Fitness of *Listeria monocytogenes* and Other *Listeria* Species Isolated from Two Nova Scotia Watersheds

by

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen known to colonize surfaces, form antimicrobial-resistant biofilms, and resist environmental stresses such as desiccation, helping it persist in food processing environments. Over 670 presumptive *Listeria* isolates collected from an urban and a rural Nova Scotia watershed were identified through sequencing of the 16S rRNA and/or *sigB* genes. *L. monocytogenes*, *L. innocua*, and *L. seeligeri* were isolated from both watersheds. *L. welshimeri* and *L. fleischmannii* were only isolated from the rural watershed. The fitness of each species was evaluated through motility, biofilm, desiccation and benzalkonium chloride (BAC) assays. *L. fleischmannii* and *L. innocua* formed significantly less biofilm. *L. monocytogenes* strains from the urban and rural watersheds were the most and least desiccation-resistant, respectively. Generally, *L. fleischmannii* was the most susceptible to BAC. This research provides greater insight into natural reservoirs of pathogenic *Listeria* and the factors that help *Listeria* persist in food processing plants.

LIST OF ABBREVIATIONS USED

BAC	Benzalkonium chloride
BHI	Brain heart infusion
BZT	Benzethonium chloride
CFU	Colony forming units
CP	Collin's Park
EPS	Extracellular polymeric substance
GB	Glycine betaine
LEB	*Listeria* enrichment broth
Lm	*Listeria monocytogenes*
Li	*Listeria innocua*
Ls	*Listeria seeligeri*
Lw	*Listeria welshimeri*
Lf	*Listeria fleischmannii*
Lm 08	*Listeria monocytogenes* strain 08-5578
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
MIC	Minimum inhibitory concentration
MM	Middle Musquodoboit
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCR	Polymerase chain reaction
PS	Peptone saline
QAC	Quaternary ammonium compound
RH	Relative humidity

rRNA	Ribosomal ribonucleic acid
RTE	Ready-to-eat
SS	Stainless steel
T_m	Membrane phase transition temperature
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSB-glu	Tryptic soy broth + 1% glucose

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CHAPTER 1 INTRODUCTION

1.1 Rationale

Listeria monocytogenes is a foodborne pathogen that poses a significant challenge for the food industry. First described by Murray et al. in 1926, *L. monocytogenes* was not identified as a foodborne pathogen until 1981 following the investigation of an outbreak in Atlantic Canada caused by contaminated coleslaw (Schlech et al., 1983). *L. monocytogenes* outbreaks are most often associated with ready-to-eat (RTE) foods such as deli meats, soft cheeses, smoked salmon, and fresh produce (Schuchat et al., 1991). Consumption of contaminated RTE food products, which either contained initial high levels of the pathogen or allowed for growth during refrigerated storage, can cause serious and potentially life-threatening illness in high-risk individuals such as pregnant women, neonates, the elderly, and immunocompromised persons (de Noordhout et al., 2014). In Canada, *L. monocytogenes* causes an estimated 178 cases of illness per year and is the 25th most common cause of foodborne illness (Thomas et al., 2013). Although the number of annual cases is low relative to other foodborne pathogens, *L. monocytogenes* is a significant concern to the health care system and food industry because of the high rates of hospitalization and death. It is estimated that 84% of listeriosis cases result in hospitalization and the mortality rate is estimated at 19% (Thomas et al., 2015).

L. monocytogenes has been isolated from many environments, including soil, water, vegetation, sewage, farms, animal feeds, and food processing facilities (Sauders and Wiedmann, 2007). Although these environments are potential reservoirs of pathogenic *Listeria* that could be part of a transmission pathway leading to human illness, most research has focused on *Listeria* in farm and food processing environments.

Once introduced into a food processing facility, *L. monocytogenes* can colonize surfaces and may persist for years (Unnerstad et al., 1996; Miettinen et al., 1999; Keto-Timonen et al., 2007). *L. monocytogenes* is able to adhere to surfaces in food processing plants, particularly those with moisture and food residues, and subsequently form biofilms that increase resistance to disinfectants (Robbins et al., 2005; Takahashi et al., 2011). Fitness, the ability of an organism to survive and reproduce under various environmental conditions, is a key reason for the long term persistence of some *L. monocytogenes* strains. Persistent strains often demonstrate relatively high fitness compared to other microorganisms under environmental stresses such as high salt concentrations, low pH, refrigeration temperatures, and desiccation (Lado and Yousef, 2007).

This study aims to explore the diversity of *Listeria* spp. including pathogenic *Listeria* in natural aqueous reservoirs, as well as provide a better understanding of the factors contributing to the persistence of *Listeria* spp. including *L. monocytogenes* in food processing environments. Such knowledge is important for the development of more effective sanitation programs that can reduce the contamination of RTE foods and subsequent outbreaks and illnesses caused by *L. monocytogenes*.

1.2 Research Objectives

This research was divided into two main studies: 1) Diversity and fitness of *Listeria* spp. in an urban and a rural Nova Scotia watershed, and 2) Effect of osmolytes on the desiccation tolerance of *Listeria monocytogenes*. The main objective of the first study was to test the hypothesis that there would be a difference in the diversity and prevalence of *Listeria* spp. isolated from two different Nova Scotia watersheds. Furthermore, this study aimed to compare the fitness of species isolated from each of the watersheds. Fitness was assessed as the ability to form biofilms, tolerate desiccation, and survive treatment with benzalkonium chloride. The main purpose of the second study was to investigate if select osmolytes would significantly impact the desiccation survival of *L. monocytogenes.* The specific objectives of each study were:

Part 1: Diversity and Fitness of *Listeria* spp. in an Urban and a Rural Nova Scotia Watershed

1. Use colony PCR and Sanger sequencing targeting the 16S rRNA and sigB genes to identify naturally occurring *Listeria* species isolated from an urban and a rural Nova Scotia watershed.
2. Assess the motility of *Listeria* spp. isolated from Nova Scotia watersheds.
3. Compare the biofilm formation of *Listeria* spp. isolated from Nova Scotia watersheds.
4. Compare the desiccation survival of *Listeria* spp. isolated from Nova Scotia watersheds.
5. Evaluate the ability of benzalkonium chloride to inhibit the growth and biofilm formation of watershed *Listeria* spp.
6. Evaluate the ability of benzalkonium chloride to eradicate preformed biofilms of watershed *Listeria* spp.

