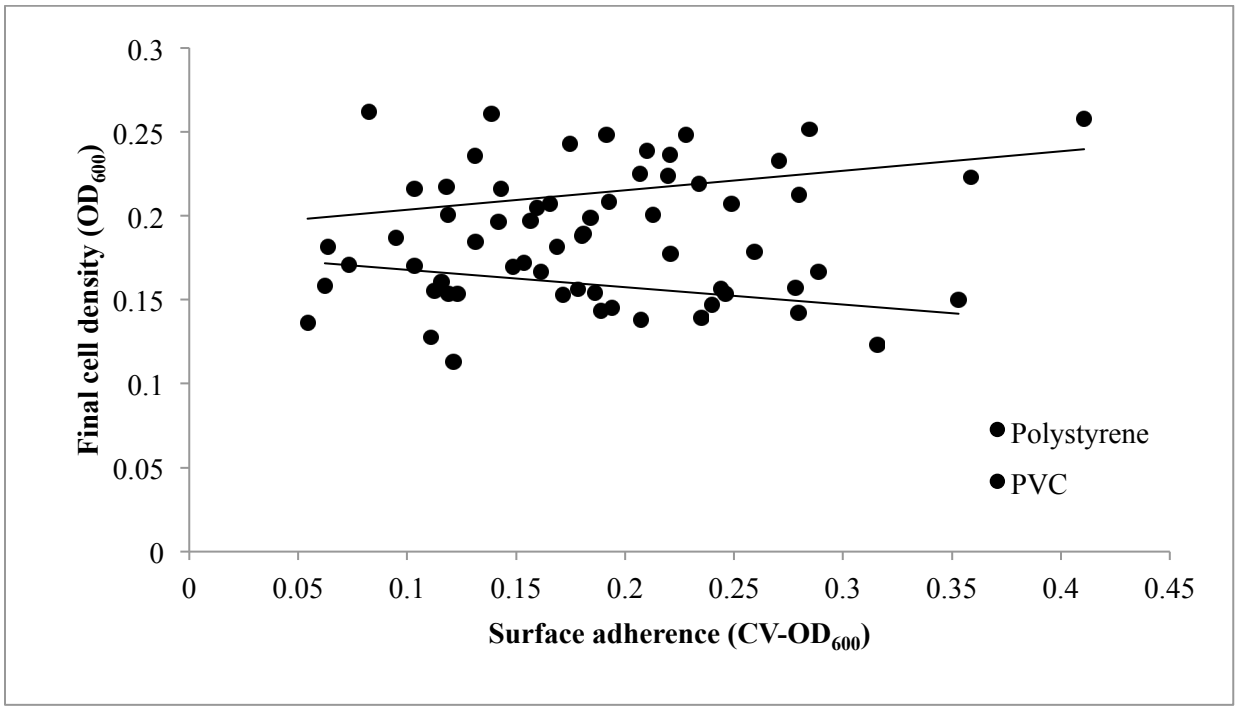


Figure 3.4 Final cell densities versus surface adherence on polystyrene (r=0.161, p=0.117; Pearson's correlation) and polyvinyl chloride (PVC) microtiter plates (r=-0.417, p=0.018; Pearson's correlation). Each data point represents the overall mean value for each isolate.

3.3.3 Sanitizer resistance

The resistance breakpoint used in this study was 10 μg/ml, two times that of the most susceptible strains (5 μg/ml). Lm 115 (BAC MIC = 20 μg/ml; E-SAN MIC = 15 μg/ml), Lm 116 (BAC MIC = 25 μg/ml; E-SAN MIC = 15 μg/ml), both from Lineage II, and Lineage I isolate Lm 368 (BAC MIC = 15 μg/ml; E-SAN MIC = 10 μg/ml), showed resistance to both BAC and E-SAN (Table 3.5). The observed resistance to QAC sanitizers for Lm 115 and Lm 116 could be explained by the presence of the BAC resistance determinant *bcrABC*. Both of these isolates were recovered sporadically from unknown FPE sources in 2012. Although Lm 368 belongs to a recurring pulsotype (LMACI.0775/LMAAI.1129), all other isolates from this pulsotype (Lm 193, Lm 200, Lm 356, and Lm 381) recovered on raw materials and in the FPE had a susceptible phenotype to both antimicrobials (MIC = 5 μg/ml). Lm 368 resistance to BAC and E-SAN could not be attributed to presence of known QAC resistance genetic determinants. MIC values were slightly higher overall for BAC ($\bar{x}$ = 6.6) compared to E-SAN ($\bar{x}$ = 5.9).

Table 3.5 Minimum inhibitory concentration (MIC) values for *Listeria monocytogenes* isolates to benzalkonium chloride (BAC) and E-SAN and genetic determinants associated with BAC resistance.

Isolate	MIC		Classification[b]	BAC resistance determinant[c]
	BAC (μg/ml)	E-SAN (μg/ml)		
Lm 115	20	15	Resistant	*bcrABC*
Lm 116	25	15	Resistant	*bcrABC*
Lm 368	15	10	Resistant	Absent
All others (n=25)[a]	5	5	Sensitive	Not tested
CDL69 (control)	20	15	Resistant	*bcrABC*

[a] See Table 3.2; [b] Resistance breakpoint: 10 μg/ml; [c] Screened for *bcrABC*, *qacH*, and *emrE*

3.3.4 Stress response

3.3.4.1 Cold stress

All isolates were able to adapt to cold shock and grow at 4°C. The mean ± standard deviation starting inoculum for isolates across two biological replicates was 3.01 ± 0.12 log_{10}CFU/ml increasing to 9.09 ± 0.09 log_{10}CFU/ml after 21 days at 4°C. Time to reach stationary phase was also delayed under cold stress compared to optimal conditions (~17 days at 4°C versus ~14 h at 30°C). Growth limitations at 4°C were not attributable to overall growth deficiencies under optimal conditions (Table 3.6). Log_{10}CFU/ml growth curves for isolates grown at 4°C fit the DmFit model well, with all modeled growth curves having an R^2>0.97.

LPD values ranged from 28 h (Lm 363, Lineage I) to 53 h (Lm 115, Lineage II), with no significant differences seen between isolates for this parameter (Figure 3.5; p=0.961; one-way ANOVA). EGR values ranged from 0.0178 (Δlog_{10}CFU/ml)/h (Lm 368, Lineage I) to 0.0223 (Δlog_{10}CFU/ml)/h (Lm 205, Lineage I). Significant differences were seen between the two slowest growers (Lm 368 and Lm 363) and the three fastest growers (Lm 356, Lm 359, and Lm 205) (Figure 3.6; p<0.05; Tukey's HSD test).

No differences were seen in EGR between lineages (p=0.848; Student's t-test) or between recurring and sporadic strains (p=0.806; Student's t-test). Differences in growth rate were seen between sources (p=0.031; one-way ANOVA) with cutting room isolates having a slower mean growth rate (0.0191 (Δlog_{10}CFU/ml)/h; n=11) compared to isolates derived from raw fish surface samples (0.0213 (Δlog_{10}CFU/ml)/h; n=4) after comparison by Tukey's HSD test.

Table 3.6 Comparison of growth kinetics of *Listeria monocytogenes* isolates under optimal conditions to growth kinetics under stress conditions.

Comparison[a]		Pearson correlation coefficient (r_p)[b]	*P* value
LPD BHI, 30°C	LPD BHI 6% NaCl, 30°C	0.262	0.178
LPD BHI, 30°C	LPD BHI, 4°C	0.221	0.258
EGR BHI, 30°C	EGR BHI 6% NaCl, 30°C	0.068	0.731
EGR BHI, 30°C	EGR BHI, 4°C	0.293	0.130

[a] Lag phase duration (LPD); Exponential growth rate (EGR); Brain heart infusion broth (BHI); [b] Overall mean of each isolate for each parameter were used for comparison

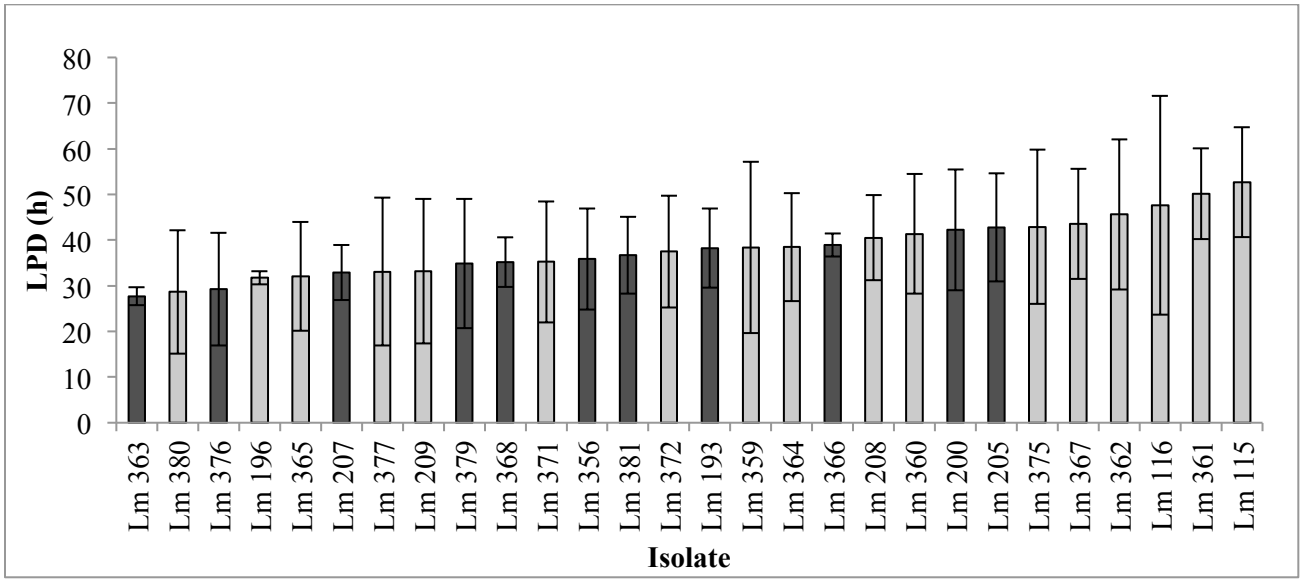


Figure 3.5 Lag phase duration (LPD) for *Listeria monocytogenes* isolates grown in BHI at 4°C. Bars represent mean ± standard deviation of two biological replicates. Dark gray bars indicate Lineage I isolates, light gray bars are Lineage II.

Comparing between isolates in the subset, increased EGR at refrigerated temperatures were seen for Lm 205, Lm 356, and Lm 359. Lm 205 belongs to Lineage I and was recovered from gull feces. An isolate from each recurring subtype (Lm 356 and Lm 359), both recovered from the surface of raw salmon, were found to have increased adaptation to refrigerated temperatures.

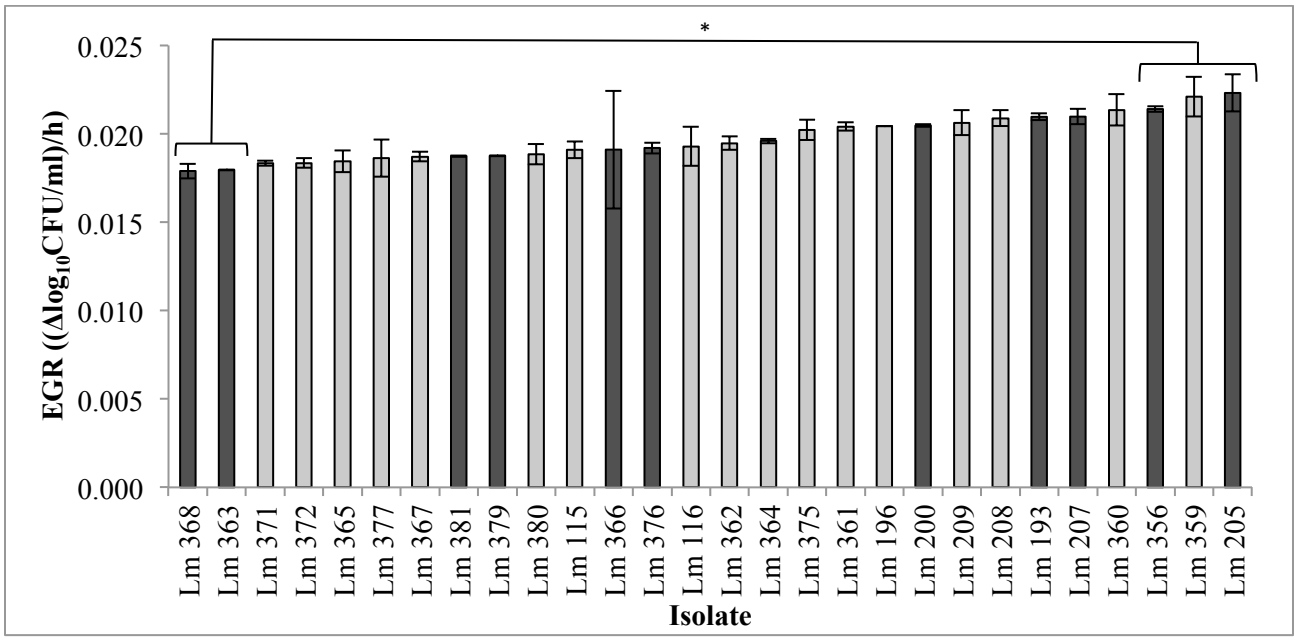


Figure 3.6 Exponential growth rate (EGR) for *Listeria monocytogenes* isolates grown in BHI at 4°C. Bars represent mean ± standard deviation of two biological replicates. Dark gray bars indicate Lineage I isolates, light gray bars are Lineage II. Bars connected with a star (*) are significantly different ($p<0.05$; Tukey's HSD).

3.3.4.2 Salt stress

All isolates in the subset were able to adapt to and grow in BHI with 6% NaCl. The mean ± standard deviation OD_{600} reading at the start of the assay was 0.0112 ± 0.006 and increased to 0.541 ± 0.086 by 24 h. Median growth rate of isolates under salt stress (0.048 ΔOD_{600}/h) was also significantly reduced ($p<0.001$; Mann-Whitney U) compared to growth under optimal conditions (0.135 ΔOD_{600}/h). Growth limitations in BHI with 6% NaCl were not attributable to overall growth deficiencies under optimal conditions (Table 3.6). OD_{600} growth curves for isolates grown under salt stress fit the DmFit model well, with all modeled growth curves having an $R^2>0.97$.

LPD values ranged from 2.9 h (Lm 205, Lineage I) to 6.1 h (Lm 366, Lineage I) (Figure 3.7). EGR ranged from 0.0382 OD_{600}/h (Lm 365, Lineage II) to 0.0666 OD_{600}/h (Lm 377, Lineage II) (Figure 3.8). Significant differences were seen between isolates on the high and low end of each range for LPD and EGR using the Tukey's HSD test ($p<0.05$; see Appendix C) on log transformed growth parameters (Figures 3.7 and 3.8). No differences in LPD were seen between lineages ($p=0.196$; Mann-Whitney U), source ($p=0.485$; Kruskal-Wallis), or between recurring and sporadic strains ($p=0.760$; Mann-Whitney U).

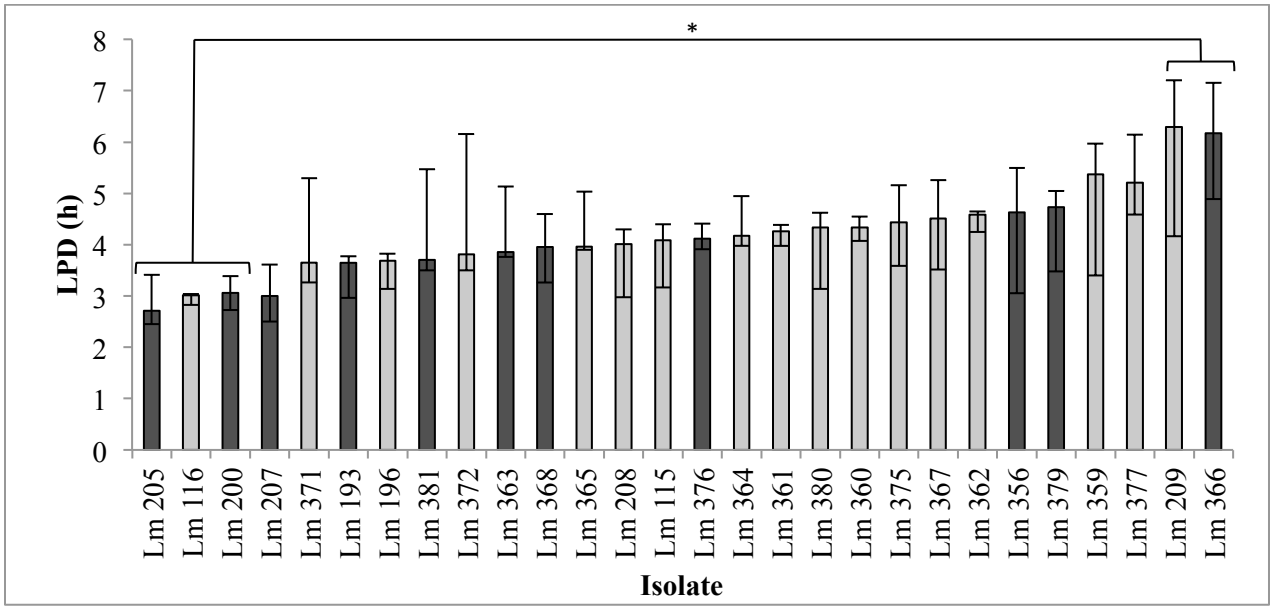


Figure 3.7 Lag phase duration (LPD) of *Listeria monocytogenes* isolates grown in BHI with 6% NaCl at 30°C. Bars represent median ± range of three biological replicates. Dark gray bars indicate Lineage I isolates, light gray bars are Lineage II. Bars connected with a star (*) are significantly different ($p<0.05$; Tukey's HSD on log transformed data).