Part 2: Effect of Osmolytes on the Desiccation Tolerance of *Listeria monocytogenes*

1. Determine if select osmolytes improve the desiccation survival of *Listeria monocytogenes.*

2. Compare the effects of osmolytes on several different strains of *Listeria monocytogenes*.
3. Compare the effects of osmolyte concentration on *L. monocytogenes* desiccation survival.

CHAPTER 2 LITERATURE REVIEW

2.1 *Listeria*

Listeria is a genus of bacteria that consists of Gram positive, non-spore forming, facultative anaerobic bacilli. There are currently 17 recognized species of *Listeria*: *L. monocytogenes*, *L. ivanovii*, *L. welshimeri*, *L. innocua*, *L. seeligeri*, *L. grayi*, *L. rocourtiae*, *L. marthii*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis, L. aquatica, L. cornellensis, L. grandensis, L. riparia*, *L. booriae*, and *L. newyorkensis* (den Bakker et al., 2014; Weller et al., 2015). Of these species, only *L. ivanovii* and *L. monocytogenes* are pathogenic in humans, with almost all cases of illness attributed to the latter (Guillet et al., 2010).

2.1.1 *Listeria* in the Environment

Listeria spp. are ubiquitous in nature and have been isolated from soil, surface waters, sewage, vegetation, animal feeds, animal feces, food processing plants, and farm environments (Wiedmann et al., 1997; Sauders et al., 2012; den Bakker et al., 2013; Leong et al., 2014; Linke et al., 2014, Stea et al., 2015). *L. monocytogenes* is the most well studied species, and has been found throughout the world including North America, South America, Europe, Asia, Africa and Oceania (Hofer et al., 2000; Sauders et al., 2012; Hmaïed et al., 2014; Linke et al., 2014; McAuley et al., 2014; Tango et al., 2014). *L. innocua*, *L. seeligeri*, and *L. welshimeri* have been isolated across several continents including North America, Europe and South America (Hofer et al., 2000; Sauders et al., 2012; Linke et al., 2014). Eleven novel *Listeria* species (*L. rocourtiae*, *L. marthii*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis, L. aquatica, L. cornellensis, L.*

grandensis, L. riparia, *L. booriae*, and *L. newyorkensis*) have been described since 2009, and many of these strains have yet to be isolated from as wide a range of environments. For example, *L. fleischmannii* has so far only been isolated from cheeses in Italy and Switzerland and environmental samples from a cattle ranch in Colorado (Bertsch et al., 2013; den Bakker et al., 2013; Chiara et al., 2015).

Depending on the type of environment, certain *Listeria* spp. will dominate. Higher rates of *L. monocytogenes* tend to be found in areas influenced by ruminants and humans such as agricultural land, urban environments, sewage, and food (MacGowan et al., 1994; Lyautey et al., 2007; Sauders et al., 2012; Linke et al., 2014). In natural environments such as soil and water, other *Listeria* spp. are more common. One study in New York State found that *L. seeligeri* and *L. welshimeri* were significantly associated with natural environments (Sauders et al., 2012). Studies in the UK found that *L. ivanovii* and *L. seeligeri* were frequently isolated from urban soil, while *L. innocua*, *L. seeligeri* and *L. welshimeri* were often isolated from fresh water sites (Frances et al., 1991; MacGowan et al., 1994). Likewise, a study in Austria found that *L. seeligeri*, *L. innocua*, and *L. ivanovii* were the dominant species in soil and water samples (Linke et al., 2014).

2.1.2 *Listeria monocytogenes* and Food Safety

Listeriosis is a disease caused by infection with pathogenic *Listeria* spp. and can be severe and life threatening in highly susceptible individuals such as pregnant women, neonates, the elderly and immunocompromised persons (de Noordhout et al., 2014). Both *L. ivanovii* and *L. monocytogenes* can cause illness in humans; however, the vast majority of human listeriosis cases are attributed to the latter (Guillet et al., 2010). Inactivation of *L. monocytogenes* can be achieved with pasteurization and cooking; therefore, outbreaks are typically associated with RTE foods that are not cooked by consumers prior to consumption, including fresh produce, deli meats, soft cheeses, and smoked salmon (Schuchat et al., 1991). With an estimated mortality rate of 19%, *L. monocytogenes* is a serious concern for the food industry and health care system (Thomas et al., 2015).

Although *L. monocytogenes* is ubiquitous in the environment, RTE foods typically become carriers through contamination in the processing plant. *L. monocytogenes* is able to adhere to surfaces in a food-processing environment, particularly those with moisture and food residues, and subsequently form biofilms (Borucki, 2003; Takahashi et al., 2011). Biofilms increase the resistance of bacterial cells to removal (Lado and Youseff, 2007). The combination of strong surface adherence, biofilm formation, and inadequate sanitation leaves food processing surfaces contaminated with the pathogen. In 2008, there was a major outbreak of *L. monocytogenes* in Canada caused by deli meats (Maple Leaf Food, Inc), which resulted in 22 deaths. Following an investigation, it was found that inadequate sanitation practices led to biofilm formation on the slicing equipment, and subsequent contamination of the deli meat (Weatherill, 2009). Although outbreaks of this magnitude are rare in Canada, *L. monocytogenes* continues to be a problem for the food industry. In 2015, the Canadian Food Inspection Agency recalled 22 products due to the finding of *L. monocytogenes* (Canadian Food Inspection Agency, 2016).

2.2 Biofilms

Bacterial biofilms are aggregates of surface-associated cells joined together by an extracellular polymeric substance (EPS) matrix (Donlan, 2002). The structure of biofilms and composition of EPS varies among bacteria. *L. monocytogenes* biofilms can range from flat multilayers to a more complex honeycomb-like structure (Marsh et al., 2003;

Hingston et al., 2013; Guilbaud et al., 2015). The thread-like EPS matrix that connects biofilm cells is composed of proteins, extracellular DNA, and carbohydrates (Marsh et al., 2003; Chae et al., 2006; Harmsen et al., 2010; Nguyen and Burrows, 2014). Biofilm cells differ from planktonic cells in their protein content. Biofilm cells were found to have a greater abundance of several proteins involved in sugar metabolism, energy generation, stress response, envelope and protein synthesis, and regulatory functions (Hefford et al., 2005; Lourenço et al., 2013). There are five stages of biofilm formation: (1) initial, reversible attachment (2) irreversible attachment, (3) proliferation of cells and production of EPS, (4) maturation of biofilm, (5) detachment and dispersion of cells (Srey et al., 2013). Biofilms are significant concern for food safety, as they allow microorganisms to resist desiccation, ultraviolet light, and antimicrobials (Robbins et al., 2005; Bernbom et al., 2011; Truelstrup Hansen and Vogel, 2011).

2.2.1 Factors Affecting Biofilm Formation

There are many factors that affect biofilm formation including strain, pH, temperature, surface properties, motility, presence of nutrients and food soils, and competition. *L. monocytogenes* attachment and biofilm formation is greater under highly acidic and highly alkaline culture conditions than under neutral conditions (Nilsson et al., 2011). Higher temperatures tend to result in greater biofilm formation, with an optimal temperature of about 30-37˚C (Duffy and Sheridan, 1997; Briandet et al., 1999; Chavant et al., 2002). Above 37˚C, the number of attached cells decreases (Mai and Conner, 2007). *L. monocytogenes* can colonize and form biofilms on many different materials, including glass, stainless, steel, rubber, and polypropylene; however, the amount of biofilm varies (Mafu et al., 1990; Blackman and Frank, 1996). Surface attachment and subsequent biofilm formation is affected by multiple properties of a surface including roughness, hydrophobicity, charge, and stiffness (Chavant et al., 2002; Lichter et al., 2008; Chaturongkasumrit et al., 2011; Song et al., 2015). Several studies comparing paralyzed-flagellum mutants and flagella-minus mutants to their respective motile, wild-type strain have found differences in the attachment and biofilm formation of immotile and motile strains (Vatanyoopaisarn et al., 2000; Lemon et al., 2007). These experimental results are explained in greater detail in section 2.5. The amount and type of nutrients present is another important factor. In studies comparing the biofilm formation of *L. monocytogenes* grown in high and low nutrient media, significant differences were observed between strains grown in nutrient-rich versus those grown in nutrient-poor conditions; however, the correlation between biofilm formation and nutrient content of the growth media varied among studies and strains (Moltz and Martin, 2005; Folsom et al., 2006; Kadam et al., 2013). One study observed that biofilm formation was enhanced in low nutrient media compared to high nutrient media (Kadam et al., 2013). Other studies have found that the relationship between nutrients and biofilm formation is strain-dependent. Moltz and Martin (2005) found that six *L. monocytogenes* strains exhibited greater biofilm formation in nutrient-poor media, while two strains exhibited greater biofilm formation in nutrient-rich media. Another study found that five strains produced more biofilm in nutrient-poor media, 14 strains in nutrient-rich media, and 11 strains produced the same amount of biofilm in each media (Folsom et al., 2006). High nutrient content can enhance or diminish biofilm formation depending on the strain. Kim and Frank (1995) studied the effects of specific nutrients on *L. monocytogenes* biofilms, and found that mannose and trehalose enhanced biofilm development. A reduction in biofilm development was observed when cells were grown in high or low levels of phosphate compared to that in modified Welshimer's broth (Kim and Frank, 1995). In food processing environments, *L. monocytogenes* is often found in mixed species biofilms. Depending on the other species present, biofilm formation by the pathogen may increase, decrease, or exhibit no significant difference from single-species *L. monocytogenes* biofilms (Carpentier and Chassaing, 2004).

2.3 Desiccation

Desiccation is the removal of a significant amount of water from a cell, and occurs when the water activity of a cell is higher than that of the surrounding environment. The surrounding environment may be an aqueous solution (osmotic stress) or a gaseous environment (matric stress) (Potts, 1994). Greater differences in water activity result in more rapid desiccation, and therefore reduced bacterial survival. In a study comparing the survival of *Listeria monocytogenes* at three relative humidities (RH), 75%, 43% and 2%, it was observed that survival rates were higher at increasing RH (Vogel et al., 2010).

Cells undergo significant changes during desiccation. The effects of desiccation on a bacterial community as a whole include change in surface area, shrinkage, salt precipitation, change in texture and shape, and change in colour due to the oxidation of pigments. The effects on an individual cell may include shrinkage of capsular layers, increase in intracellular salt levels, crowding of macromolecules, changes in cell volumes, changes in biophysical properties such as surface tension, reduced fluidity, damage to external layers such as pili and membranes, and changes in physiological processes (Potts, 1994). The cellular changes associated with desiccation can be lethal for many bacteria. Certain bacteria, including *Deinococcus radiodurans* and *Mycobacterium tuberculosis,* are able to survive air-drying to a point of nearly complete dehydration, and are therefore considered desiccation tolerant (Billi and Potts, 2002). *L. monocytogenes* is a desiccation tolerant bacterium, and was found to survive desiccation conditions for 91 days in a study mimicking food-processing environments (Vogel et al., 2010).