As stressful conditions can cause morphological changes in *L. monocytogenes* which may affect the relationship between OD_{600} and viable cell counts (Francois et al., 2005), LPD and EGR determined by OD_{600} growth curve data were validated by comparing the findings of isolates

from high and low extremes for each parameter with results obtained from a standard culture-based growth assay. Differences between high (Lm 366) and low (Lm 205) LPD isolates remained significant (p=0.003; Student's t-test), whereas no significant difference was noted for isolates with high (Lm 377) and low (Lm 365) EGR values using the culture-based method (p=0.279; Student's t-test; Figure 3.9).

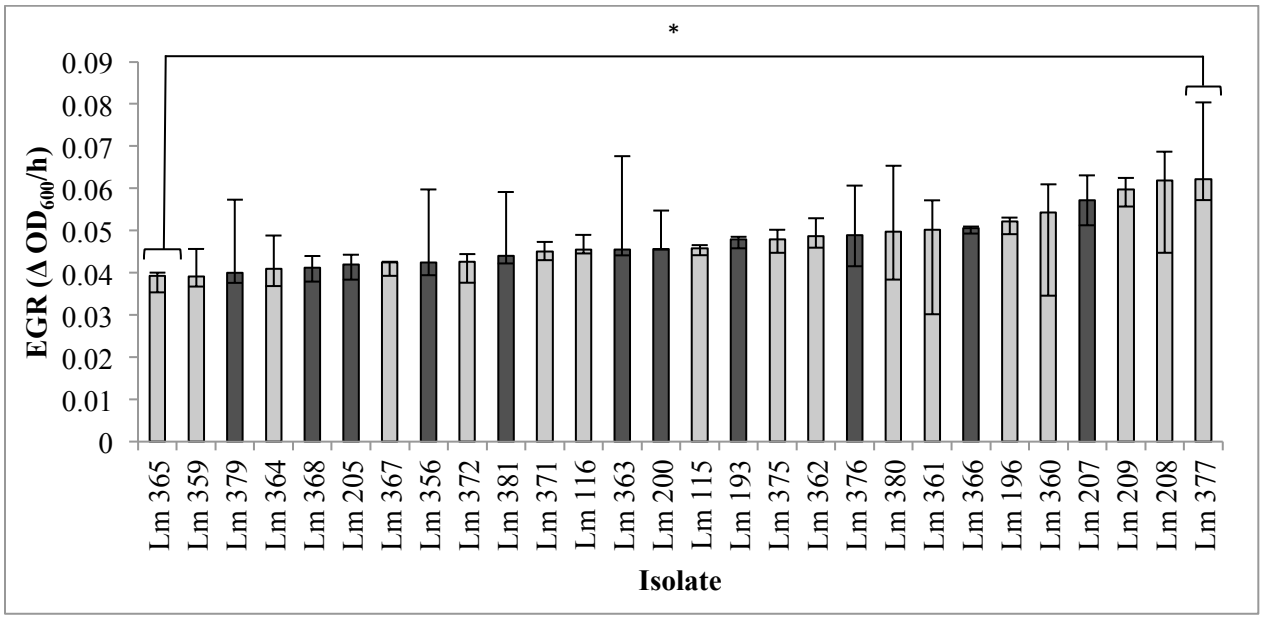


Figure 3.8 Exponential growth rate (EGR) of *Listeria monocytogenes* isolates grown in BHI with 6% NaCl at 30°C. Bars represent median ± range of three biological replicates. Dark gray bars indicate Lineage I isolates, light gray bars are Lineage II. Bars connected with a star (*) are significantly different ($p<0.05$; Tukey's HSD on log transformed data).

Lm 116, Lm 200, and Lm 205 were fast adapters in 6% NaCl compared to the other isolates tested in this study. Lm 116 and Lm 200 were recovered in the processing plant, where Lm 205 was isolated from gull feces. Two of the three fast adapting isolates were Lineage I strains. Although Lm 377 had a significantly higher EGR than Lm 365 (Figure 3.8), this isolate was not classified as a fast grower in salt as this significant difference was not seen when these isolates were compared using the colony count growth assay (Figure 3.9).

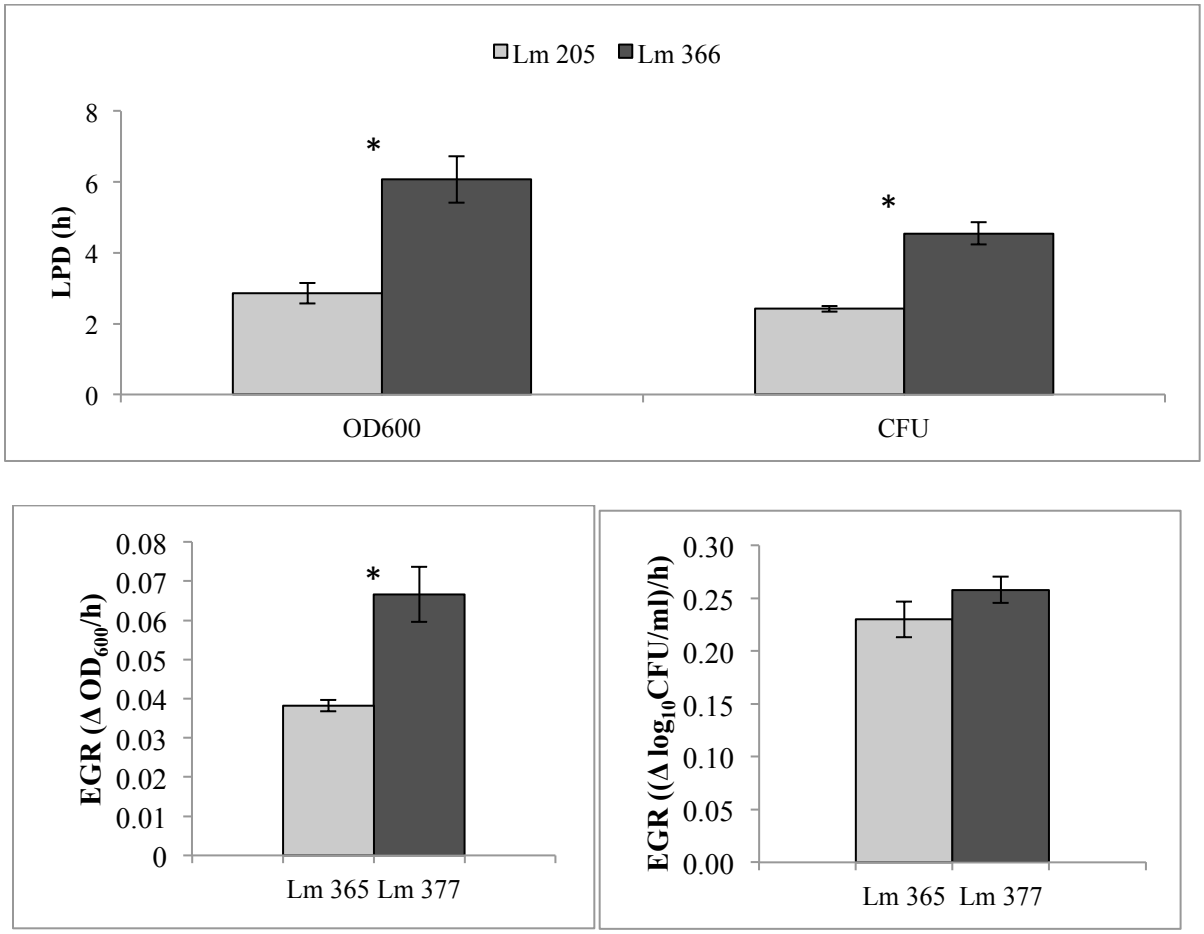


Figure 3.9 Comparison of lag phase duration (LPD) and exponential growth rate (EGR) values derived for *Listeria monocytogenes* isolates on the high and low end of each range using optical density microtiter plate growth assay (OD_{600}) or colony count growth assay (CFU). Starred graphs (*) indicate a significant difference between paired bars ($p<0.05$; Student's t-test).

3.3.5 Comparison of persistence attributes

To determine if any of the isolates possessed more than one attribute favorable to persistence in the FPE, or survival and growth in foods, isolates with high adherence (to polystyrene and/or PVC), enhanced stress response, and resistance to QACs were compared (Figure 3.10). One isolate, Lm 116 (sporadic subtype, unknown source, Lineage II), had high surface adherence,

short LPD in salt, and increased resistance to QACs. No other isolates showed enhanced abilities for more than one persistence attribute.

Additionally, the relationship between LPD values under cold temperature and salt stress, and EGR values under cold temperature and salt stress were assessed. No relationship was seen between isolate LPD (Figure 3.11; r_p=-0.200, p=0.309) or EGR (Figure 3.12; r_p=0.094, p=0.634) under these two stresses.

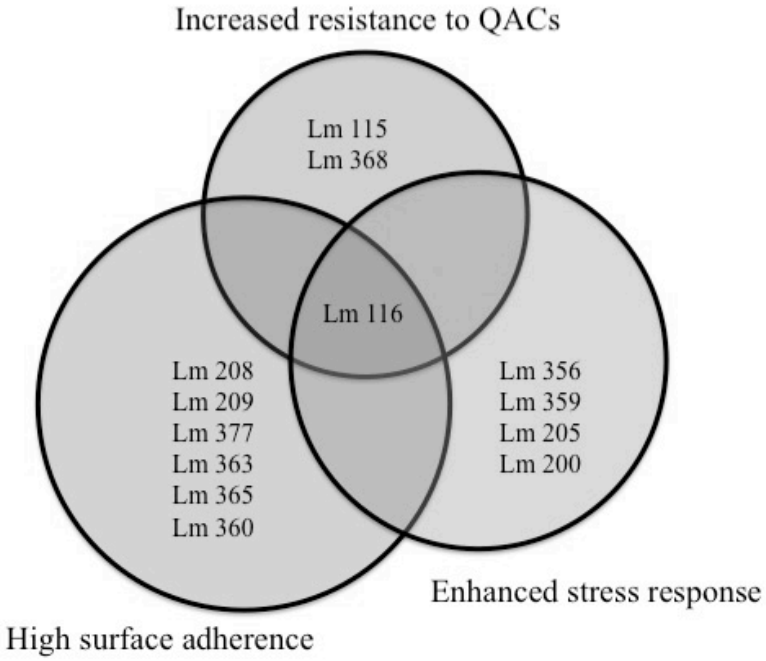


Figure 3.10 Venn diagram representing *Listeria monocytogenes* isolates with enhanced ability to survive in food processing environments and/or grow in RTE food products. High surface adherers were isolates that had significantly higher CV-OD_{600} values compared to the lowest values in the subset on polystyrene and/or polyvinyl chloride microtiter plates. Enhanced stress response includes isolates with significantly shorter lag phase duration under salt stress (6% NaCl) or significantly faster exponential growth rate when grown at 4°C, compared to isolates on the opposite end of the respective ranges. Isolates that possessed increased resistance to quaternary ammonium compounds (QACs) were able to grow in the presence of benzalkonium chloride and E-SAN at concentrations > 10 μg/ml.

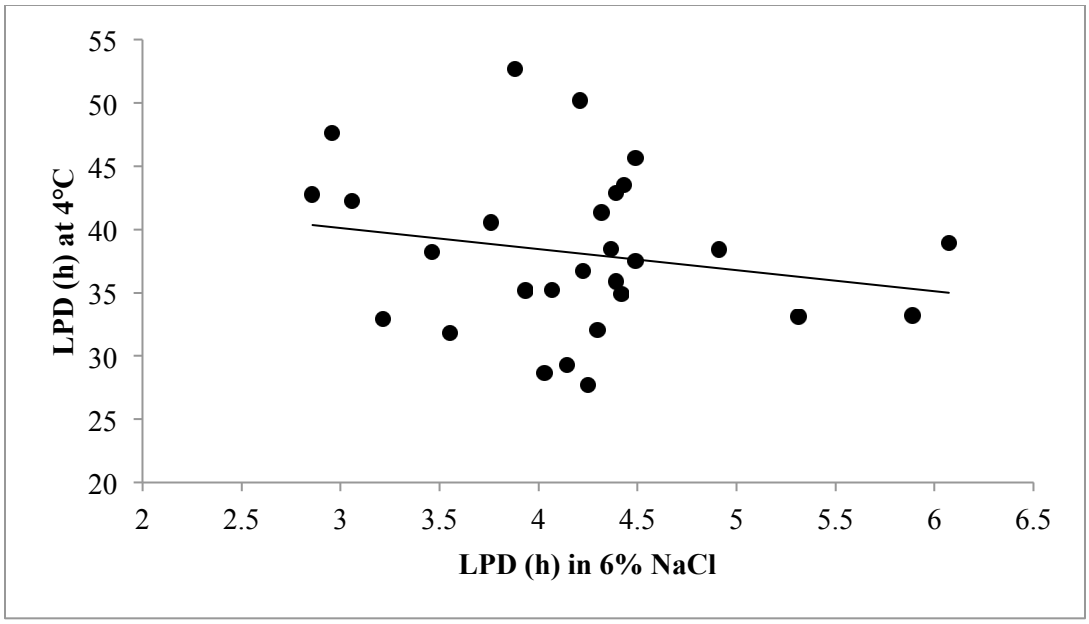


Figure 3.11 Lag phase duration (LPD) of *Listeria monocytogenes* isolates grown in BHI with 6% NaCl versus LPD of isolates grown at 4°C (r=-0.200, p=0.309; Pearson's correlation). Each data point represents the overall mean for each isolate.

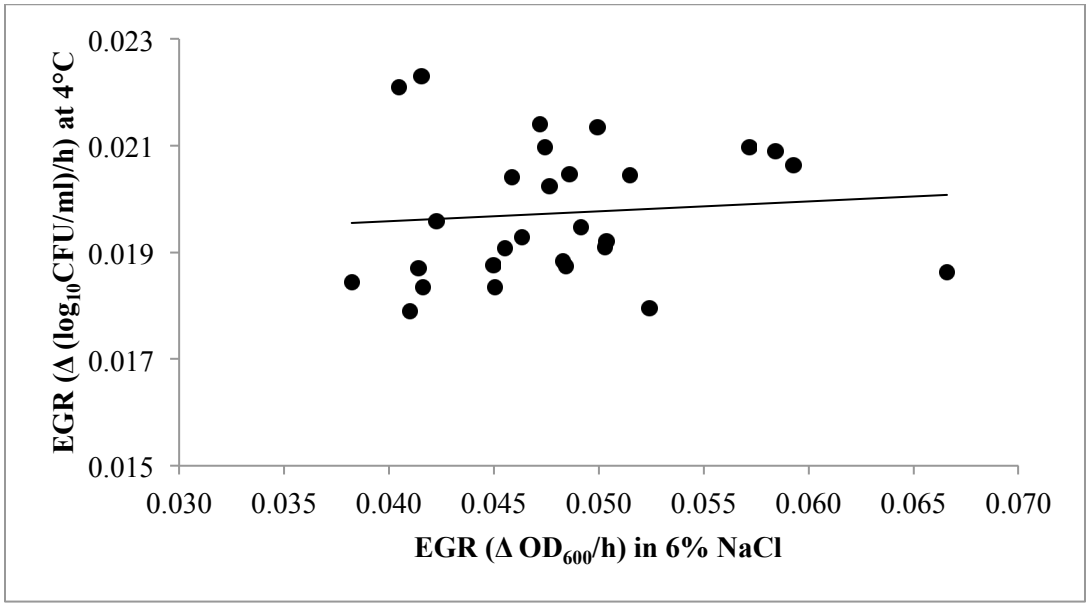


Figure 3.12 Exponential growth rate (EGR) of *Listeria monocytogenes* isolates grown in BHI with 6% NaCl versus EGR of isolates grown at 4°C (r=0.094, p=0.634; Pearson's correlation). Each data point represents the overall mean for each isolate

3.4 Discussion and conclusions

3.4.1 Lineage typing

L. monocytogenes can be divided into four phylogenetic lineages (Orsi et al., 2011; Valderrama and Cutter, 2013). Assignment of *L. monocytogenes* to their respective lineages has been done using molecular means including, but not limited to, ribotyping, PFGE, and multilocus sequence typing (MLST) (Brosch et al., 1994; Fugett et al., 2006; Nadon et al., 2001; Orsi et al., 2011; Ragon et al., 2008; Ward et al., 2004; Wiedmann et al., 1997). This study used PCR-based lineage typing described by Ward et al. (2004) wherein lineages are assigned based on genetic differences present in the *prfA* virulence cluster. This inexpensive and straightforward method of lineage typing was previously shown to be accurate, with 100% sensitivity and specificity in assessing lineage of 112 isolates (Ward et al., 2004). In this study, all isolates could be typed with this method into either Lineage I or Lineage II. The absence of Lineages III and IV strains was not surprising, as strains from these lineages are rare (Orsi et al., 2011). Overall, more Lineage II strains were isolated from both inside and outside the plant. These findings are in line with the general trend of Lineage II strains more often associated with food and FPEs compared to Lineage I strains, which are more often recovered from clinical samples (reviewed by Orsi et al., 2011).

3.4.2 Surface adherence

In FPEs it is suggested that *L. monocytogenes* biofilm formation on abiotic surfaces may allow enhanced survival or persistence of certain strains (da Silva and De Martinis, 2013; Valderrama and Cutter, 2013). *L. monocytogenes* strains vary in their ability to adhere to a variety of surfaces (Valderrama and Cutter, 2013). In this study, biofilm formation was indirectly assessed using a

CV surface adherence assay widely accepted for *L. monocytogenes* biofilm research (Borucki et al., 2003; Chang et al., 2012; Djordjevic et al., 2002; Harvey et al., 2007; Lemon et al., 2007). Surface adherence was assessed by measuring absorbance of solubilized CV dye, where the absorbance reading is proportional to the biomass adhered to the surface (Pantanella et al., 2013). While this method provides a relatively quick and easy comparison of *L. monocytogenes* surface adherence, the CV method is known for having relatively low reproducibility (Borucki et al., 2003; Chang et al., 2012; Harvey et al., 2007; Marsh et al., 2003; Pantanella et al., 2013). In addition, slight changes in experimental design can have a dramatic effect on the outcome of the assay (Borucki et al., 2003; Chang et al., 2012; Harvey et al., 2007; Marsh et al., 2003; Pantanella et al., 2013).