2.3.1 Factors Affecting Desiccation Tolerance

The presence of a biofilm, as well as its maturity, can impact *L. monocytogenes* desiccation survival. The formation of biofilms has been found to improve *L. monocytogenes* desiccation tolerance, with higher survival rates seen in mature biofilms than in immature biofilms (Truelstrup Hansen and Vogel, 2011; Hingston et al., 2013). The EPS material connecting biofilm cells is hygroscopic in nature, which slows the desiccation of bacterial cells (Roberson and Firestone, 1992; Ophir and Gutnick, 1994).

The downregulation of flagella has been found to increase desiccation tolerance in bacteria such as *L. monocytogenes*, *Salmonella*, and *Pseudomonas putida* (van de Mortel and Halverson, 2004; Li et al., 2012; Hingston et al., 2015). Conversely, other organisms like *Bradyhizobium japonicum* upregulate flagella production in response to desiccation (Cytryn et al., 2007). Flagella production is a high-energy investment, which may explain the advantage of downregulation under environmental stresses like desiccation (Guttenplan and Kearns, 2013). Flagella production and motility can also indirectly affect desiccation tolerance by influencing biofilm formation (section 2.2.1), which in turn affects desiccation tolerance (Vatanyoopaisarn et al., 2000; Lemon et al., 2007).

The cell membrane is a lipid-based structure that acts as a selective barrier between the cell and external environment, and is thought to have an impact on desiccation tolerance (Nikaido and Vaara, 1985; Potts, 1994). Membranes can exist in two different phases: liquid crystalline and gel. In the liquid crystalline state, water molecules are positioned between lipid head groups, helping the membrane maintain fluidity. When water molecules are removed, the lipids are packed tightly and the membrane becomes less fluid and enters the gel state (Tardieum et al., 1973; Chapman 1994). The change from one state to another can be related to the membrane phase transition temperature (T_m). Above T_m the membrane in the liquid crystalline state, and below T_m the membrane is in the gel state (Tokumasu et al., 2002). When close to the transition temperature, these two phases can co-exist, resulting in very high membrane permeability that can be disastrous for cells when free water is available for solute transfer (Crowe and Crowe, 1986; Crowe and Crowe, 1992). Desiccation causes T_m to increase, which is problematic for cells when T_m rises to ambient temperature, and the two membrane phases co-exist. The presence of sugars such as trehalose and sucrose can minimize the damage caused by desiccation. The sugar will replace water molecules, allowing the lipids to maintain their fluid structure, and prevent the increase in T_m that would normally occur during desiccation (Leslie et al., 1995). Though mechanisms that are not fully understood, it is believed that desiccation-tolerant cells have means of resisting the rise in T_m caused by desiccation (Potts, 1994). Some bacteria alter membrane composition in response to environmental stress. Scherber et al. (2009) found that the percent composition of saturated fatty acids in *E. coli* membranes increased during desiccation, and then decreased upon rehydration. Hingston et al. (2015) created a library of *L. monocytogenes* transposon mutants and screened for mutants showing an altered desiccation tolerance relative to the wild-type. Of the desiccation-sensitive mutants, two had interruptions in genes related to lipid biosynthesis and two related to membrane transport. Interruptions in three genes related to membrane lipid biosynthesis, and one gene related to membrane transport led to mutants becoming desiccation-tolerant. These results indicate that the structure and composition of the lipid membrane is important for the desiccation tolerance of *L. monocytogenes*.

Extracellular food components can impact desiccation tolerance. Studies have examined the effect of food components ranging from specific nutrients such as amino acids, sugars and salt, to more general food soils including cabbage and ground pork (Chaibenjawong and Foster, 2011; Takahashi et al., 2011; Hingston et al., 2013). Under environmental stress conditions, some bacteria will accumulate high concentrations of certain compounds called compatible solutes, which help improve survival. A wide variety of compounds can act as compatible solutes including sugars, amino acids and their derivatives, and polyols (da Costa et al., 1998). Amino acids and their derivatives such as glycine betaine, carnitine and proline, have been found to improve the desiccation tolerance of certain bacteria. Glycine betaine was found to improve the desiccation tolerance of *L. monocytogenes,* and *Staphylococcus aureus* (Dreux et al., 2008; Chaibenjawong and Foster, 2011; Huang et al., 2015). The desiccation survival of *L. monocytogenes* increases in the presence of carnitine and proline; however, the effects of proline are less significant than glycine betaine and carnitine (Huang et al., 2015). Sugars trehalose and sucrose have both been found to improve the desiccation survival or *S. aureus* and *Salmonella enterica* (Chaibenjawong and Foster, 2011; Gruzdev et al., 2012). The role of compatible solutes in the stress tolerance of *L. monocytogenes* and other bacteria is explored further in section 2.6. Various types of food soils have been found to improve the desiccation tolerance of *L. monocytogenes* including fresh and cold-smoked salmon, tuna, cabbage, ground pork, carrot, nori, milk, and soy milk (Vogel et al., 2010; Takahashi et al., 2011; Takashi et al., 2015). Other studies have focused on the effects of more specific food components such as salt and fats. While high concentrations of salt can be inhibitory to microorganisms, multiple studies have observed that the presence of 5% NaCl significantly improves *L. monocytogenes* desiccation survival (Vogel et al., 2010; Hingston et al., 2013). The presence of certain fats can also impact desiccation tolerance. Hingston et al. (2013) found that the presence of animal lard (20-60%) improved the desiccation survival of *L. monocytogenes*, while the presence of canola oil (5-10%) had no significant effect.