Knowing the limitations of the CV assay, two sets of controls were used to assure that the results of this experiment were meaningful. While one set of control strains had expected surface adherence results, the 10403S wild-type strain and 10403SΔ*flaA* pair had surface adherence opposite to expected results on PVC if compared to what Lemon et al. (2007) observed with these strains. The opposite results seen on PVC in this study may be explained by the differences in PVC plates used. Researchers have reported large differences in adhesion between different lots of microtiter plates (An and Friedman, 2000). Lemon et al. (2007) used plates manufactured by Falcon and Costar manufactured microtiter plates were used in this study. Differences in experimental methodology may also account for the differences seen. Takahasi et al. (2009) demonstrated that the use of different culture medium could have a marked effect on surface adherence of identical strains. Lemon et al. (2007) grew the strains in a defined minimal media while this study grew strains in rich, undefined TSB. While the results of these isolates did

not match the expectation for these strains, having a second set of control strains that did match expectations provides support for the validity of the surface adherence results presented in this study.

Significant negative correlation between final planktonic cell density and surface adherence on PVC was observed in this study. With a higher cell density one might expect higher surface adherence values, as more cells would be able to interact with the surface. The negative correlation seen in this study is opposite to this expectation. At 20°C in TSB under the experimental conditions described in this study *L. monocytogenes* will have reach stationary phase after 48 h of growth, therefore all isolates should have reached a constant cell density. At constant density, high adherers would have more cells associated with the surface, causing a decrease in planktonic cells, possibly explaining the significant negative correlation seen in this study. This data suggests that although there may be a slight effect of growth on surface adherence to PVC, surface adherence differences observed on both surfaces were most likely due to factors other than ability of strains to grow in TSB.

Variation in the ability of isolates to adhere to polystyrene and PVC was reduced when compared to other studies. Median CV-OD_{600} values on polystyrene ranged from 0.09 (Lm 205, Lineage I) to 0.39 (Lm 365, Lineage II) and mean CV-OD_{600} ranged from 0.08 (Lm 205, Lineage I) to 0.32 (Lm 363, Lineage I) on PVC. In comparison, Harvey et al. (2007) saw a range of CV-OD_{595} values from less than 0.6 to over 1.2 on polystyrene using similar methods to this study. Djordjevic et al. (2002) also reported a wide range of adherence values from 0.3 to 1.4 on PVC plates with similar methods. The lower overall surface adherence values seen in the present study

could be due to differences in methodology (e.g., more efficient and consistent washing may have been achieved using the plate washer where others use a pipet for this step, or different media used to grow cultures), or perhaps this subset consists of only low adhering strains. While Harvey et al. (2007) did see a wide range of surface adherence values, most (92%, n=138) had values less than 0.6, offering support for the second explanation, especially with the much smaller subset tested in this study.

Comparing between surface adherences on the two materials used, CV-OD_{600} values were significantly higher on polystyrene than PVC. Bacterial surface adherence is influenced by many factors, including the environment, surface materials, and characteristics of the bacterium (Borucki et al., 2003; Di Bonaventura et al., 2008; Folsom et al., 2006). Surface material was the only variable factor between these two experiments, suggesting that the characteristics of these materials may account for the differences seen in surface adherence. Absolom et al. (1983) reported that for most bacteria, including *L. monocytogenes*, surface adherence decreases with an increase in surface tension. This relationship can be explained as increase in surface tension decreases interaction of the aqueous phase with that material, decreasing opportunity for contact and adherence of bacterial cells in solution to that surface. Looking at the surface tension of polystyrene (34 mJ/m^2) and PVC (37.9 mJ/m^2) taking the above mentioned relationship into account suggests that the higher surface tension of PVC may explain the decrease in surface adherence seen on this surface compared to polystyrene in this study (Diversified Enterprises, 2009).

Despite being lower on PVC, surface adherence on this surface showed a significant positive correlation to surface adherence on polystyrene. Studies on surface adherence have used a variety of methods and surfaces to assess this characteristic, making it difficult to compare between significant findings derived from this research (Valderrama and Cutter, 2013). While previous research has shown that there is a correlation between *L. monocytogenes* surface adherence on stainless steel and PVC (Djordjevic et al., 2002) and glass and polystyrene (Marsh et al., 2003), this same correlation has not previously been demonstrated for PVC and polystyrene, the two plastics most often used for assessment of surface adherence using the CV method (reviewed by Valderrama and Cutter, 2013). The significant correlation in surface adherence for these two surfaces found in this study suggests that results derived from these studies may be comparable.

Previous studies on *L. monocytogenes* surface adherence have found that certain phylogenetic lineages of this bacterium can adhere to abiotic surfaces better than others, although these findings are conflicting. Djordjevic et al. (2002), Folsom et al. (2006), and Takahashi et al. (2009) all found that mean biofilm production was higher for Lineage I strains compared to Lineage II and III strains. In contrast, both Borucki et al. (2003) and Harvey et al. (2007) found that Lineage II strains possessed better surface adherence compared to Lineage I strains. Borucki et al. (2003) suggest that these conflicting findings may arise from differences in methodology, sample sizes, and specific strains used. In this study, which compared surface adherence after 48 h for 28 isolates derived from sampling in and around a seafood processing environment via CV assay, Lineage II had significantly higher surface adherence compared to Lineage I strains. Aside from Lm 363, all isolates in the upper 50% of surface adherence values belonged to Lineage II

on both polystyrene and PVC. These findings may in part help to explain why Lineage II is more often found in food and FPE than Lineage I (Orsi et al., 2011).

In the FPE the environmental conditions, nutrient availability and surfaces are varied. This research provided a general assessment of surface adherence for a subset of *L. monocytogenes* recovered in and around a seafood processing facility in BC under laboratory conditions. While hydrophobic materials, such as plastics used in this study, are common in food processing environments (including gaskets, conveyor belts, cutting boards, trays, etc.) many of the surfaces in these environments are constructed from hydrophilic materials such as stainless steel (Harvey et al., 2007). It is suggested that results of surface adherence on hydrophobic materials cannot predict the same trait on hydrophilic materials (Harvey et al., 2007), although this is not always the case (Djordjevic et al., 2002). Additionally, Di Bonaventura et al. (2008) found that surface adherence and subsequent biofilm formation varies with temperature; at 22°C and 37°C biofilms were complex, dense structures, while at 4°C and 12°C they consisted of sparse collections of cells with minimum amounts of EPS. This study assessed surface adherence at 20°C, but the facility that these isolates were derived from has working temperatures at or below 12°C. Some studies use minimal nutrient content media, as this is thought to better reflect the conditions in the FPE, although in some areas (e.g., drains) this may not be the case. This study used rich undefined media to grow cells due to the ease of preparation and because previous work had successfully used this media for a similar purpose (Harvey et al., 2007). Interestingly, Harvey et al. (2007) and Folsom et al. (2006) both found that *L. monocytogenes* grown in nutrient reduced medium produced more dense biofilms than those grown in TSB, with biofilm formation suspected to be a part of the starvation stress response. Therefore, in this study, monitoring

growth in a rich medium may have under estimated biofilm forming ability of the subset tested. Finally, in the FPE biofilms are often more complex consisting of multiple species and organisms (Zottola and Sasahara, 1994), where this study assessed mono-species biofilm forming capabilities. Interactions with bacteria commonly found in the FPE, such as *Pseudomonas* and *Enterococcus,* may enhance biofilm-forming ability of *L. monocytogenes* (Ferreira et al., 2014; Fox et al., 2014).

3.4.3 Sanitizer resistance

Some *L. monocytogenes* are resistant to sub-lethal concentrations of QACs, sanitizers widely used in the FPE (Elhanafi et al., 2010; Lundén et al., 2003; Müller et al., 2013). This resistance has been suggested to contribute to survival and persistence of certain strains in this environment despite controls in place (Ferreira et al., 2014; Lundén et al., 2003). QACs are a class of molecules characterized by a nitrogen atom linked to four organic groups (cations) paired with an anion (usually chloride) (Marriott, 2006). QACs act on *L. monocytogenes'* outer cell membrane resulting in solubilization of hydrophobic components, causing destabilization of the membrane barrier, leakage, and enzyme inhibition (Gilbert and Moore, 2005; Marriott, 2006). While these compounds are desirable to the food industry for their many advantageous properties, because QACs are relatively stable these compounds may be present in the environment for extended periods at sub-lethal concentrations (Allen et al., 2015; Müller et al., 2013).

This study used an agar-based MIC method to determine resistance to BAC and E-SAN. MIC is the method most commonly used for screening of bacterial resistance to sanitizers (Soumet et al.,

2005). Unlike antibiotics, sanitizer MIC testing does not have standardized criteria for resistance breakpoints, and therefore strain resistance is often arbitrarily assigned (Soumet et al., 2005). For example, some researchers have assigned resistance if MIC values are two times higher than MIC values for the most susceptible strains (Aase et al., 2000; Soumet et al., 2005), a convention that was adopted for this study. For both BAC and E-SAN, resistance was assigned to isolates that had an MIC of ≥ 10 μg/ml. This value is similar to that used by Soumet et al. (2005), which assigned resistance to isolates with an MIC value higher than 7.5 μg/ml BAC and the same as the breakpoint used by Mullapudi et al. (2008).

Of the 28 isolates tested three (11%) were resistant to both BAC and E-SAN. Resistance to BAC was higher compared to E-SAN, although BAC consists of C12 and C14 alkyl derivatives that are known to have the greatest biocide activity (Mistral Industrial Chemicals, 2015). E-SAN may be more effective due to formulation that includes both BACs and BAC derivative N-alkyl dimethyl ethyl benzyl ammonium chloride. Addition of QAC derivatives can be used to extend the biocide spectrum of a sanitizer and enhance the efficacy of BAC-based products (Mistral Industrial Chemicals, 2015).

Using a breakpoint of 7 μg/ml Mereghetti et al. (2000) found 7 out of 97 strains to be less susceptible to BAC. In contrast, 46% of *L. monocytogenes* (n=123) isolated from a turkey processing plant possessed BAC resistance using a breakpoint of 10 μg/ml (Mullapudi et al., 2008). Similar to the present study, Soumet et al. (2005) evaluated BAC resistance in *L. monocytogenes* from smoked seafood processing facilities. With a breakpoint of 7.5 μg/ml the authors found 48% of isolates (n=254) possessed reduced susceptibility to BAC, with most of

these strains isolated from salmon processing facilities. While resistance was reported in this research the ratio of resistant to sensitive strains was low compared to results of previous studies, and compared to other smoked salmon processors.

This data provides information on low-level resistance to QACs. The concentrations tested (0 to 25 ppm) and breakpoints used for classifying resistance (≥ 10 ppm) are well below the recommended concentration for use in the FPE for these sanitizers (200 ppm on food-contact surfaces and 1000 ppm on non-food contact surfaces). Some have stated that although MIC is widely used for sanitizer resistance assessment, the determination of the bactericidal effect at the manufacturer's recommended concentration of a sanitizer is of more interest to the food industry (Sundheim et al., 1998). That being said, knowing that QACs are no-rinse sanitizers, stable in FPEs, and may be present in sub-lethal concentrations, MIC data provides useful information on whether certain strains may have enhanced ability to survive in these circumstances; these findings are of interest to food processors. In fact, repeat exposure to sub-lethal concentrations of QACs has been shown to reversibly increase MIC values for some strains of *L. monocytogenes* (Kastbjerg and Gram, 2012; Lundén et al., 2003).

Resistance to sanitizers has been suggested as a potential mechanism of *L. monocytogenes* persistence in FPE, as persistent strains have been recovered in these environments after cleaning and sanitation activities (Lundén et al., 2003; Mędrala et al., 2003; Soumet et al., 2005). Lundén et al. (2003) showed that persistent strains had increased MIC to QACs, results supported by findings from others (Fox et al., 2011; Nakamura et al., 2013; Ortiz et al., 2014a). However, like most research into persistence mechanisms, others have found no link between persistence and

sanitizer resistance (Earnshaw and Lawrence, 1998; Heir et al., 1995; Holah et al., 2002; Kastbjerg and Gram, 2012). This study supports the latter with no link found between recurring subtypes and resistance to sanitizers. Interestingly, Lm 368 belongs to a recurring pulsotype (LMACI.0775/LMAAI.1129), but Lm 193, Lm 200, Lm 356, and Lm 381 from the same pulsotype did not possess resistance to BAC or E-SAN. Differing MIC values for QACs has previously been reported for the same *L. monocytogenes* strains (Romanova et al., 2002). While PFGE is a highly discriminatory method for subtyping *L. monocytogenes*, small genetic variances that go undetected in PFGE typing may result in phenotypic differences between strains that appear to be clonal using this subtyping method (Romanova et al., 2006).

To understand the genetic basis for the resistance to BAC and E-SAN observed in this study, the three isolates possessing resistance were screened for genetic resistance determinants *bcrABC*, *qacH*, and *emrE*. All three genes encode efflux pumps that have been linked with increased resistance to BAC (Elhanafi et al., 2010; Gilmour et al., 2010; Müller et al., 2013). Lm 115 and Lm 116 possessed *bcrABC*, explaining the resistance seen in these strains, while none of the resistance determinants were found in Lm 368. The lack of *qacH* in this subset is not surprising, as this genetic element is more common in European isolates, whereas *bcrABC* seems to be more commonly present in North American strains (Dutta et al., 2013).

The lack of a resistance determinant in Lm 368 despite a resistant phenotype to BAC and E-SAN is a curious finding. While slightly lower MIC values were seen for this strain compared to Lm 115 and Lm 116, this strain also possessed resistance to both sanitizers. Some potential explanations for the resistance in this isolate could include a novel resistance determinant or

stress-induced mutagenesis allowing increased ability of this strain to adapt to sanitizers. In a separate study, Lm 368 was the only isolate that showed elevated spontaneous mutation frequencies when plated on BHI with rifampicin (Appendix D). Hyper-mutable phenotypes have been shown to correlate with enhanced ability of some bacteria to survive environmental stresses (Gonzalez et al., 2008, 2012).

3.4.4 Stress response

L. monocytogenes can adapt to changing environmental conditions and survive under various stress conditions, including high salt, low temperature, and low pH (Gandhi and Chikindas, 2007; Valderrama and Cutter, 2013). In cold-smoked seafood processing *L. monocytogenes* are subjected to two stresses on a regular basis: refrigerated temperatures and high salt concentrations (Thomas et al., 2012). In this study, growth kinetics for recurring and sporadically isolated *L. monocytogenes* derived from in and around a cold-smoked seafood processor at 4°C in BHI or at 30°C in BHI containing 6% NaCl were assessed.

3.4.4.1 Cold stress adaptation and growth

Cold temperatures present many challenges to bacterial growth and survival. A decrease in membrane fluidity results in issues with nutrient uptake, an increase in DNA supercoiling challenges replication and transcription, secondary structures in RNA cause problems in translation and inefficient protein folding and cold temperatures reduce enzyme activity (Chan and Wiedmann, 2009). For *L. monocytogenes* to survive and grow at cold temperatures the bacterium must be able to overcome these obstacles (Chan and Wiedmann, 2009; Tasara and Stephan, 2006; Thieringer et al., 1998).

The response of *L. monocytogenes* to cold temperatures can be divided into two stages. The initial stage of cold shock is where synthesis of all proteins is halted except for those proteins responsible for the cold shock response, followed by an acclimation and transition stage into cold adapted status where synthesis of non-cold shock proteins is resumed (Thieringer et al., 1998). Phenotypic heterogeneity among strains of *L. monocytogenes* has been repeatedly observed in strains grown at refrigerated temperatures (Arguedas-Villa et al., 2014, 2010; Kovacevic et al., 2013a; Lianou et al., 2006; Nufer et al., 2007). The greatest variability in growth between strains subjected to cold shock is often seen in the initial cold shock phase, as measured by LPD (Arguedas-Villa et al., 2010). Kovacevic et al. (2013a) assessed LPD for 33 isolates from unprocessed food, FPEs, and in RTE food in BC and arbitrarily divided the isolates into three categories: fast (LPD < 70 h), intermediate (70 h < LPD < 200 h), and slow (LPD > 200 h) adapters. Similar groups were assigned by Arguedas-Villa et al. (2010) where fast adapters had a LPD ranging from 7 to 30 h, intermediate strains had LPD between 54 and 140 h, and 231 to 423 h for slow adapters. In this study LPD for 28 isolates ranged from 28 to 53 h, though no significant difference in LPD was seen between isolates.

Using the grouping suggested by Kovacevic et al. (2013a), all isolates in the subset assessed in this study could be designated as fast adapters. As these isolates were recovered primarily from the refrigerated seafood processing environment, rather than from a variety of sources, the observation of fast adapting strains of *L. monocytogenes* in this environment is not surprising. The ability to rapidly adapt to and grow in a cold environment is a beneficial trait to *L.*

monocytogenes in the FPE as cells are competing for nutrients with other microorganisms in this FPE (Durack et al., 2013).

Interestingly, more variability was seen in the growth rate of isolates compared to the LPD in this subset with mean EGR values ranging from 0.0179 (Lm 368, Lineage I) to 0.0223 ($\Delta log_{10}CFU/ml)/h$ (Lm 205, Lineage I). Arguedas-Villa et al. (2014) reported EGR values for isolates derived from foods and FPE in Canada and Switzerland ranging from approximately 0.001 to around 0.020 ($\Delta log_{10}CFU/ml)/h$ using similar methodology to this study. The EGR values from this study more closely resemble those of the Swiss *L. monocytogenes* tested by Arguedas-Villa et al. (2014), which had significantly higher EGR compared to isolates from Canadian sources. As there was no correlation between cold growth EGR and EGR of isolates in BHI at 30°C inherent growth characteristics of these strains does not explain the variability in EGR observed in this study. Instead, the differences seen in growth rate of isolates at 4°C could be due to differences in expression of cold shock proteins (Csps) and cold acclimation proteins (Caps), though further understanding of these potential differences would require additional studies on the gene expression in these strains after cold growth acclimation.

3.4.4.2 Salt stress adaptation and growth

This study assessed salt stress adaptation and growth by subjecting each isolate to 6% NaCl in BHI at 30°C over 24 h. Increases in extracellular solute concentration causes movement of water out of the cell through osmosis, resulting in cell dehydration and inhibition of substrate and cofactor transport into the cell (Durack et al., 2013). *L. monocytogenes* responds to osmotic stress through the accumulation of compatible solutes (Bae et al., 2012; Csonka, 1989). Several

studies have shown *L. monocytogenes'* ability to adapt to and grow under osmotic stress to be varied and diverse (Adrião et al., 2008; Bergholz et al., 2010; Faleiro et al., 2003; Ribeiro et al., 2014; Vialette et al., 2003). Although many have researched osmotolerance and osmoadaptation in *L. monocytogenes* the methods used vary in their design making it difficult to compare growth kinetic results across studies.

The findings in the present study show that all strains isolated from the FPE and from gull feces could grow at NaCl concentrations higher than what would be encountered in the cold-smoked salmon produced at this facility (2 to 3% water phase NaCl). All isolates were able to quickly adapt to the salt stress, with some isolates better able to adapt compared to others, possibly due to more efficient uptake of compatible solutes to regain osmotic balance. Although there was a difference seen in LPD, the narrow range seen between isolates revealed most isolates to be the same in this regard. Perhaps more differences between strains would have been seen at a higher salt concentration, as some methods have used 10 to 12.5% NaCl (Adrião et al., 2008; Durack et al., 2013; Ribeiro et al., 2014). This study used 6% to reflect salt concentrations closer to what would be expected in smoked seafood products, which typically ranges between 2% and 8% water phase salt content (Hwang et al., 2009).

While EGR values were significantly different when values derived from turbidity-based growth curves were compared, the significant differences between strains were not observed after validation was performed using the standard culture-based growth assay approach for isolates on the high and low end of the EGR range. Others have reported differences between growth rates calculated from plate count data and results obtained from optical density readings (Francois et

al., 2005). This is especially true for assessing growth kinetics for isolates in stressful conditions, as morphological changes in the cell may result in optical density values that do not reflect the actual cell numbers (Francois et al., 2005). For example, under salt stress conditions *L. monocytogenes* cells elongate (Choi and Yoon, 2013) potentially changing the relationship between optical density readings and cell density in this experiment, and thus misrepresenting the growth rate of the cells under this stress. Nonetheless, using cell density has been demonstrated to be a quick and easy method for general screening of isolate growth kinetics in the presence of stress (Faleiro et al., 2003; Francois et al., 2006), although culture-based methods allows for a more accurate depiction of growth under stress.

3.4.4.3 Relationship between cold and salt stress response

Researchers have noted that uptake of compatible solutes through membrane transporters, including BetL, Gbu, and OpuC, is a main contributor to both cold temperature and salt stress response in *L. monocytogenes* (Sleator et al., 2003a, 2003b). In addition, Durack et al. (2013) found that similar genetic mechanisms operate in response to these two stresses and under both stresses the general stress response regulator, sigma B (σ^B), is suppressed and genes controlled by alternative σ^L (RpoN) and CodY regulators are induced (Durack et al., 2013). As mechanisms involved in cold and salt stress response are similar, despite differing physiological effects of the two stresses (Durack et al., 2013; Schmid et al., 2009), this study assessed the correlation between cold and salt stress LPD and EGR values for each isolate. In this study no relationship could be found in regards to the ability of isolates to adapt to and grow in 6% NaCl and at 4°C. This lack of connection could be due to the limited variation between isolates LPD and EGR in the subset for both stresses and limited subset size.

3.4.5 Comparison of persistence attributes across isolates

Little overlap was seen between isolates with high adherence to abiotic surfaces, sanitizer resistance, and enhanced stress response. However, one isolate (Lm 116, Lineage II) did possess all three attributes. Interestingly, this isolate was only isolated once in 2012 and did not seem to persist in the processing environment despite having a suite of characteristics that may favor survival and growth in the FPE. While this study looked at each attribute individually it is important to note that combined these characteristics may have a synergistic effect. Pan et al. (2006) found that *L. monocytogenes* in biofilms were more resistant to sanitizers than the same strains in planktonic state. Additionally, numerous studies have shown that exposure to one stress may enhance adaptation or tolerance of *L. monocytogenes* to other environmental stresses (Bergholz et al., 2010; Faleiro et al., 2003; Lundén et al., 2003). Bergholz et al. (2010) reported enhanced adaptation of *L. monocytogenes* to salt stress after being exposed to 7°C. Knowing that some strains possess attributes that may enhance their survival in the FPE and in foods, it would be interesting to see, for example, if the high surface adherence seen in Lm 116 would contribute to further resistance to sanitizers and/or enhanced stress response.

The overall lack of association between recurring strains and traits that may enhance FPE survival and growth in this study is not surprising. Difficulty classifying persistent strains has often been cited as a limitation in research looking at mechanisms of *L. monocytogenes* persistence (Ferreira et al., 2014). Specifically, some recurring strains classified as persistent may not actually be persisting but instead are continuously reintroduced into the FPE (Ferreira et al., 2014). Conversely, sporadically classified strains may possess traits associated with enhanced survival and growth in the FPE or may be persisting but this persistence is not captured

through environmental monitoring (Ferreira et al., 2014). The two strains in this study classified as recurring were routinely isolated in the processing facility over five and 13-month periods. These strains were linked to raw materials from a specific supplier and were only present in the facility after processing of fish from this supplier. This observation suggests that these strains were being reintroduced, rather than persisting, in this facility. This would explain why no specific traits were seen in these isolates compared to strains isolated on one occurrence in the plant. That being said, understanding *L. monocytogenes* characteristics relevant to food processing, regardless of whether the strain is persisting or not, can provide a better picture of the population of *L. monocytogenes* in that FPE. This information could aid processors in development of plant-specific control strategies targeting characteristics specific to the *L. monocytogenes* at that facility improving food safety by reducing or eliminating these potentially persistent subtypes.

3.4.6 Conclusions

This study aimed to characterize *L. monocytogenes* isolated from a BC seafood processing facility for attributes associated with FPE persistence and enhanced survival and growth in RTE foods. While this research did not show a link between subtype recurrence and ability to adhere to abiotic surfaces, resist sanitizers, or adapt to common FPE stresses, the findings presented here did demonstrate that isolates from a BC seafood processing environment possessed traits associated with enhanced survival and growth in this environment and in RTE foods. All *L. monocytogenes* assessed could adhere to abiotic surfaces commonly found in the FPE, with some isolates possessing higher adherence compared to others in the subset. In addition, three isolates had low-level resistance to QACs commonly used as sanitizers in the FPE and all isolates could

quickly adapt to and grow at refrigerated temperatures and under osmotic stress. Resistance to QACs could be linked to the genetic determinant *bcrABC* in two resistant isolates, while one resistant isolate lacked known resistance determinants for QACs. Lineage II isolates were more often isolated from the FPE and isolates from this genetic group were associated with characteristics imparting enhanced tolerance to FPE stresses. These results suggest that *L. monocytogenes* in BC seafood processing environments may be able to adapt to the stresses in these environments and confirm the need for comprehensive sanitation measures and routine testing to ensure that control measures are effective in eliminating *L. monocytogenes* from the FPE.

Chapter 4: *Listeria monocytogenes* isolated from a ready-to-eat seafood processing facility in British Columbia possess characteristics associated with increased risk

4.1 Introduction

Listeria monocytogenes is a bacterial pathogen that can cause a severe human disease, listeriosis, in susceptible populations such as the elderly, immunocompromised individuals, and pregnant women (Health Canada, 2011a). Although the incidence of listeriosis is rare, this disease is linked to high morbidity and mortality with 94% of those developing invasive listeriosis hospitalized, and mortality ranging from 20 to 40% (Health Canada, 2011a; Scallan et al., 2011). From a public health perspective, the presence of *L. monocytogenes* in the food processing environment (FPE) is cause for concern because contamination of ready-to-eat (RTE) foods, and consequent cases of listeriosis, are most often associated with *L. monocytogenes* in these environments (Lappi et al., 2004). Three main risk factors are associated with developing listeriosis: (*i*) number of bacteria ingested, (*ii*) the strain causing infection, and (*iii*) the host (FDA/FSIS/CDC, 2003; Vázquez-Boland et al., 2001).

Foods containing less than 100 CFU/g *L. monocytogenes* pose little risk of causing listeriosis (Chen et al., 2003). The Health Canada policy on *L. monocytogenes* in RTE foods aims to limit the amount of this pathogen at consumption to 100 CFU/g through inspection, finished product testing, and environmental monitoring activities (Health Canada, 2011a). In this policy, RTE foods are categorized by their relative risk. Category 1 are foods that can support the growth of *L. monocytogenes*, defined as an increase of more than 0.5 $\log_{10}$CFU/g over the product shelf life (Health Canada, 2012b). Category 2 products pose less risk and can be subdivided into Category 2A (foods that can support the growth of *L. monocytogenes* but the levels would not exceed 100

CFU/g over the shelf life) or Category 2B (foods that do not support the growth of *L. monocytogenes*). The presence of any *L. monocytogenes* in a product under Category 1 is unacceptable under this policy whereas Category 2 products destined for retail can contain up to 100 CFU/g. Although it is well known that *L. monocytogenes* can survive and grow in some RTE products (FDA/FSIS/CDC, 2003; Jami et al., 2014; Tocmo et al., 2014), if the refrigerated shelf life is less than five days these products are considered Category 2A, as the narrow shelf life would likely limit the opportunity of *L. monocytogenes* to increase by more than 0.5 $\log_{10}$CFU/g (Health Canada, 2012b).

Under the Health Canada policy, all *L. monocytogenes* pose equal risk to the population, though research has shown that strains vary in their ability to cause listeriosis (Cantinelli et al., 2013; Kathariou, 2002; Liu, 2006; Nightingale et al., 2008, 2005). For instance, only three of the 13 serotypes, 1/2a, 1/2b, and 4b, have been implicated in the majority of listeriosis cases (Swaminathan and Gerner-Smidt, 2007). Van Stelten et al. (2011) suggest that *L. monocytogenes* be divided into two groups based on their capacity to cause disease. The first group includes epidemic clone strains that have been associated with large-scale outbreaks worldwide (Cantinelli et al., 2013). In Canada, one epidemic clone, ECV, has been causing human illness for more than two decades (Gilmour et al., 2010; Knabel et al., 2012). The second group includes *L. monocytogenes* that possess attenuated virulence characteristics and are only associated with listeriosis on rare occasions (Van Stelten et al., 2011). Mutations often linked with attenuated virulence result in a premature stop codon (PMSC) in *inlA*, resulting in truncated and secreted InlA (Jacquet et al., 2004; Nightingale et al., 2005). As InlA is required for invasion of the host intestinal barrier, these PMSCs result in an attenuated virulence phenotype (Chen et al., 2011;

Van Stelten et al., 2011). Together, these markers can be used to better understand strain specific risks associated with a population of *L. monocytogenes.*

Treatment of invasive listeriosis often requires antibiotic intervention (Lungu et al., 2011; Schlech, 2000). In general, resistance to clinically relevant antibiotics is not common in *L. monocytogenes* (Lungu et al., 2011). However, repeated exposure of *L. monocytogenes* to stresses in the food chain may select for resistance mechanisms or promote horizontal transfer of genetic material conferring antibiotic resistance (Allen et al., 2015; Gómez et al., 2014; Lungu et al., 2011). For this reason regular surveillance of antibiotic resistance in *L. monocytogenes* isolated from the FPE is important for detecting potential resistance that may be cause for concern from the public health perspective (Allen et al., 2015; Granier et al., 2011).

This study assessed the potential public health risk of *L. monocytogenes* isolated from a RTE seafood processing facility in British Columbia (BC). As this facility produces cold-smoked salmon with a refrigerated shelf life of three days (Category 2A product) a challenge study was used to estimate the number of bacteria ingested in a product after this shelf life. In addition, markers previously linked to virulence of strains, as described above, were assessed for a subset of *L. monocytogenes*, and resistance of these strains to clinically relevant antibiotics was determined.

4.2 Materials and methods

4.2.1 Challenge study

4.2.1.1 Product characteristics

Wet-cured cold-smoked salmon finished product manufactured in a BC RTE seafood processing facility was used in this study. This product is frozen after packaging and stored at <-18°C until thawed by the consumer. Frozen, vacuum-sealed products were obtained from the processor and stored at -20°C until use, approximately three months from the manufacturing date.

4.2.1.2 Bacterial strains and inoculum preparation

Isolates (Table 4.1) frozen in 20% glycerol tryptic soy broth (TSB) at -80°C were streaked onto tryptic soy agar (TSA) and incubated at 37°C for 24 h. A single colony of each strain was cultured individually in 5 ml TSB for 18 h at 37°C with shaking, and then sub-cultured (100 µl) in 5 ml TSB for 7 days at 7°C under static conditions. Cultures were standardized to OD_{600} = 0.65 (BioSpec-mini, Shimadzu, Tokyo, Japan), centrifuged (10,000 $\times g$, 2 min), supernatants were removed, pellets were suspended in peptone water, and each strain was combined in equal proportions to produce a cocktail. The cocktail was serially diluted in peptone water to the appropriate cell concentrations to achieve <2 $\log_{10}$CFU/g and ~3 $\log_{10}$CFU/g target inoculation levels in the cold-smoked salmon products.

Table 4.1 *Listeria monocytogenes* strains used in challenge study cocktail.

Isolate	Characteristics
08-5578	Reference strain, Lineage II, implicated in outbreak (Gilmour et al., 2010)
Lm 193	Recurring strain, Lineage I, isolated from cutting board
Lm 361	Sporadic strain, Lineage II, isolated from cutting board
Lm 372	Recurring strain, Lineage II, isolated from cutting board
Lm 376	Sporadic strain, Lineage I, isolated from cutting board

4.2.1.3 Growth assessment of *L. monocytogenes* in cold smoked salmon

On the day before the experiment, the vacuum seal of each package was broken and products were thawed at 4°C overnight (as per package instructions). Once thawed, this product has a shelf life of three days, as indicated on the label. Cold-smoked salmon was weighed (25 ± 0.5 g) into sterile WhirlPak bags. The *L. monocytogenes* cocktail described above was pipetted (0.1 ml) onto the surface of each sample and spread by gently massaging the bag by hand for 30 sec. Samples were incubated at 7°C for the duration of the study. Enumeration on Oxford (OXA) and Palcam (PAL) selective agar was completed on days 0, 3 (end of shelf life), and 5 after 1:5 dilution in pre-chilled peptone water and stomaching (230 rpm, 2 min), following MFLP-77 (Health Canada, 2011c). Three batches of product were used for each inoculum level with three technical replicates analyzed at each time point for each batch. Un-inoculated day 0 samples were examined for presence of *Listeria* spp. following MFHPB-30 protocol (Health Canada, 2011b).

4.2.1.4 Physicochemical analysis

In addition to microbiological assessment, physicochemical attributes (pH, water activity, moisture content, and salt content) of each batch were tested. Three technical replicates were measured for each batch on each plating day for all attributes. pH was assessed by inserting a calibrated surface pH probe directly into the product. Water activity was assessed by placing ~ 0.5 g of sample in a plastic dish and reading the sample on a water activity meter (AquaLab 3, Decagon Devices, Inc., Pullman, WA). Moisture content (wet basis) was determined using standard air-drying protocol (105°C, 24 h). Salt content was determined by diluting 10 g of product in 90 ml of boiling water in a filter bag, stomaching (230 rpm, 5 min), and measuring the

chloride concentration with high range Chloride QuanTab test strips (Hach, Loveland, CO). Water phase salt (WPS) content of each batch was calculated following the guidelines by Hilderbrand (2000).

4.2.1.5 Statistical analysis

One-way analysis of variance (ANOVA) and Student's t-test were used to assess differences in growth between batches and inoculum levels. If there were significant differences found using ANOVA, Tukey's HSD test was employed to assess differences within the group. Pearson's correlation was used to assess relationships in data. All statistics were performed using Minitab Version 17, with differences with $p<0.05$ considered significant.

4.2.2 Virulence potential

4.2.2.1 Bacterial isolates

A total of 28 *L. monocytogenes* isolates were selected to assess virulence potential. This subset was subtyped by pulsed-field gel electrophoresis (PFGE) and ribotyping (Chapter 2) and characterized (Chapter 3) in previous studies. All unique pulsotypes were represented in this subset in order to assess inter-strain differences, with two pulsotypes including more than one isolate to measure intra-strain differences.

4.2.2.2 Serotyping

The Listeriosis Reference Service for Canada generously completed serotyping on all isolates in the subset. Serotyping was done for both O- and H-antigens using agglutination according to the manufacturer's instructions (Denken Seiken Co., Ltd., Tokyo, Japan).

4.2.2.3 *inlA* gene sequencing

The 2,400 bp i*nlA* gene was sequenced for all isolates in the subset to determine if these isolates possessed a PMSC as an indicator of attenuated virulence. Conventional polymerase chain reaction (PCR) was used to amplify *inlA* as described by Ragon et al. (2008) for each isolate. A 100 µl reaction mixture was prepared with 250 ng template DNA (prepared as per section 3.2.1.1) and included 1X AmpliTaq buffer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$ 0.5 µM of inlA-F and inlA-R primers (Table 4.2), and 2.5 U AmpliTaq 360 DNA polymerase. Thermocycling was carried out under the following conditions: 94°C for 10 min, 35 cycles of 94°C for 30 sec, 55.2°C for 30 sec, 72°C for 90 sec, followed by a final extension at 72°C for 10 min. PCR products were purified with the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Oakville, ON), following the manufacturer's protocol, and then sequenced offsite with Sanger sequencing (GENEWIZ Inc., South Plainfeld, NJ) using primers outlined in Table 4.2. Nucleotide sequences were assembled and analyzed for the presence of PMSCs by comparing obtained i*nlA* sequence data to that of the *L. monocytogenes* EGDe reference strain (Glaser et al., 2001) with Geneious software (version 6.0.6, Biomatters Ltd.).

Table 4.2 Primers used for *inlA* profiling**.**

Use	Primer	Primer sequence (5'-3')	Product size (bp)
PCR and sequencing	inlA-F	CGG ATG CAG GAG AAA ATC C	2,564
	inlA-R	CTT TCA CAC TAT CCT CTC C	
Internal sequencing primers (ISP)	inlA-ISP-F1	GAT ATA ACT CCA CTT GGG	N/A
	inlA-ISP-R1	GCT CTA AGT TAG TGA GTG CG	
	inlA-ISP-F2	GTG GAC GGC AAA GAA AC	N/A
	inlA-ISP-R2	GAG ATG TTG TTA CAC CGT C	

Primers described by Ragon et al. (2008). N/A: Not applicable.