2.4 Resistance to Benzalkonium Chloride

Benzalkonium chloride (BAC) is a quaternary ammonium compound (QAC) commonly used as a disinfectant in food production environments. QACs are surfactants, which disrupt cell wall integrity and cause fatal changes in membrane permeability (Wessels and Ingmer, 2013). They are primarily used to target Gram-positive bacteria, but are also effective against Gram-negative bacteria, some viruses, fungi and protozoans (Tezel and Pavlostathis, 2015).

Resistance to disinfectants is a major concern in food production facilities, since inadequate sanitation practices can lead to cross-contamination of food products and subsequent illness. Continued exposure to sub-inhibitory concentrations of disinfectants has been linked to the development of sanitation resistance (To et al., 2002). Cells in biofilms tend to be more resistant to sanitation than planktonic cells; therefore, the use of disinfectants at a concentration that is inhibitory to planktonic cells may be sub-inhibitory to biofilm cells (Pan et al., 2006). Several studies have suggested there is a connection between resistance to BAC and enhanced biofilm formation. Exposure to BAC at sub-inhibitory concentrations enhanced biofilm formation in *E. coli* (Pagedar et al., 2012). In a study comparing persistent and transient *L. monocytogenes* strains isolated from a fish processing plant, persistent strains exhibited greater biofilm formation and resistance to BAC than transient strains (Nakamura et al., 2013). Another study comparing persistent and transient *L. monocytogenes* found that persistent strains had the highest resistance to two QACs, benzethonium chloride (BZT) and cetylpyridinium chloride (Fox et al., 2011). Expression of certain stress response genes have been liked to both biofilm formation and BAC resistance in *L. monocytogenes* including: *SigB*, *HrcA* and *DnaK* (van der Veen and Abee, 2010a; van der Veen and Abee, 2010b). Fox et al. (2011) found that in the presence of BZT, *L. monocytogenes* upregulates several genes involved in peptidoglycan biosynthesis. Since QACs disrupt bacterial cell walls, the upregulation of genes related to peptidoglycan biosynthesis may be an important mechanism in resisting the effects of QACs. Another way *L. monocytogenes* becomes resistant to QAC is through the overexpression of multidrug efflux pumps that export antimicrobials that enter the cell (Mereghetti et al., 2000; Romanova et al., 2006). The overexpression of efflux pumps can be induced by exposure to QAC or QAC-induced stress (Tezel and Pavlostathis, 2015). This mechanism is of great interest to researchers since the efflux pumps confer resistance to multiple antimicrobials and is transferable through horizontal gene transfer (Xu et al., 2014).

Exposure to sub-inhibitory concentrations of QAC is not limited to food processing facilities. After use, QACs are either directly released into the environment or enter the environment in trace amounts after passing through wastewater treatment. Consequently, QACs become present in the environment at sub-inhibitory concentrations (Tezel and Pavlostathis, 2015).

2.5 Motility

Listeria monocytogenes is motile through the production of four to six peritrichous flagella per cell (Schirm et al., 2004). Flagella synthesis is temperature-dependent. At 37˚C, MogR inhibits flagellin synthesis through the repression of flagellar genes, but at 30˚C and below, flagella are produced due to the repression of MogR (Kathariou et al., 1995; Shen et al., 2006).

Several studies suggest that flagellar motility contributes to surface attachment and formation of biofilms. The attachment of *L. monocytogenes* to stainless steel was found to be 10-fold lower in an immotile, flagellin mutant than the motile, wild type (Vatanyoopaisarn et al., 2000). Lemon et al. (2007) compared a flagella-minus mutant and a paralyzed-flagellum mutant to a wild-type strain. The two motility mutants behaved similarly, exhibiting decreased attachment and biofilm formation compared to the wild-type. Chang et al. (2012) performed transposon mutagenesis on *L. monocytogenes* Scott A, and found mutations in 5 flagella related genes that resulted in decreased biofilm production. Other studies suggest that immotility may be advantageous. In a study of *L. monocytogenes* desiccation survival, a library of transposon mutants was created using strain 568, and several motility mutants were selected (Hingston et al., 2015). Seven immotile mutants were found to have greater desiccation tolerance than the wild-type strain. One of these immotile mutants, *Lm* 568 Δ *flaA*, was also found to form more biofilm at the bottom of a microtiter plate than the wild-type, while no biofilm formed in a peg lid assay (Piercey et al., 2016). This difference in biofilm formation likely reflects different methods of adherence needed to form biofilms on a peg suspended in broth compared to a flat surface underneath the broth culture. This relationship between flagellar downregulation and desiccation tolerance has been observed in other organisms including *Salmonella* and *Pseudomonas putida* (van de Mortel and Halverson, 2004; Li et al., 2012). Interestingly, many newly identified *Listeria* species are naturally immotile including: *L. rocourtiae*, *L. weihenstephanensis*, *L. cornellensis*, *L. riparia*, *L. grandensis*, *L. fleischmannii*, *L. aquatica*, *L. floridensis*, *L. booriae*, and *L. newyorkensis* (den Bakker et al., 2014; Weller et al., 2015).

2.6 Osmolytes and Compatible Solutes

Osmolytes are compounds that affect osmosis. Bacteria can accumulate certain osmolytes under environmental stress conditions to improve survival. These osmolytes are called compatible solutes, and can be accumulated in high concentrations without interfering with essential metabolic processes. Compatible solutes balance osmolarity, stabilize enzyme function, and help maintain membrane integrity (Sleator and Hill, 2001). A wide variety of compounds can act as compatible solutes including sugars, amino acids and their derivatives, and polyols (da Costa et al., 1998).

2.6.1 Amino Acids and their Derivatives

Several amino acid and amino acid derivatives have been identified as compatible solutes for *L. monocytogenes* including glycine betaine, carnitine, acetylcarnitine, proline, proline betaine, γ-butyrobetaine, and 3-dimethylsulphoniopropionate (Bayles and Wilkinson, 2000; Huang et al., 2015). Acetylcarnitine, proline betaine, γ-butyrobetaine, and 3-dimethylsulphoniopropionate act as osmo- and cryoprotectants for *L. monocytogenes* (Bayles and Wilkinson, 2000); however, their effects during desiccation have yet to be studied.

Glycine betaine (GB) is an amino acid derivative most often found in foods of plant origin. It acts as a compatible solute for *L. monocytogenes* under high osmolarity, low temperature, and desiccation (Ko et al., 1994; Bayles and Wilkinson, 2000; Angelidis and Smith, 2003; Huang et al., 2015). Dreux et al. (2008) observed that the improvement of *L. monocytogenes* desiccation survival on parsley leaves was positively correlated to GB concentration. This mirrors the results of a study on the effects of different concentrations of osmolytes on the desiccation survival of *Staphylococcus aureus* (Chaibenjawong and Foster, 2011). *L. monocytogenes* cannot synthesize GB and must import it from the external environment. Uptake of GB is predominantly mediated by the osmolyte transport systems Gbu and BetL, and to a lesser degree OpuC. Although GB cannot be synthesized, uptake may not be required to improve desiccation survival. Dreux et al. (2008) observed that GB had similar effects on both the wild-type strain and a *L. monocytogenes* mutant strain with deletions in all three-transporter genes, suggesting that either the protective effect of extracellular GB was independent of intracellular accumulation, or that GB was imported through an alternative route.

Carnitine is an amino acid derivative mainly found in foods of animal origin. Carnitine is a well-recognized compatible solute utilized by microorganisms such as *Lactobacillus plantarum, Lactococcus lactis*, *Bacillus subtilis*, and *L. monocytogenes* (Wood et al., 2001; Huang et al., 2015). Carnitine has been found to act as an osmo- and cryoprotectant for *L. monocytogenes*, as well as improve desiccation survival (Bayles and Wilkinson, 2000; Huang et al., 2015). *L. monocytogenes* is unable to synthesize carnitine, and uses the OpuC transporter for uptake (Angelidis and Smith, 2003).

Proline is an amino acid that is found in foods of plant origin, eggs, dairy and fish (Sosulski and Imafidon, 1990). Proline is known to act as a compatible solute in *L. monocytogenes* under high osmolarity, low temperature and desiccation (Beumer et al., 1994; Bayles and Wilkinson, 2000; Huang et al., 2015). Unlike glycine betaine and carnitine, proline can be synthesized by the cell (Beumer et al., 1994). An *L. monocytogenes proBA* deletion mutant incapable of synthesizing proline displayed decreased osmo- and barotolerance compared to the wild type suggesting that synthesis of proline is important for stress tolerance (Sleator et al., 2001; Considine et al., 2011). *L. monocytogenes* is also able to uptake exogenous proline. The presence of exogenous proline has been found to improve osmotolerance and desiccation tolerance in *L. monocytogenes*, but to a lesser degree than glycine betaine and carnitine (Beumer et al., 1994; Bayles and Wilkinson, 2000; Huang et al., 2015).

2.6.2 Sugars

There is limited research on the role of sugars as compatible solutes for *L. monocytogenes*. Lactose, a disaccharide found in milk, has been found to act as a cryoprotectant for several microorganisms including *L. monocytogenes*, *Escherichia coli*, *Lactobacillus delbruekii*, *Saccharomyces cerevisiae*, *Lactobacillus casei*, and *Pseudomonas fluorescens* (Doebbler, 1966; El-Kest and Marth, 1991; Panoff et al., 2000; Hubálek, 2003; Nag and Das, 2013; Cabrefiga et al., 2014). The effect of lactose on *L. monocytogenes* desiccation tolerance has yet to be investigated.

Trehalose is a well-studied compatible solute naturally found in mushrooms, honey, shellfish, and foods containing brewer’s or baker’s yeast (Richards et al., 2002). It can minimize desiccation damage by stabilizing membranes and proteins (Leslie et al., 1995). Trehalose acts as a cryoprotectant for *E. coli*, *Bacillus thuringiensis*, and *Lb. casei,* as well as improves the survival of *Salmonella enterica* during desiccation (Leslie et al., 1995; Gruzdev et al., 2012; Nag and Das, 2013). Less is known about trehalose as a compatible solute for *L. monocytogenes.* In a study of *L. monocytogenes* trehalose metabolism, it was observed that mutations in the *treA* gene led to an accumulation of trehalose in the cytosol, which resulted in greater resistance to thermal, desiccation and osmotic stress compared to wild-type cells (Ells and Truelstrup Hansen, 2011). However, their results suggest that *L. monocytogenes* is unable to naturally accumulate trehalose. Some compounds can improve stress tolerance without being imported into the cell (Dreux et al., 2008). It is unknown if exogenous trehalose can improve the desiccation tolerance of *L. monocytogenes* without uptake.

Sucrose is a disaccharide consisting of glucose and fructose joined by an α-1,2 glycosidic linkage. It is also a non-reducing sugar naturally found in fruits and vegetables, and used as a sweetener in processed foods (Yuan et al., 2015). Sucrose acts as a cryoprotectant for a variety of bacteria including *E. coli, B. thuringiensis, and Lactobacillus delbrueckii* subsp. *bulgaricus* (Leslie et al., 1995; Huang et al., 2006). The presence of sucrose has been shown to improve the desiccation survival of *S. aureus* and *Salmonella enterica* (Chaibenjawong and Foster, 2011; Gruzdev et al., 2012). In a study of the metabolome of *L. monocytogenes* grown at different temperatures, it was found that the intracellular concentration of sucrose was 7.2-fold greater at 8˚C than at 35˚C (Singh et al., 2011).