4.2.2.4 Comparison of subset isolates to epidemic clone subtypes

To assess if any of the strains in the study subset may be related to strains implicated previously in listeriosis outbreaks, ribotypes of isolates in the subset were compared to published epidemic clone ribotypes (Table 4.3).

Table 4.3 Ribotypes associated with epidemic clones of *Listeria monocytogenes*.

Epidemic clone	*MLST type*	*Serotype*	*Associated ribotype(s)*
I	CC1	4b	DUP-1038 DUP-1038B
II	CC6	4b	DUP-1044 DUP-1044A
III	CC11	1/2a	DUP-1053A
IV/Ia	CC2	4b	DUP-1042 DUP-1042B

Adapted from Chen et al. (2007).

4.2.3 Antibiotic resistance screening

Resistance to antibiotics was assessed by disc diffusion assay for all isolates in the subset. Isolates were grown at 37°C for 18 h in TSB with shaking (180 rpm), diluted to approximately 10^7 CFU/ml in tempered 0.75% agar, mixed gently by vortexing, and poured onto Muller-Hinton agar (MHA). Once solidified, antibiotic susceptibility test discs (BBL Sensi-Discs, Franklin Lakes, NJ) were applied and plates were incubated at 37°C for 24 h. Similar to Kovacevic et al. (2013b), a panel of 17 antibiotic agents representing 12 antibiotic classes were selected for screening (Table 4.4). After incubation, zones of inhibition were measured to the nearest millimeter. Isolates were classified as resistant, intermediate (reduced susceptibility), or sensitive (susceptible) to each antibiotic based on EUCAST (2012) and CLSI (2012) guidelines. CLSI staphylococci diameter breakpoints were applied for antibiotics without *L. monocytogenes* criteria available. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains.

Table 4.4 Antibiotics used in resistance screening.

Class	*Agent*	*Abbreviation*	*Disc potency (μg)*
Aminoglycosides	Amikacin	AMK	30
	Gentamicin	GEN	10
	Kanamycin	KAN	30
	Streptomycin	STR	10
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	SXT	10
	Trimethoprim	TMP	5
Quinolones	Ciprofloxacin	CIP	5
	Nalidixic acid	NAL	30
Pencillin	Ampicillin	AMP	10
Cephalosporin	Cefoxitin	FOX	30
Phenicol	Chloramphenicol	CHL	30
Oxazolidinone	Linezolid	LZD	30
Lincosamide	Clindamycin	CLI	2
Macrolide	Erythromycin	ERY	15
Carbapenems	Imipenem	IPM	10
Ansamycin	Rifampicin	RIF	5
Tetracyclines	Tetracycline	TET	30

4.3 Results

4.3.1 Challenge study

Un-inoculated cold-smoked salmon samples tested negative for the presence of *Listeria* spp. following MFHPB-30. Cold-smoked salmon product supported the growth of *L. monocytogenes* strains derived from a BC seafood processing facility (Figure 4.1). At a starting inoculum of <2 $\log_{10}$CFU/g, *L. monocytogenes* was able to reach approximately 3 $\log_{10}$CFU/g after three days and 4 $\log_{10}$CFU/g after five days. At a starting inoculum of ~3 $\log_{10}$CFU/g, these strains were able to reach approximately 4.5 $\log_{10}$CFU/g after three days and 5.5 $\log_{10}$CFU/g in five days.

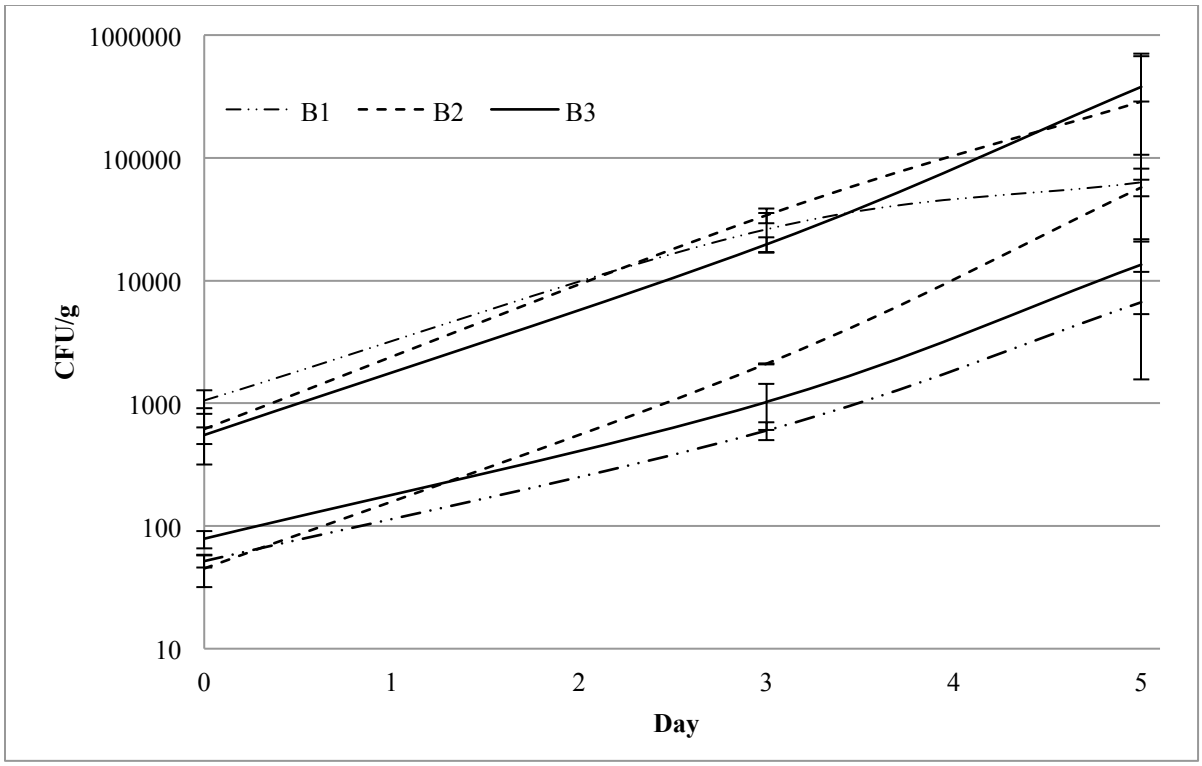


Figure 4.1 Growth of *Listeria monocytogenes* cocktail in cold-smoked salmon at 7°C. Data points are mean CFU/g and error bars represent standard deviation. B1, B2, and B3 indicated three different batches of product.

Table 4.5 Log_{10}CFU/g increase in *Listeria monocytogenes* in cold-smoked salmon at 7°C.

Target Inoculum level	***Batch[a]***	***Starting Inoculum (log_{10}CFU/g)***	***log_{10}CFU/g increase from day 0*** **Day 3**	**Day 5**
<2 log_{10}CFU/g	B1	1.71 ± 0.05	1.06 ± 0.07	2.11 ± 0.32
	B2	1.64 ± 0.12	1.67 ± 0.01	3.11 ± 0.07
	B3	1.89 ± 0.07	1.12 ± 0.17	2.24 ± 0.35
~3 log_{10}CFU/g	B1	3.01 ± 0.10	1.40 ± 0.15	1.78 ± 0.37
	B2	2.75 ± 0.25	1.74 ± 0.06	2.67 ± 0.76
	B3	2.74 ± 0.07	1.55 ± 0.06	2.84 ± 0.40

Values represent mean ± standard deviation. [a] B1, B2, and B3 represent different batches of cold-smoked salmon.

The inoculum level did not have an effect on growth potential of *L. monocytogenes* in the products. The mean log_{10}CFU/g increase from the start of the experiment to day three across all batches was higher for ~3 log_{10}CFU/g (1.575 log_{10}CFU/g) starting level compared to <2 log_{10}CFU/g (1.280 log_{10}CFU/g) but this difference was not significant (p=0.053; Student's t-

test). Additionally, the mean $\log_{10}$CFU/g increase after five days was not significantly different between the two inoculum levels ($p=0.519$; Student's t-test).

There was, however, a difference in growth seen between batches inoculated with <2 $\log_{10}$CFU/g, where B2 had a significantly higher $\log_{10}$ CFU/g increase after both three ($p=0.006$; one-way ANOVA, Tukey's HSD) and five ($p=0.006$; one-way ANOVA, Tukey's HSD) days of incubation at 7°C compared to B1 and B3. This difference was not observed for samples initially inoculated with ~3 $\log_{10}$CFU/g ($p>0.15$; one-way ANOVA).

Although there were not enough data points collected to run comprehensive regression analysis to assess effects of physicochemical attributes on *L. monocytogenes* growth in these products, some general observations can be made from the physicochemical characteristics gathered in this study. Overall, there was little change in pH, water activity, moisture content, and water phase salt over the five-day test period (Table 4.6). However, water phase salt was significantly lower for B2 compared to B1 and B3 on all three days (Table 4.6; $p<0.001$; one-way ANOVA). B2 was also a sliced product, whereas B1 and B3 were whole cold-smoked fillets (Table 4.6).

4.3.2 Virulence potential

Fourteen PFGE pulsotypes were recovered in the processing facility, two from 2012, and 12 from January 2013 to June 2014. The predominant serotype was 1/2a (59%), followed by 4b (25%), and one each of 1/2b and 1/2c (Figure 4.2A). During this same sampling period, eight pulsotypes were recovered in gull dropping in the parking lot exterior to the plant. Among

isolates collected from gull feces, the majority of strains were 1/2a (63%), with the remainder being 4b (37%; Figure 4.2B).

Table 4.6 Product characteristics and physicochemical properties of cold-smoked salmon over the study duration.

Characteristic		***B1***	***B2***	***B3***
Product ingredients		Pacific Coho salmon, salt, natural wood smoke		
Shelf life		Three days after thawing		
Product type		Whole fillet	Sliced fillet	Whole fillet
pH	Day 0	6.03 ± 0.03	6.03 ± 0.01	5.97 ± 0.03
	Day 3	6.11 ± 0.01	6.00 ± 0.04	6.06 ± 0.02
	Day 5	6.10 ± 0.04	6.04 ± 0.05	6.10 ± 0.04
Water activity	Day 0	0.961 ± 0.005	0.958 ± 0.010	0.962 ± 0.008
(a_w)	Day 3	0.947 ± 0.005	0.953 ± 0.006	0.943 ± 0.006
	Day 5	0.951 ± 0.002	0.951 ± 0.008	0.953 ± 0.001
Moisture	Day 0	72.90 ± 0.44	72.09 ± 0.52	66.02 ± 0.77
(% wet basis)	Day 3	73.21 ± 1.44	68.34 ± 2.24	68.30 ± 1.79
	Day 5	73.32 ± 0.77	70.84 ± 1.79	65.07 ± 0.68
Water phase salt	Day 0	2.44 ± 0.05	1.33 ± 0.12	2.78 ± 0.07
(% WPS)	Day 3	2.43 ± 0.02	1.40 ± 0.13	2.70 ± 0.09
	Day 5	2.43 ± 0.06	1.36 ± 0.15	2.82 ± 0.09

Values are mean ± standard deviation of three technical replicates. B1, B2, and B3 represent different batches of cold-smoked salmon.

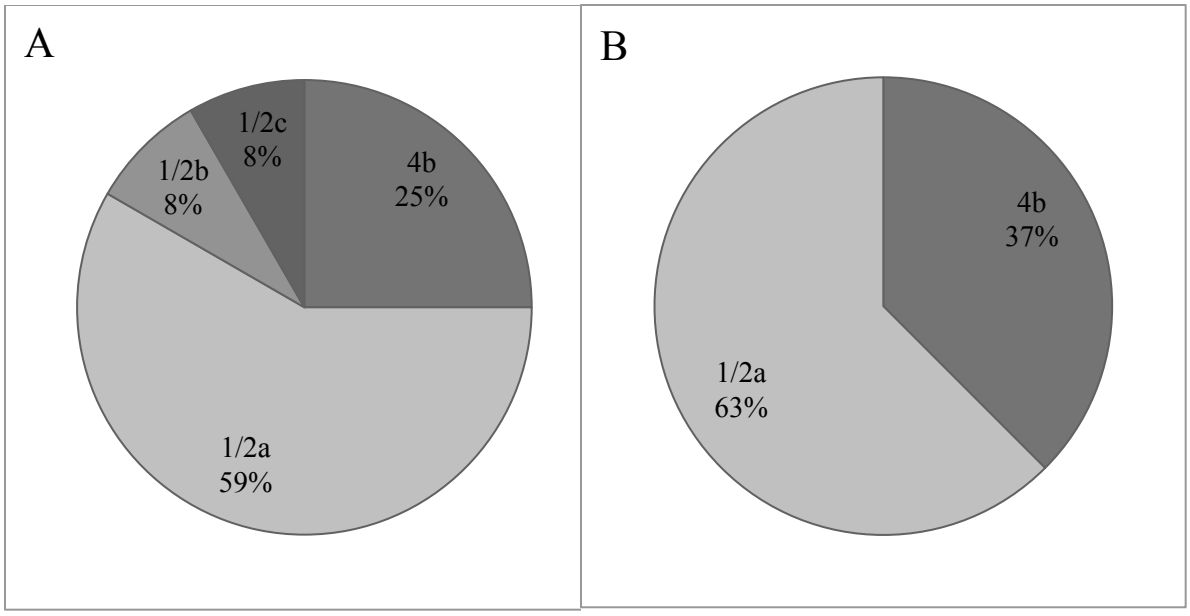


Figure 4.1 Distribution of *Listeria monocytogenes* serotypes for strains isolated over 18 months from (A) inside the seafood processing plant (n=12) and (B) exterior the facilities in gull feces (n=8).

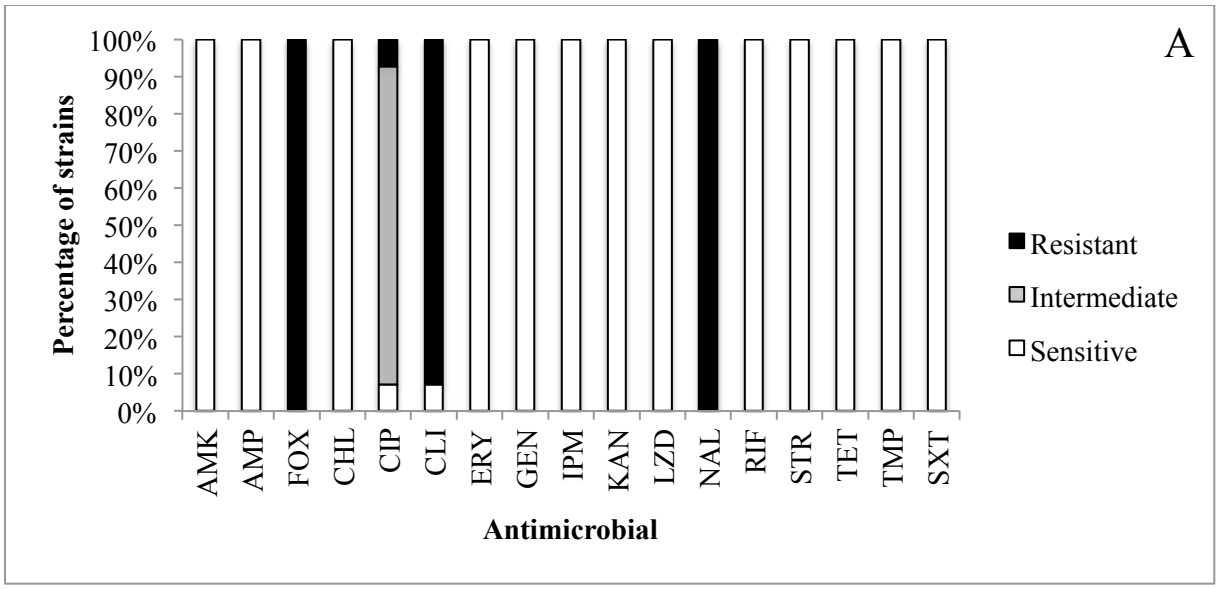
All five isolates from recurring pulsotype LMACI.0775/LMAAI.1129 and the single isolate from LMACI.0060/LMAAI.0857, both serotype 4b, had a three-codon deletion in *inlA* from amino acid 738 to 740, relative to EGDe. Both of these strains were found in the processing facility, with LMACI.0775/LMAAI.1129 recovered on both raw salmon surface and the cutting boards, and LMACI.0060/LMAAI.0857 isolated from finished product. Lm 360 (1/2c; recovered from raw salmon surface) possessed a PMSC in *inlA* at amino acid 760. The remaining strains encoded full-length InlA protein (see Appendix E).

Lm 376 (4b; cutting board) and Lm 366 (4b; gull feces) belong to ribotype DUP-1038 associated with epidemic clone I strains. Lm 205 (4b; gull feces) and Lm 363 (1/2b; cutting board) were ribotype DUP-1044, one of the ribotypes associated with epidemic clone II *L. monocytogenes*. No other ribotypes from the study subset could be linked to the epidemic clones associated ribotypes listed in Table 4.1.

4.3.3 Antibiotic resistance screening

The results for controls used in the study were within the acceptable limits defined by CLSI (2012). To assess intra-strain variation in antibiotic resistance, isolates from the same pulsotypes were compared. Three isolates (Lm 359, Lm 372, and Lm 380) from LMACI.0083/LMAAI.0086 possessed the same profile (Appendix E). Lm 193 and Lm 356 (both LMACI.0775/LMAAI.1129) varied slightly in their antibiotic resistance profiles. Lm 193 displayed resistance to CIP and Lm 356 had intermediate resistance to CLI. All other isolates from pulsotype LMACI.0775/LMAAI.1129 (Lm 200, Lm 368, and Lm 381) showed resistance to CLI, FOX, and NAL, and decreased susceptibility to CIP (Appendix E).