2.6.3 Other Compatible Solutes

Inositol is a polyol found in foods such as beans, citrus fruit, cantaloupe, and whole grain bread (Clements and Darnell, 1980). The role of inositol and inositol derivatives as compatible solutes is not as well studied as compounds such as glycine betaine or trehalose. Myo-inositol has been shown to act as a compatible solute for renomedullary cells, dehydrated collembolans, and hibernating beetles (Michell, 2007).

There is currently a lack of research on the effect of inositol on bacterial cells. Though inositol has not been investigated as a compatible solute for *L. monocytogenes*, it has been shown that the intracellular concentration of inositol is 2.1 fold greater at 8˚C than 37˚C, suggesting that the cell may accumulate inositol as a response to cold stress (Singh et al., 2011).

Choline is a nutrient frequently grouped with B-complex vitamins and is found in a wide variety of foods including legumes, cruciferous vegetables, wheat germ, eggs, and liver (Zeisel et al., 2003). Accumulation of choline has been shown to have osmoprotective effects in *Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa,* as well as thermoprotective effects in *Bacillus subtilis* (Boch et al., 1994; Hoffmann and Bremer, 2011; Lamark et al., 1996; Fitzsimmons et al., 2012). Choline is a precursor to glycine betaine, and it is thought that the protective effect of choline is dependent on its conversion to glycine betaine (Wood et al., 2001). In a study of osmoregulation in *E. coli*, mutants unable to convert choline to glycine betaine were not protected against osmotic stress by choline (Le Rudulier et al., 1984). Little is known about the effects of choline under desiccation conditions, as well as its potential protective effects on *L. monocytogenes*.

CHAPTER 3 DIVERSITY AND FITNESS OF *LISTERIA* SPP. IN AN URBAN AND A RURAL NOVA SCOTIA WATERSHED

3.1 Introduction

Listeria is a genus of Gram positive, non-spore forming, facultative anaerobic bacilli that are ubiquitous in the environment. *Listeria* spp. have been isolated from a wide range of environments including soil, water, vegetation, sewage, farms, animal feeds, and food processing facilities (Sauders and Wiedmann, 2007). The genus has grown significantly in recent years. Presently, there is a total of 17 recognized *Listeria* species, 11 of which have been discovered since 2009. Prior to that, the last species added to the genus was *L. ivanovii* in 1985 (Seeliger et al., 1984). Of the 17 species, only *L. ivanovii* and *L. monocytogenes* are pathogenic in humans, with nearly all illnesses attributed to the latter (Guillet et al., 2010). *L. monocytogenes* is a foodborne pathogen associated with ready-to-eat foods including fresh produce, deli meats, soft cheeses, and smoked fish (Schuchat et al., 1991). As the most significant pathogen in this genus, *L. monocytogenes* has become the main focus of *Listeria*-related research.

There have been many studies on the ecology and fitness of *L. monocytogenes* in an effort to better understand the transmission pathways that lead to human illness, and the factors that help *L. monocytogenes* persist in food processing facilities. This information is important for reducing the risk of contamination of foods with *L. monocytogenes* and subsequent illness. There are limited studies comparing the fitness of other *Listeria* species, and most use older species such as *L. welshimeri*, *L. innocua*, and *L. ivanovii* (Ells and Truelstrup Hansen, 2006; Ells and Truelstrup Hansen, 2010; Jamali et al., 2015). There have been several studies on the diversity of *Listeria* spp. isolated from different sources such as soil, water, feces, vegetation and food; however, most of the isolates were found to belong to the older species (MacGowan et al., 1994; Sauders et al., 2012; Linke et al., 2014). Samples from these studies were collected in 2009 or earlier, before many newer *Listeria* species were identified; therefore, some of the techniques used to isolate *Listeria* may not have been conducive to the selection of certain species. For example, Sauders et al. (2012) used motility as a screening method since, at the time, all known *Listeria* species were motile. Since 2009, eleven new species of *Listeria* have been identified, 10 of which are immotile. The isolation of many of the newly identified species has been geographically limited. For example, *L. booriae* and *L. weihenstephanensis* have so far only been isolated from samples collected in the United States and Germany, respectively (Lang Halter et al., 2013; Weller et al., 2015). Gaining a more thorough knowledge of all *Listeria* species will help provide a more complete understanding of the evolution, ecology, genomics and phenotypic characteristics of pathogenic and non-pathogen *Listeria* spp.

This study aims to provide a better understanding of natural reservoirs of pathogenic and commensal *Listeria* species and the factors contributing to their survival in food processing environments. Colony PCR and Sanger sequencing targeting the 16S ribosomal RNA (rRNA) and *sigB* genes were used to identify naturally occurring *Listeria* species isolated from an urban and a rural Nova Scotia watershed. For each of the detected *Listeria* species, two isolates were selected from each watershed, followed by an evaluation of their fitness through assays testing motility, biofilm formation, desiccation tolerance and resistance to benzalkonium chloride.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Culture Conditions

In a previous study, a total of 1,314 *Listeria* isolates were obtained from water samples from two Nova Scotia watersheds: the rural Musquodoboit River watershed and the urban Lake Fletcher watershed, which serve as the source water for the drinking water treatment plants in Middle Musquodoboit (MM) and the Collin's Park (CP) subdivision, respectively. Maps of each watershed including sampling sites can be found in Appendix A. To isolate *Listeria* spp. from water samples, Stea et al (2015) used *Listeria* enrichment broth (LEB), followed by Fraser broth, and then PALCAM agar. Presumptive *Listeria* colonies were selected from PALCAM plates (up to eight colonies per plate), and the isolates were stored at -80˚C in brain heart infusion (BHI) broth with 15% glycerol. The isolates were previously classified by their colony morphology on RAPID'*L.mono* agar as *L. monocytogenes*, *L. ivanovii*, *innocua* group (*L. innocua*, *L. marthii*, and *L. grayi*), or *welshimeri* group (*L. welshimeri*, *L. seeligeri*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis*, *L. riparia*, *L. booriae*, and *L. newyorkensis*) The identity of *L. monocytogenes* isolates was also confirmed by PCR detection of the *prfA* gene (Stea et al., 2015). In the present study, identification based on sequencing was completed on 679 strains (52%) randomly selected from the original library.