All strains in the study subset were resistant to FOX and NAL. Resistance to CLI was present in all but one strain (Lm 377; isolated from cutting board) (Figure 4.3). Intermediate resistance to CIP was observed for all strains except Lm 196, which was resistant, and Lm 376, which was sensitive (Figure 4.3; Appendix E). Multiple resistance phenotypes (i.e., resistance to $\geq$ 4 different classes of antibiotics) were not observed for any of the strains in this subset (Cerf et al., 2010). Cross-resistance, or resistance to different antibiotics belonging to the same class (Cerf et al., 2010), was seen in the quinolones for all but one isolate.



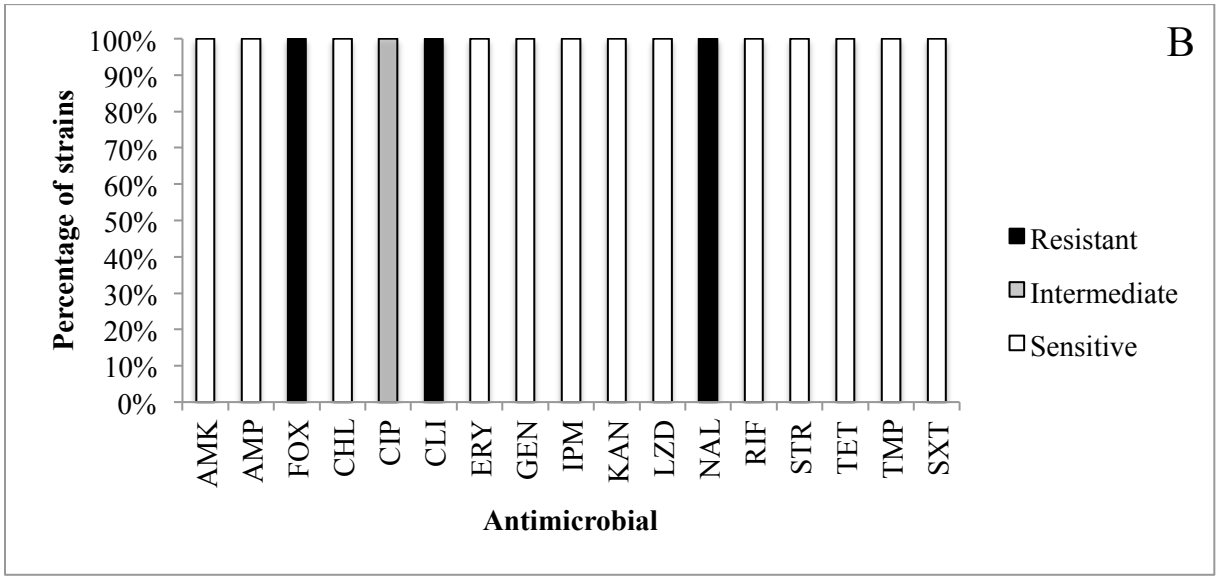


Figure 4.3 Antibiotic resistance of *Listeria monocytogenes* recovered from (A) inside a BC seafood processing facility and (B) exterior to the facility in gull feces (see Table 4.4 for list of abbreviations).

4.4 Discussion and conclusions

4.4.1 Challenge study

Cold-smoked salmon supported growth of a cocktail of five *L. monocytogenes* strains from <2 $\log_{10}$CFU/g to around 3 $\log_{10}$CFU/g over the indicated refrigerated shelf life of the product (three

days). The mean $\log_{10}$CFU/g increase from starting inoculum after three days (± standard deviation) was 1.28 ± 0.34, well over the 0.5 $\log_{10}$CFU/g increase that indicates a product able to support the growth of this pathogen under the Health Canada definition (Table 4.5).

These results are comparable to what others have observed. In a study by Kang et al. (2012) cold-smoked salmon inoculated with approximately 4.5 $\log_{10}$CFU/g *L. monocytogenes*, previously adapted to nutrient-deprivation and low temperature stress, had no lag phase (similar to this study; Figure 4.1) and a mean growth rate of 0.26 $\log_{10}$CFU/g/day, equivalent to a 0.78 $\log_{10}$CFU/g increase in three days. Hwang and Sheen (2009) inoculated cold-smoked salmon with 1 to 2 $\log_{10}$CFU/g non-adapted *L. monocytogenes* and stored product at 8°C. In contrast to the present study and the results from Kang et al. (2012), their study reported a lag phase duration ranging between 86 h and 115 h, most likely due to the non-adapted physiological state of the cells used by these investigators. However, the growth rate, 0.0163 $\log_{10}$ CFU/g/h (equivalent to 1.17 $\log_{10}$ CFU/g increase after three days), was comparable. Both studies reported a growth slightly lower than findings of the present study. Research has shown that *L. monocytogenes* grow faster under aerobic conditions compared to anaerobic conditions in nutrient rich broth (Romick et al., 1995) and therefore this lower growth rate may be explained by the anaerobic growth conditions of these studies compared to aerobic conditions used in this study to mimic the conditions of storage for this particular product.

While these results may cause reason for concern, it is important to note that inoculation of the product was done after thawing. Therefore, this study was not reflective of conditions that the bacteria would endure through frozen storage and distribution steps. Studies have shown that,

although *L. monocytogenes* can survive frozen storage, these conditions can injure bacterial cells (Dalgaard and Vigel Jørgensen, 1998; Embarek, 1994; Flanders and Donnelly, 1994; Kang et al., 2012). This provides an explanation for no or less than expected growth in naturally contaminated cold-smoked salmon compared to studies that inoculate product after thawing. On the other hand, the thawing step may actually aid in growth of *L. monocytogenes* in cold-smoked salmon (Kang et al., 2012) as freezing causes muscle structural damage in the product resulting in release of nutrients and increased surface area that may accelerate growth of *L. monocytogenes* upon thawing (Sigurgisladottir et al., 2000).

A variety of factors have been cited to influence growth of *L. monocytogenes* in challenge studies. Comparison of the results of this study to others, as done above, is difficult due to experimental methodologies that differ in extrinsic factors (e.g., temperature, oxygen availability), *L. monocytogenes* strains, inoculation levels and physiological state of cells (Beaufort et al., 2014; Cornu et al., 2006; Health Canada, 2012b). This study closely followed the guidelines by Health Canada (2012b) in order to best reflect the circumstances the product would encounter in the hands of the consumer. A cocktail of strains representing different lineages and classifications of *L. monocytogenes* was used and inoculated at two levels, one under the regulatory limit of 100 CFU/g (or 2 $\log_{10}$CFU/g) and the other at ~3 $\log_{10}$CFU/g. Although some research suggests that an increase in starting levels could overwhelm the preservation systems in the product causing an increase in growth potential (Dalgaard and Vigel Jørgensen, 1998), no significant difference was seen between the two starting inoculums and $\log_{10}$CFU/g increase after three and five days of growth in this study.

Product specific factors may also influence growth of *L. monocytogenes* including intrinsic factors (e.g., pH, water activity, moisture content, salt content, phenolic compounds) and associated microflora. Cold-smoked salmon typically has a pH between 5.9 to 6.3 and water activity ranging from 0.95 to 0.98 and contains 65% to 78% moisture (wet basis) and 2% to 8% water phase salt (Cornu et al., 2006; Hwang et al., 2009). The cold-smoked salmon used in this study was within these ranges for all intrinsic characteristics tested over the five-day study duration, with the exception of water phase salt values for B2 product, which was significantly lower than B1 and B3 (Table 4.6). B2 also had a significantly higher $\log_{10}$CFU/g increase through the study. It is tempting to suggest that the lower water phase salt in this product may be a factor in the growth differences seen, as others have found variability in water phase salt to have a great impact on growth potential of *L. monocytogenes* (Cornu et al., 2006). However, other factors that were not measured, including associated microflora (Rorvik et al., 1991) and phenolic compounds resulting from smoking (Dalgaard and Vigel Jørgensen, 1998), may also play a role in the differences observed. In addition, B2 was a sliced product, compared to B1 and B3, which were both whole fillets. Perhaps the physical characteristics of the sliced product (e.g., increased surface area and access to nutrients) may have played into the increased growth observed for this specific batch.

As there is no kill step in the cold-smoking process and typical hurdle strategies used in cold-smoked salmon production (e.g., curing, smoking, and vacuum-packing) are not sufficient to inhibit *L. monocytogenes* growth (Cornu et al., 2006; Tang et al., 2013), contamination of this product may pose serious risks to consumers. Health Canada (2012b) recommends processors consider product reformulation to reduce the risk of *L. monocytogenes* growth through additional

antimicrobial hurdles. Some researchers suggest addition of bactericidal or bacteriostatic antimicrobial agents, such as nisin, potassium lactate, and sodium diacetate as these agents are effective at reducing *L. monocytogenes* population in cold-smoked salmon (Nilsson et al., 1997; Soni and Nannapaneni, 2010; Tang et al., 2013; Vogel et al., 2006). Processors, though, may not favour the use of chemical preservatives as consumers demand products that do not contain additives (Tomé et al., 2007). Additionally, in Canada, many of these agents are not approved for used in cold-smoked salmon (Health Canada, 2013). Other hurdle technologies, such as the use of bio-preservatives, anti-*Listeria* bacteriophage, and modified atmosphere packaging have also been explored in RTE products, including cold-smoked salmon (Huss et al., 2000; Soni and Nannapaneni, 2010; Soni et al., 2014; Tomé et al., 2007). In contrast, Tomé et al. (2007) propose that additives or processing aids may not be necessary, and instead suggests altering the cold-smoking process and intrinsic properties to provide optimal conditions for the growth of lactic acid bacteria (LAB) naturally in the product. LAB has been shown to inhibit growth of *L. monocytogenes* through both production of bacteriocins and through microbial antagonism (Tomé et al., 2007). Each intervention mentioned above has pros and cons (Huss et al., 2000), but should be considered on their own, or together, to reduce levels of *L. monocytogenes* in these high-risk products.

4.4.2 Assessment of virulence potential

Although there are 13 different serotypes of *L. monocytogenes,* serotype 1/2a, 1/2b and 4b strains cause over 95% of listeriosis in the United States (Swaminathan and Gerner-Smidt, 2007). Historically the majority of listeriosis outbreaks worldwide have been caused by 4b strains, suggesting that this serotype may be more virulent than others (Swaminathan and Gerner-Smidt,

2007), however in Canada 1/2a strains seem to cause the most illness (Knabel et al., 2012). Swaminathan and Gerner-Smidt (2007) observed that many countries are seeing a shift from outbreaks primarily caused by 4b serotypes to blood associated diseases associated with 1/2a and 1/2b serotypes. The authors cite that increased sensitivity of blood testing and a decrease in large-scale 4b outbreaks could explain this shift (Swaminathan and Gerner-Smidt, 2007). In Canada, the predominance of 1/2a *L. monocytogenes* in listeriosis cases may also be linked to an epidemic clone (referred to as epidemic clone V) of this serotype implicated in many outbreaks over the last 20 years (Knabel et al., 2012).

Inside the seafood processing plant, 92% of isolates were serotype 4b, 1/2a, or 1/2b, with 1/2a predominating in this subset (Figure 4.2). *L. monocytogenes* isolated from gull feces in the parking lot of this facility were mainly serotype 1/2a (63%) and the remainder were 4b. From a risk characterization standpoint, these findings suggest that isolates present in the FPE may be able to cause disease if they were to contaminate food products at this establishment. As 1/2a are most often associated with the FPE (Orsi et al., 2011) and the majority of seafood isolates are 1/2a (Dauphin et al., 2001; Gudmundsdóttir et al., 2005), the findings of this study are not surprising.

Sequencing of the virulence gene *inlA* encoding the 800 amino acid protein InlA required for internalization of *L. monocytogenes* during infection also provides useful information on the virulence potential of different strains (Jacquet et al., 2004; Nightingale et al., 2005). Single nucleotide polymorphisms in *inlA* resulting in PMSCs upstream the LPXTG motif result in the expression of truncated forms of InlA that are secreted, rather than anchored to the bacterial cell

wall (Chen and Nightingale, 2013; Felício et al., 2007; Jonquieres et al., 1998; Nightingale et al., 2005). These mutations result in loss of InlA functionality and attenuated virulence (Jonquieres et al., 1998; Nightingale et al., 2005). Many investigators have supported the notion that PMSCs in *inlA* result in reduced invasion phenotype, with 20 different *inlA* PMSCs identified in *L. monocytogenes* from around the world to-date (Cantinelli et al., 2013; Ferreira et al., 2011; Kovacevic et al., 2013a; Ortiz et al., 2014b; Roche et al., 2005).

In this study, one strain (LMACI.0148/LMAAI.1187, 1/2c, isolated from raw salmon surface) possessed a PMSC in *inlA* at amino acid 760. This PMSC has not been described previously in the literature. The finding of a PMSC in the only serotype 1/2c in this subset is not unexpected, as previous work has shown PMSCs in *inlA* are more likely to belong to serotype 1/2a and 1/2c, rather than serotype 4b or 1/2b (Jacquet et al., 2004). In fact, 100% (n=25) of 1/2c strains screened by Jacquet et al. (2004) had *inlA* PMSCs. Although invasion assays were not performed to confirm that this PMSC would result in reduced invasiveness of this isolate, a PMSC at amino acid 760, upstream the LPXTG motif (amino acids 767-771), suggests that this PMSC would result in a truncated and secreted InlA protein and decreased invasion capabilities.

The observation that between 35% and 45% of *L. monocytogenes* isolated from FPEs and RTE foods possess PMSCs in *inlA* (Chen et al., 2011; Kovacevic et al., 2013a) has led many to suggest that these mutations may provide a fitness advantage to isolates in the FPE (Cruz et al., 2014; Roche et al., 2012). This hypothesis has been challenged, though not ruled out, by the finding that isolates with full-length InlA have increased ability to adapt to cold shock compared to isolates with *inlA* PMSCs (Kovacevic et al., 2013a). In BC, *L. monocytogenes* with *inlA*

PMSCs have been isolated from the FPE and RTE foods, with 33% (n=39) of seafood associated *L. monocytogenes* having a PMSC in *inlA*. The low incidence of *inlA* PMSCs in this study is a stark contrast to these findings. However, the low prevalence is in agreement with others, who also found most *L. monocytogenes* isolated from RTE seafood in Japan (Handa-Miya et al., 2007) and in retail delicatessen establishments in the United States (Wang et al., 2015) possess genes encoding full-length InlA. The lower prevalence of mutations in the *inlA* gene in this study might be explained by the small number of isolates within the subset analyzed originating from a single processing facility. Of five BC fish processing facilities examined in the study by Kovacevic et al. (2013a) three facilities had isolates lacking PMSCs while two facilities had PMSCs in the majority of recovered isolates.

This study also found six isolates, representing two unique pulsotypes (LMACI.0775/LMAAI.1129, 4b, isolated from raw salmon surface and cutting board; LMACI.0060/LMAAI.0857, 4b, isolated from finished product), with a three-codon deletion from amino acid 738 to 740. This three-codon deletion was previously described in *L. monocytogenes* isolates from a variety of sources, including epidemic clone II isolates (Cantinelli et al., 2013; Ferreira et al., 2014; Kovacevic et al., 2013a; Larivière-Gauthier et al., 2014; Roche et al., 2012). Ferreira et al. (2011) noted decreased invasion of Caco-2 cells by one strain possessing this three-codon deletion, but suggested that this mutation was not the cause of the decreased invasion phenotype. This in-frame deletion is in the pre-anchor region of the InlA protein and therefore would not interfere with the functional domain or anchoring motif (LPXTG) (Cantinelli et al., 2013). The finding of highly invasive isolates possessing this three-codon deletion provides support for this idea (Cantinelli et al., 2013; Kovacevic et al., 2013a).

Interestingly, most of the strains that have been found with this three-codon deletion belong to serotype 4b, a finding reflected in this study, although Kovacevic et al. (2013a) did describe one 1/2a isolate with the same three-codon deletion.

Finally, as ribotyping data is available for some epidemic clone strains in the literature (Chen et al., 2007), these ribotypes were compared to those in this study subset to determine if any of the strains within this subset may be associated with epidemic clone strains previously implicated in listeriosis outbreaks (Cantinelli et al., 2013; Chen et al., 2007). Although some strains in the study subsets were ribotypes that matched those of epidemic clones, it is difficult to infer that these strains are epidemic clones from this data alone. As previously mentioned, ribotyping is not as discriminatory as other subtyping methods, especially for serotype 4b isolates (Corcoran et al., 2006; Graves et al., 2007). A more discriminatory method, such as MLST or MvLST, or the multiplex PCR method targeting genes specific to each epidemic clone as described by Chen et al. (2007) should be considered for further comparison of these strains to genetic traits of known epidemic clones.

In summary, virulence potential for strains derived from a BC RTE seafood processing facility was approximated through assessment of genetic and phenotypic attributes associated with virulence or attenuated virulence of *L. monocytogenes* in the literature. The majority of isolates from this facility possessed traits associated with virulence, similar to what has been observed in other seafood processing environments (Gudmundsdóttir et al., 2006; Handa-Miya et al., 2007; Kovacevic et al., 2013a). Nearly all *L. monocytogenes* strains were from serotypes most frequently associated with disease, possessed full-length *inlA*, and had ribotypes associated with epidemic clones implicated in multiple outbreaks of listeriosis.

Although this subset possessed traits associated with virulence, it cannot be concluded that these strains are virulent based on this data alone. While nearly all the *L. monocytogenes* in this subset encoded full-length InlA, this gene is only one of many involved in virulence of this pathogen, any of which could be compromised by a mutation. For example, Roche et al. (2005) characterized 26 *L. monocytogenes* with low virulence phenotypes and found mutations resulting in loss of function in virulence genes *prfA, plcA,* and/or *plcB*. In addition, research has shown that even with a PMSC in *inlA*, listeriosis may occur through InlA independent mechanisms (Jacquet et al., 2004; Van Stelten et al., 2010), though most strains do show attenuated virulence with this mutation.

4.4.3 Antibiotic resistance screening

Treatment of invasive listeriosis often requires antibiotic intervention (Lungu et al., 2011; Schlech, 2000; Swaminathan and Gerner-Smidt, 2007). Antibiotics that are most commonly used for treatment of listeriosis include AMP in combination with an aminoglycoside such as GEN, or SXT (Lemoy et al., 2012; Painter and Slutsker, 2007; Schlech, 2000; Swaminathan and Gerner-Smidt, 2007). All isolates tested were susceptible to these three antibiotics. These results correspond with the general observation that resistance to clinically relevant treatment options is not common in *L. monocytogenes*, and previous findings reported for food chain derived *L. monocytogenes* in BC (Allen et al., 2015; Kovacevic et al., 2013b, 2012b; Lungu et al., 2011).

It is well established that *L. monocytogenes* have reduced susceptibility or resistance to a number of antibiotic classes: sulfomethoxazole, cephalosporins, and first-generation quinolones (Troxler

et al., 2000). Therefore, the resistance of all isolates to FOX (cephalosporin) and NAL (first-generation quinolone) was expected. However, the high proportion of resistance to CLI (95%) and reduced susceptibility (91%) or resistance (5%) to CIP in the 22 strains screened is surprising, as *L. monocytogenes* is typically susceptible to these antibiotics (Troxler et al., 2000). Similar resistance profiles were observed in a different set of *L. monocytogenes* derived from BC FPEs and RTE foods, though the present study did not show reduced susceptibility to STR and LZD or resistance to TET as did Kovacevic et al. (2012b).

CLI belongs to the lincosamide class of antibiotic agents that interfere with bacterial ribosomes to inhibit translation activities (Walsh, 2003). Resistance of *L. monocytogenes* to CLI has been reported previously (Chen et al., 2010; Davis and Jackson, 2009; Kovacevic et al., 2012b; Prazak et al., 2002). Kovacevic et al. (2012b) found that of 54 *L. monocytogenes* isolated from BC FPEs and RTE foods 33% were resistant and 67% had reduced susceptibility to CLI. The findings of this study report higher prevalence of resistance in this particular subset (95%) compared to these findings. However, the results from this present study are similar to other reports of CLI resistance in *Listeria* spp., including *L. monocytogenes* (Chen et al., 2010; Prazak et al., 2002).

CIP, a second generation cephalosporin, is a bactericidal agent that disrupts synthesis of peptidoglycan in Gram-positive organisms through inhibition of penicillin binding proteins required for crosslinking of peptidoglycan (Walsh, 2003). Kovacevic et al. (2012b) found resistance to CIP in 7% and reduced susceptibility in 67% of *L. monocytogenes* derived from BC FPE and RTE foods. Additionally, others have reported low prevalence of *L. monocytogenes* resistance to CIP (Davis and Jackson, 2009). In this study, most (91%) strains had reduced

susceptibility to CIP, with one strain possessing resistance. These findings are in agreement with the previous reports of CIP resistance.

Antibiotic resistance in *L. monocytogenes* is caused primarily through horizontal transfer of resistance genes via plasmids or transposons, or through the over-expression of efflux pumps (Charpentier et al., 1999; Godreuil et al., 2003; Lungu et al., 2011). Resistance for both CIP and CLI is mediated primarily by efflux pumps (Godreuil et al., 2003; Lismond et al., 2008; Mata et al., 2000), though some evidence suggests other mechanisms may contributing to resistance seen towards these antibiotic agents (Kovacevic et al., 2012b). Further evaluation of resistance mechanisms that may account for the intermediate and full resistance to CLI and CIP in the strains assessed in this study is needed.

4.4.4 Conclusions

Risk of contracting listeriosis depends on many factors, including the number of *L. monocytogenes* ingested, the virulence potential of the strain, and host susceptibility. The first two factors were assessed in this study to estimate the risk of listeriosis resulting from BC-manufactured cold-smoked salmon finished product *L. monocytogenes* contamination. Results showed that a cocktail of five *L. monocytogenes* strains starting at an acceptable concentration (<100 CFU/g) could grow to unsafe numbers in this product over the three-day refrigerated shelf life. While none of the strains in the subset could be definitively linked to epidemic clones, these strains possessed traits associated with virulence and listeriosis including majority being serotype 1/2a, 4b, or 1/2b and 95% encoding full-length InlA. These isolates did not possess resistance to AMP, GEN, or SXT, antibiotics commonly administered for treatment of invasive listeriosis.

However, uncharacteristic resistance to CIP and CLI was noted, similar to what has previously been seen in FPE and RTE food isolates from BC.

These results show that *L. monocytogenes* strains recovered from a BC seafood processing facility may be capable of causing listeriosis if transferred to RTE foods at this seafood processing facility. These findings highlight the need for processors of RTE seafood products to be vigilant in their control of *L. monocytogenes* in the processing plant environment to minimize risk of finished product contamination and potential threat of listeriosis to consumers of their product.

Chapter 5: Conclusion

Presence of *Listeria* spp., including pathogenic *Listeria monocytogenes,* have been observed in seafood processing environments and ready-to-eat (RTE) seafood in British Columbia (BC). Although efforts have been made to characterize strains isolated from these samples, there is limited data on the source of contamination and potential persistence of *L. monocytogenes* in BC seafood processing environments, as well as the risks that finished product contamination by these strains would pose to consumers. Building on current knowledge, this research aimed to evaluate the source, persistence attributes, and risk of *L. monocytogenes* strains isolated from a RTE seafood processing facility in BC.

Knowing that *L. monocytogenes* control is a challenge for RTE seafood processors, this study hypothesized that *Listeria* spp. could be isolated from the processing environment and finished products of a seafood processing facility. Of the 2,959 samples taken from raw materials, the processing environment, finished products, and the facility exterior of a single seafood processor in BC over 18 months, 121 (4.1%) were positive for *Listeria* spp. with *L. monocytogenes* making up 36% of these positive samples. Compared to other *Listeria* spp. prevalence findings in seafood processing environments available, the prevalence findings of this study were low, though comparisons like this are limited by differing study methodologies, and the fact that each plant seems to have unique contamination prevalence and profiles. In this processing plant, *L. monocytogenes* was primarily isolated from raw materials and pre-smoking area cutting boards, though *L. monocytogenes* contamination was not seen past these first two steps, aside from three occurrences of finished product contamination. Taken together this knowledge demonstrates that *L. monocytogenes* continues to be a challenge for BC seafood processors. However, the control

strategies in place at this facility were effective at eliminating this pathogen early in the process, though on rare occasions finished product contamination was observed.

Another challenge to processors of RTE seafood is persistence of certain strains of *L. monocytogenes* in the processing environment. Subtyping of *L. monocytogenes* recovered in this study together with statistical tools were used to investigate the hypothesis that *L. monocytogenes* was persisting in this particular BC seafood processing plant resulting in finished product contamination. Two persistent pulsotypes in the food processing environment (FPE) were observed, with this persistence supported by quantitative comparison of persistent ribotype frequencies to two publically available reference distributions. In addition, one of the persistent strains was responsible for finished product contamination. However, raw salmon surface and pre-operational FPE sampling provided evidence that raw materials were the primary source of contamination in this facility, and that strain reintroduction, rather than persistence, was the reason for repeated isolation of these two pulsotypes. These findings highlight the limitations of persistence categorization used in *L. monocytogenes* persistence research and point to the importance of raw materials testing in persistence research to rule-out reintroduction. In addition, the lack of persistence seen in this plant suggests that the control strategies used are effective at minimizing strain persistence.

Considering persistence from a different perspective, all unique *L. monocytogenes* strains isolated from this facility were assessed for characteristics putatively associated with *L. monocytogenes* persistence in the literature: surface adherence, sanitizer resistance, and response to environmental stresses. The findings of these experiments indicated that all strains adhered to

abiotic surfaces and adapted quickly to cold and salt stresses. In addition, three isolates had low-level resistance of quaternary ammonium compounds, though genetic resistant determinants were not found in one isolate. This study was unique in that all three attributes commonly linked to persistence were analyzed and compared. Within the study subset, strains typically possessed one attribute favorable to persistence in the FPE. The small sample size and lack of variability seen in these traits, though, made it difficult to categorize strains into groups for each of the characteristics assessed. Moreover, the lack of persistent strains in this study did not allow for any comparison of persistence and the attributes tested.

Finally, this research hypothesized that *L. monocytogenes* isolated from the FPE of a RTE seafood processor in BC have the capacity to pose risk to public health. The results of this study showed that *L. monocytogenes* strains recovered from a BC seafood processing facility may be capable of causing listeriosis if transferred to RTE foods. Cold-smoked salmon produced at this facility supported the growth of *L. monocytogenes* over the product shelf life from acceptable to unacceptable levels when inoculated post-thawing, based on Health Canada standards. Although worrying, the implication of these findings was limited, as they did not reflect the true conditions *L. monocytogenes* would endure through product distribution and storage. Risk approximation by serotyping and *inlA* profiling revealed that strains isolated from this plant were associated with serotypes causing listeriosis and that nearly all (93%; n=14) strains possessed full length *inlA*, a gene encoding InlA protein required for invasion of epithelial cells during the infection cycle. One strain possessed a novel pre-mature stop codon (PMSC) mutation, which is predicted to result in a truncated and secreted InlA protein. Though the indicators used in this evaluation

suggest that these strains may be able to cause listeriosis in humans, they did not show resistance to antibiotics commonly used to treat this disease.

While these results add to the understanding of *L. monocytogenes* in seafood processing facilities, more work is needed to develop a comprehensive understanding of *L. monocytogenes* populations in these environments. Firstly, this research emphasized the need for a more precise definition of persistence, as the current models for persistence categorization do not provide criteria that would rule out reintroduction of strains. Taking this into account, future work on source and persistence of *L. monocytogenes* in FPEs should be undertaken in additional facilities in order to assess how *L. monocytogenes* populations in these establishments compared to the findings of this study. Additionally, as the control strategies at the plant assessed in this study seemed to be effective at eliminating *L. monocytogenes* contamination, it may be interesting to compare control strategies and prevalence across other plants to see what methods are most effective at controlling this pathogen. While this study used well-known indicators for approximation of risk, there are many other tools that can be utilized to better estimate risks associated with these strains. For example, a challenge study designed to better mimic distribution and storage conditions endured by *L. monocytogenes* is needed to better estimate the behavior of this pathogen in contaminated product. Additionally, profiling of other virulence genes or other genetic profiling tools may be useful in providing a better picture of virulence potential for these strains. Finally, this study isolated unique strains, a quaternary ammonium compound resistant strain lacking a known genetic determinant and a strain possessing a novel *inlA* PMSC mutation. More work is needed to determine the characteristics of these strains and how they contribute to strain persistence and virulence.

This report highlights the importance of processor-level control strategies to minimize *L. monocytogenes* FPE persistence, product contamination, and risk to consumers. While *L. monocytogenes* strains were not persisting in the FPE at this facility over the study duration, the finding that this pathogen was routinely introduced on raw materials and possessed traits associated with persistence points to potential for strain persistence. In addition, the findings that cold-smoked salmon produced at this facility can support the growth of *L. monocytogenes* over the shelf life, and that these strains possess elements associated with virulence, demonstrate that finished product contamination poses risk to consumers. While these results improve understanding of *L. monocytogenes* in food environments from a scientific perspective, this research also provides valuable information to the processor on the source, persistence, and risk associated with *L. monocytogenes* in their facility. In this way, the findings of this research directly contribute to reducing the risks associated with *L. monocytogenes* in a BC seafood processing facility.

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Appendices

Appendix A: Environmental sampling sites

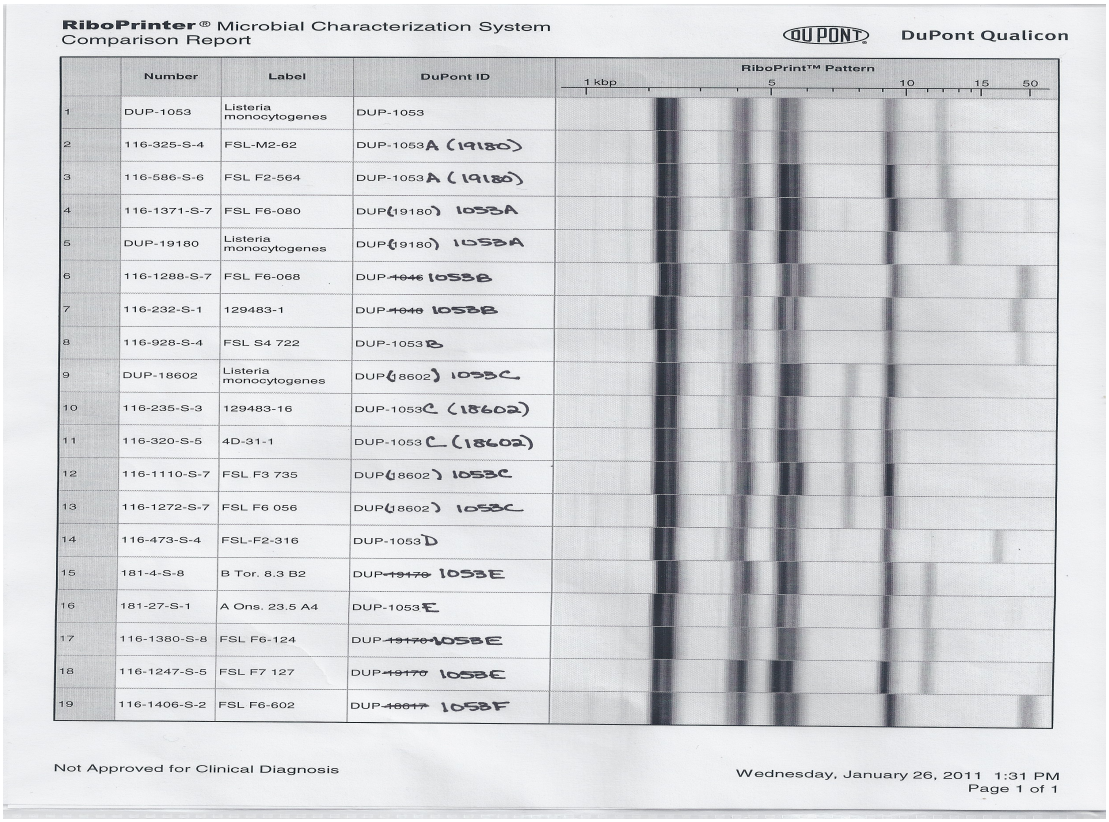
Table A.1 List of sampling sites used in environmental monitoring.

Sample Site	Room	Area	Sample Site	Room	Area
1	Blast Freezer	Floor	61	Shipping/Receiving	Hoses
2	Boxing Room	Vacuum packing machine	62	Shipping/Receiving	Forklift
3	Boxing Room	Floor mats	63	Shipping/Receiving	Ramps
4	Boxing Room	Scale	64	Shipping/Receiving	Drain
5	Boxing Room	Doors	65	Shop	General
6	Change Rooms	General	66	Slicing Room	Carts
7	Chill Room	Evaporators	67	Slicing Room	Cleaning tools
8	Chill Room	Tubs	68	Slicing Room	Slicing machine NFCS
9	Chill Room	Floors	69	Slicing Room	Evaporators
10	Chill Room	Carts	70	Slicing Room	Hand tools
11	Chill Room	Doors	71	Slicing Room	Bins
12	Clean Up Area	Cleaning tools	72	Slicing Room	Hand tools
13	Clean Up Area	Racks	73	Slicing Room	Tables
14	Clean Up Area	Doors	74	Slicing Room	Slicing machine FCS
15	Clean Up Area	Vents	75	Slicing Room	Styrofoam tray
16	Cold Storage	Plastic door cover	76	Slicing Room	Cutting boards
17	Cool Room	Mesh sheets	77	Slicing Room	Personnel
18	Cool Room	Floor	78	Slicing Room	Wall
19	Cool Room	Wall	79	Slicing Room	Levers
20	Cool Room	Doors	80	Slicing Room	Step stool
21	Cutting Room	Hand tools	81	Slicing Room	Hoses
22	Cutting Room	Bins	82	Smoker Room	Racks
23	Cutting Room	Cutting boards	83	Smoker Room	Cleaning tools
24	Cutting Room	Skinning machine FCS	84	Smoker Room	Smoker unit
25	Cutting Room	Brine injector FCS	85	Smoker Room	Maintenance tools
26	Cutting Room	Cleaning tools	86	Smoker Room	Floor
27	Cutting Room	Maintenance tools	87	Smoker Room	Drain
28	Cutting Room	Cutting tables	88	Smoker Room	Doors
29	Cutting Room	Carts	89	Thaw Room E	Racks
30	Cutting Room	Pinbone machine framework	90	Thaw Room E	Cooling unit
31	Cutting Room	Skinning machine framework	91	Thaw Room E	Drain
32	Cutting Room	Brine injector framework	92	Thaw Room E	Doors
33	Cutting Room	Evaporators	93	Thaw Room E	Drain
34	Cutting Room	Hand wash station	94	Thaw Room W	Cooling unit
35	Cutting Room	Drain	95	Thaw Room W	Wall
36	Cutting Room	Chairs/stools	96	Thaw Room W	Floor
37	Cutting Room	Hoses	97	Thaw Room W	Fish surface
38	Cutting Room	Clipboard holder	98	Tubbing Area	Tubs with plastic
39	Cutting Room	Garbage bins	99	Tubbing Area	Tubs
40	Cutting Room	Personnel	100	Tubbing Area	Drains
41	Exterior	General	101	Tubbing Area	Doors
42	Hallway A	Tubs with plastic	102	Tubbing Area	Pallet jack
43	Hallway A	Hoses	103	Tuna Room	Cleaning tools
44	Hallway A	Hand wash station	104	Tuna Room	Band saw framework
45	Hallway B	Tubs with plastic	105	Tuna Room	Tuna grinder framework
46	Hallway B	Pallets	106	Tuna Room	Tables
47	Hallway B	Carts	107	Tuna Room	Band saw machine FCS
48	Label Room	General	108	Tuna Room	Tuna grinder FCS

Sample Site	Room	Area	Sample Site	Room	Area
49	Lunch Room	General	109	Tuna Room	Bins
50	Mechanical Room	General	110	Tuna Room	Hand wash station
51	Office	General	111	Tuna Room	Pipes
52	QA Lab	General	112	Tuna Room	Drain
53	Racking Area	Racks	113	Vacpacking Room	Packing machine
54	Racking Area	Personnel	114	Vacpacking Room	Tables
55	Racking Area	Bins	115	Vacpacking Room	Step stool
56	Racking Area	Forklift	116	Vacpacking Room	Pallets
57	Racking Area	Floor	117	Vacpacking Room	Floor
58	Shipping/Receiving	Floors	118	Warehouse	General
59	Shipping/Receiving	Doors	119	Storage	Pallets
60	Shipping/Receiving	Pallets	120	Storage	Doors

General: sample was taken in various locations in room. FCS: Food contact surface. NFCS: Non-food contact surface.

Appendix B: DUP-1053 Riboprint pattern comparison



RiboPrinter® Microbial Characterization System
Comparison Report

DuPont Qualicon

	Number	Label	DuPont ID	RiboPrint™ Pattern (1 kbp, 5, 10, 15, 50)
1	DUP-1053	Listeria monocytogenes	DUP-1053	
2	116-325-S-4	FSL-M2-62	DUP-1053A (19180)	
3	116-586-S-6	FSL F2-564	DUP-1053A (19180)	
4	116-1371-S-7	FSL F6-080	DUP(19180) 1053A	
5	DUP-19180	Listeria monocytogenes	DUP(19180) 1053A	
6	116-1288-S-7	FSL F6-068	DUP~~1048~~ 1053B	
7	116-232-S-1	129483-1	DUP~~1048~~ 1053B	
8	116-928-S-4	FSL S4 722	DUP-1053B	
9	DUP-18602	Listeria monocytogenes	DUP(18602) 1053C	
10	116-235-S-3	129483-16	DUP-1053C (18602)	
11	116-320-S-5	4D-31-1	DUP-1053C (18602)	
12	116-1110-S-7	FSL F3 735	DUP(18602) 1053C	
13	116-1272-S-7	FSL F6 056	DUP(18602) 1053C	
14	116-473-S-4	FSL-F2-316	DUP-1053D	
15	181-4-S-8	B Tor. 8.3 B2	DUP~~19170~~ 1053E	
16	181-27-S-1	A Ons. 23.5 A4	DUP-1053E	
17	116-1380-S-8	FSL F6-124	DUP~~19170~~ 1053E	
18	116-1247-S-5	FSL F7 127	DUP~~19170~~ 1053E	
19	116-1406-S-2	FSL F6-602	DUP~~18017~~ 1053F	

Not Approved for Clinical Diagnosis

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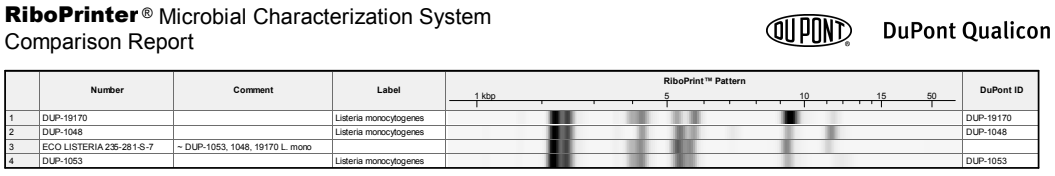
Figure B.1 Riboprint patterns for DUP-1053 strains from Food Microbe Tracker database.



RiboPrinter® Microbial Characterization System
Comparison Report

DuPont Qualicon

	Number	Comment	Label	RiboPrint™ Pattern (1 kbp, 5, 10, 15, 50)	DuPont ID
1	DUP-19170		Listeria monocytogenes		DUP-19170
2	DUP-1048		Listeria monocytogenes		DUP-1048
3	ECO LISTERIA 235-281-S-7	~ DUP-1053, 1048, 19170 L. mono			
4	DUP-1053		Listeria monocytogenes		DUP-1053

Figure B.2 Riboprint patterns for DUP-1053 strains from the study subset.

Appendix C: Summary of characterization results

Table C.1 Summary of mean or geometric mean* surface adherence and stress response data for each *Listeria monocytogenes* isolate.

Isolate	Surface adherence (CV-OD_{600})		Cold stress response		Salt stress response	
	Polystyrene*	PVC	LPD (h)	EGR (log_{10}CFU/ml)/h	LPD (h)*	EGR (OD_{600})/h*
Lm 115	0.220^{ABCDEF}	0.159^{ABCD}	52.7^{A}	0.0191^{ABCDE}	3.85^{ABCD}	0.0450^{AB}
Lm 116	0.314^{ABC}	0.217^{ABCD}	47.6^{A}	0.0193^{ABCDE}	2.95^{AB}	0.0463^{AB}
Lm 193	0.162^{CDEFG}	0.135^{BCD}	38.2^{A}	0.0210^{ABCDE}	3.44^{ABCD}	0.0474^{AB}
Lm 196	0.144^{EFG}	0.137^{BCD}	31.8^{A}	0.0204^{ABCDE}	3.54^{ABCD}	0.0514^{AB}
Lm 200	0.169^{CDEFG}	0.132^{BCD}	42.3^{A}	0.0205^{ABCDE}	3.05^{AB}	0.0484^{AB}
Lm 205	0.095^{G}	0.075^{D}	42.8^{A}	0.0223^{A}	2.83^{A}	0.0415^{AB}
Lm 207	0.140^{EFG}	0.112^{CD}	32.9^{A}	0.0210^{ABCDE}	3.17^{ABC}	0.0570^{AB}
Lm 208	0.273^{ABCDE}	0.217^{ABCD}	40.5^{A}	0.0209^{ABCDE}	3.71^{ABCD}	0.0575^{AB}
Lm 209	0.254^{ABCDE}	0.166^{ABCD}	33.2^{A}	0.0206^{ABCDE}	5.74^{CD}	0.0592^{AB}
Lm 356	0.192^{BCDEF}	0.157^{ABCD}	35.9^{A}	0.0214^{ABC}	4.34^{ABCD}	0.0464^{AB}
Lm 359	0.289^{ABCD}	0.257^{ABC}	38.4^{A}	0.0221^{AB}	4.78^{ABCD}	0.0403^{AB}
Lm 360	0.297^{ABCD}	0.281^{AB}	41.4^{A}	0.0214^{ABCD}	4.27^{ABCD}	0.0491^{AB}
Lm 361	0.241^{ABCDE}	0.220^{ABCD}	50.2^{A}	0.0204^{ABCDE}	4.14^{ABCD}	0.0455^{AB}
Lm 362	0.225^{ABCDEF}	0.250^{ABC}	45.7^{A}	0.0195^{ABCDE}	4.37^{ABCD}	0.0485^{AB}
Lm 363	0.380^{AB}	0.320^{A}	27.7^{A}	0.0180^{DE}	4.21^{ABCD}	0.0515^{AB}
Lm 364	0.262^{ABCDE}	0.217^{ABCD}	38.5^{A}	0.0196^{ABCDE}	4.31^{ABCD}	0.0420^{AB}
Lm 365	0.416^{A}	0.195^{ABCD}	32.0^{A}	0.0184^{CDE}	4.27^{ABCD}	0.0382^{A}
Lm 366	0.120^{FG}	0.108^{CD}	38.9^{A}	0.0191^{ABCDE}	6.00^{D}	0.0498^{AB}
Lm 367	0.223^{ABCDEF}	0.250^{ABC}	43.6^{A}	0.0187^{BCDE}	4.37^{ABCD}	0.0414^{AB}
Lm 368	0.155^{DEFG}	0.125^{BCD}	35.2^{A}	0.0179^{E}	3.89^{ABCD}	0.0409^{AB}
Lm 371	0.219^{ABCDEF}	0.199^{ABCD}	35.2^{A}	0.0183^{CDE}	3.98^{ABCD}	0.0442^{AB}
Lm 372	0.235^{ABCDEF}	0.196^{ABCD}	37.5^{A}	0.0184^{CDE}	4.49^{ABCD}	0.0415^{AB}
Lm 375	0.199^{BCDEF}	0.161^{ABCD}	42.9^{A}	0.0202^{ABCDE}	4.35^{ABCD}	0.0476^{AB}
Lm 376	0.138^{EFG}	0.100^{CD}	29.3^{A}	0.0192^{ABCDE}	4.14^{ABCD}	0.0499^{AB}
Lm 377	0.300^{ABCD}	0.231^{ABCD}	33.1^{A}	0.0186^{CDE}	5.27^{BCD}	0.0659^{B}
Lm 379	0.120^{FG}	0.112^{CD}	34.9^{A}	0.0188^{BCDE}	4.35^{ABCD}	0.0442^{AB}
Lm 380	0.245^{ABCDE}	0.214^{ABCD}	28.6^{A}	0.0188^{BCDE}	3.98^{ABCD}	0.0503^{AB}
Lm 381	0.170^{CDEFG}	0.132^{BCD}	36.7^{A}	0.0187^{BCDE}	4.20^{ABCD}	0.0479^{AB}
10403S (control)	-	-	39.5	0.0179	-	-
08-5578 (control)	-	-	-	-	3.36	0.0533

For each column values with the same letter are the same ($p<0.05$; Tukey's HSD). For data that was not normally distributed (indicated by *), values were log transformed to achieve normal distribution and log transformed data was used for comparison. The antilog of mean values derived from the Tukey'

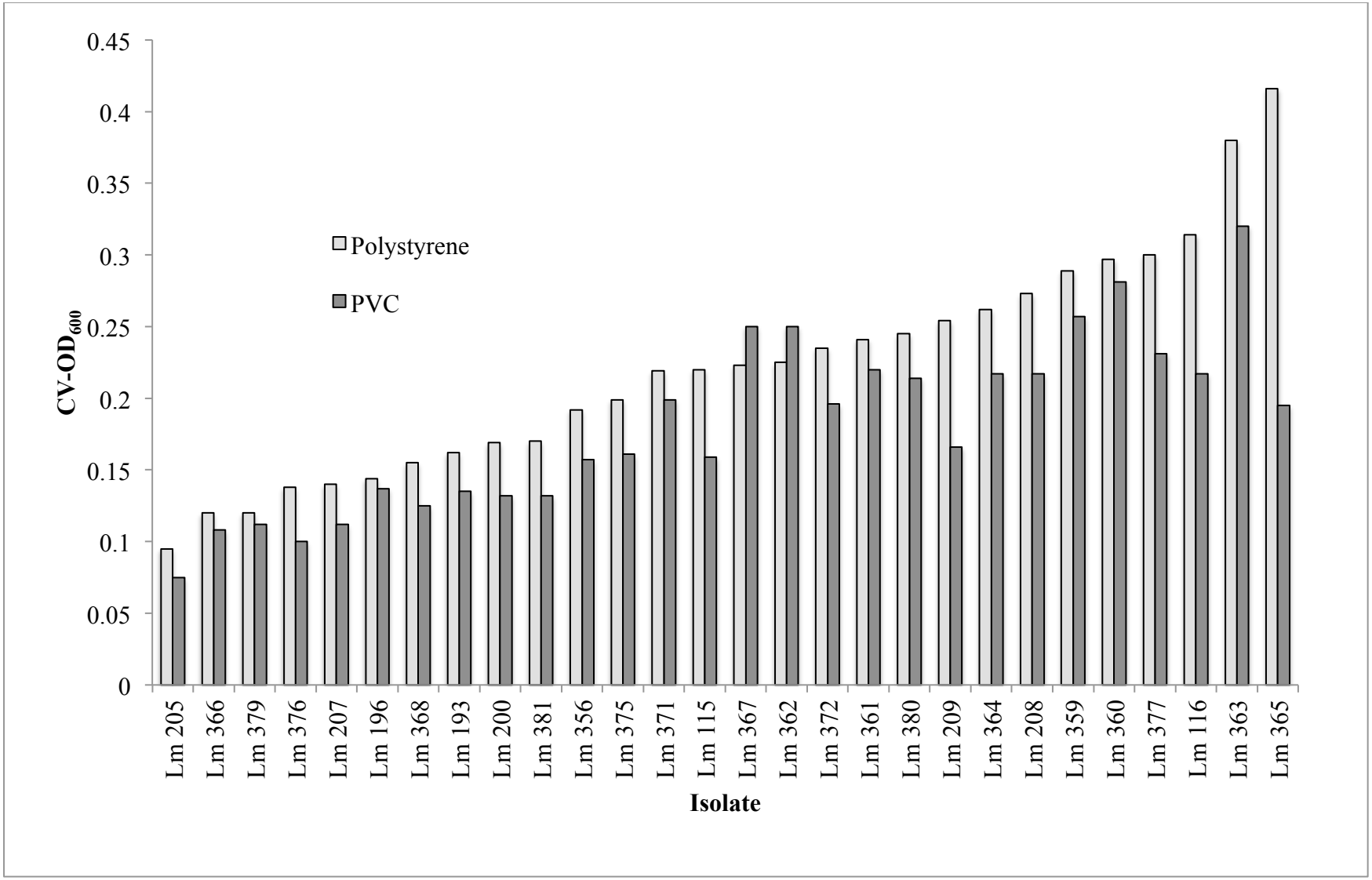


Figure C.1 Comparison of surface adherence ($CV\text{-}OD_{600}$) for each isolate on polystyrene and polyvinyl chloride (PVC) surfaces. Bars represent mean (PVC) or geometric mean (polystyrene) for each isolate.

Appendix D: Mutability results

Table D.1 Mutation frequency as determined by plating isolates on BHI with rifampin (RIF) and observing number of resistant colonies, as per methods outlined by Kovacevic et al. (2013).

Isolate	Number of RIF resistant colonies (mean ± SEM)
Lm 115	7 ± 1
Lm 116	2 ± 0
Lm 193	6 ± 1
Lm 196	1 ± 0
Lm 200	6 ± 2
Lm 205	10 ± 2
Lm 207	6 ± 1
Lm 208	8 ± 1
Lm 209	8 ±1
Lm 356	4 ± 1
Lm 359	1 ± 0
Lm 360	0 ± 0
Lm 361	1 ± 1
Lm 362	2 ± 1
Lm 363	2 ± 1
Lm 364	3 ± 1
Lm 365	5 ± 2
Lm 366	4 ± 1
Lm 367	4 ± 1
Lm 368	306 ± 13
Lm 371	1 ± 0
Lm 372	2 ± 1
Lm 375	3 ± 1
Lm 376	4 ± 1
Lm 377	4 ± 1
Lm 379	8 ± 1
Lm 380	2 ± 1
Lm 381	6 ± 1

Appendix E: Summary of risk approximation results

Table E.1 Summary of *Listeria monocytogenes* isolate characteristics used to assess potential risk and antibiotic resistance profiles.

Isolate	Pulsotype		Ribotype[a]	Source	Serotype	inlA profile[b]	AMR profile[c]
	*Asc*I fingerprint pattern	*Apa*I fingerprint pattern	DUP-Similarity				
Lm 364	LMACI.0059	LMAAI.0122/0005	= DUP-1030	Gull feces	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 365	LMACI.0334	LMAAI.0197	= DUP-1035	Gull feces	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 376	LMACI.0370	LMAAI.0048	= DUP-1038	Cutting board	4b	Full-length	CLI^R, FOX^R, NAL^R
Lm 366	LMACI.0163	LMAAI.0140	= DUP-1038	Gull feces	4b	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 367	LMACI.0170	LMAAI.0512	= DUP-1039	Gull feces	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 205	LMACI.0688	LMAAI.0996	= DUP-1044	Gull feces	4b	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 379	LMACI.0060	LMAAI.0857	= DUP-18611	Finished product	4b	3CD (aa738-740)	CLI^R, FOX^R, NAL^R, CIP^I
Lm 209	LMACI.0809	LMAAI.1180	= DUP-18615	Cutting board	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 377	LMACI.0211	LMAAI.1198	= DUP-18645	Cutting board	1/2a	Full-length	FOX^R, NAL^R, CIP^I
Lm 371	LMACI.0036	LMAAI.0671	= DUP-19165	Cutting board	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 375	LMACI.0577	LMAAI.1197	= DUP-19165	Gull feces	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 360	LMACI.0148	LMAAI.1187	= DUP-19165	Raw salmon surface	1/2c	PMSC (aa760)	CLI^R, FOX^R, NAL^R, CIP^I
Lm 116	LMACI.0364	LMAAI.0682	= DUP-19165	Unknown	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 362	LMACI.0015	LMAAI.0024	= DUP-19169	Cutting board	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 361	LMACI.0711	LMAAI.0693	= DUP-19169	Cutting board	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 208	LMACI.0041	LMAAI.0033	= DUP-19186	Gull feces	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 196	LMACI.0007	LMAAI.0014	= DUP-19186	Shipping area floor	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 115	LMACI.0774	LMAAI.1128	= DUP-20233	Unknown	1/2b	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 363	LMACI.0171/0063	LMAAI.0945	~ DUP-1044	Cutting board	1/2b	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 207	LMACI.0107	LMAAI.0915	~ DUP-1052	Gull feces	4b	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 372	LMACI.0083	LMAAI.0086	~ DUP-1053	Cutting board	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 380	LMACI.0083	LMAAI.0086	~ DUP-1053	Finished product	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 359	LMACI.0083	LMAAI.0086	~ DUP-1053	Raw salmon surface	1/2a	Full-length	CLI^R, FOX^R, NAL^R, CIP^I
Lm 193	LMACI.0775	LMAAI.1129	~ DUP-19187	Cutting board	4b	3CD (aa738-740)	CLI^R, FOX^R, NAL^R, CIP^R
Lm 368	LMACI.0775	LMAAI.1129	~ DUP-19187	Cutting board	4b	3CD (aa738-740)	CLI^R, FOX^R, NAL^R, CIP^I
Lm 381	LMACI.0775	LMAAI.1129	~ DUP-19187	Cutting board	4b	3CD (aa738-740)	CLI^R, FOX^R, NAL^R, CIP^I
Lm 200	LMACI.0775	LMAAI.1129	~ DUP-19187	Raw salmon surface	4b	3CD (aa738-740)	CLI^R, FOX^R, NAL^R, CIP^I
Lm 356	LMACI.0775	LMAAI.1129	~ DUP-19187	Raw salmon surface	4b	3CD (aa738-740)	CLI^I, FOX^R, NAL^R, CIP^I

[a] Ribotype patterns were identical (=) to ribogroups identified or within 85% similarity (~). [b] three-codon deletion (3CD), pre-mature stop codon (PMSC), amino acid (aa).
[c] Resistance (R) and intermediate (I) resistance assigned using CLSI (2012) and EUCAST (2013) breakpoints.