Routine culturing was carried out in Tryptic Soy Broth (TSB, BD Canada, Oakville, ON, Canada) or TSB with 1% glucose (TSB-glu, Fisher Scientific, Whitby, ON, Canada), or TSB with 1.5% (w/w) technical agar (BD). Strains were stored at -80˚C in BHI broth (BD) with 20% glycerol (Fisher Scientific).

3.2.2 Identification of *Listeria* Isolates

Frozen cultures were refreshed in TSB and incubated at 37˚C overnight. Broth cultures were streaked onto TSA (Bacto, BD Canada, Oakville, ON) and PALCAM (Oxoid, Nepean, ON, Canada) agar and incubated at room temperature for 48 hours. Colony polymerase chain reaction (PCR) was used to speciate the watershed samples. Each 25 µl reaction contained 0.5 µl forward primer (10 mmol), 0.5 µl reverse primer (10 mmol), 12.5 µl Taq polymerase (NEB, Ipswich, MA, USA), 11.5 µl distilled water, and one bacterial colony from the TSA plate. Table 1 contains a list of the primers used and their nucleotide sequence.

Table 1. Primers used for colony PCR

Target	Primer Name	Sequence 5' to 3'	Reference
16S rRNA[a]	fD1	AGAGTTTGATCCTGGCTCAG	Weisburg et al., 1991
16S rRNA	rP2	ACGGCTACCTTGTTACGACT	Weisburg et al., 1991
sigB[b]	*sigB*degF	ATGAAAAGCAGGTGGAGGAGAATGC	den Bakker et al., 2013
sigB	*sigB*degR	CCSGTTTCTTTTGACTRCGRTTTTC	den Bakker et al., 2013

[a] product size = ~1514bp
[b] product size = ~704bp

The following PCR cycle was performed using a Biometra T-Gradient thermocycler (Biometra, Göttingen, Germany): initial denaturation at 95˚C for 10 min, followed by 30 cycles of denaturation at 95˚C for 1 min, annealing at 61˚C for 1 min and extension at 72˚C for 1 min, followed by a final extension at 72˚C for 10 min. To purify PCR products, 0.2 µl Exonuclease I (ExoI, 20,000 U/ml, NEB) and 0.2 µl Calf Intestinal Phosphate (CIP, 10,000 U/ml, NEB) was added to each PCR reaction, then incubated at 37˚C for 15 minutes, followed by a 15 minute incubation at 80˚C to inactivate ExoI. PCR products were sequenced commercially using the Sanger method (Quintara Biosciences, Allston, MA, USA). Sequences were assembled using CLC Genomics Workbench 9 software (CLC Bio-Qiagen, Aarhus, Denmark) and identified using Blastn online software (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

3.2.3 Selection of Fitness Assay Strains

For each species of *Listeria* that was identified as being present in the sequenced library, two strains were selected from each watershed to be used in the motility, biofilm, desiccation, and benzalkonium chloride (BAC) assays (Table 2). The two strains selected were isolated from different sampling sites. Furthermore, strains selected for the assays had their identity assigned based on sequencing of both the 16S rRNA and *sigB* genes.

Table 2. *Listeria* strains used in motility, biofilm, desiccation, and BAC assays.

Species	Rural MM strains		Urban CP strains	
L. monocytogenes	MM 4-17-5	MM 1-13-7	CP 4-5-1	CP 5-2-3
L. innocua	MM 2-14-5	MM 1-14-8	CP 1-15-2	CP 4-14-3
L. seeligeri	MM 1-2-4	MM 4-10-6	CP 5-4-1	CP 4-14-6
L. welshimeri	MM 4-4-6	MM 2-8-1	*	*
L. fleischmannii	MM 3-14-3	MM 4-14-5	*	*

3.2.4 Motility Assay

Following the protocol outlined by Piercey et al. (2016), a sterile inoculation needle was used to pick colonies and then stab them into semi-solid BHI agar (0.3%). The agar plates were divided into sections (approximately 2.5 × 2.5 cm), with one stab per section. A positive and negative control was used on each plate, and samples were tested in triplicate. Lm 568, a motile strain of *L. monocytogenes,* was used as the positive control, and the immotile mutant Lm 568 Δ*flaA* (Piercey et al., 2016) was used as the negative control. Following inoculation, plates were incubated at 15˚C for 72 hours.

Lateral growth from the point of inoculation was observed after 24, 48 and 72 hours of incubation, and motility was rated relative to the positive and negative controls on the same plate.

3.2.5 Biofilm Assay

The biofilm assay was completed following protocols outlined by Piercey et al (2016). Frozen cultures were refreshed in TSB-glu and incubated at 15˚C for 48 hours. Following incubation, cultures were standardized to an absorbance ($A_{450\ nm}$) of ~ 1.0. To standardize cultures, cells were harvested by centrifugation (10,000 × *g*, 10 min), then re-suspended in TSB-glu to achieve an absorbance ($A_{450\ nm}$) of 1.0 and a final cell concentration of approximately 10^9 CFU/ml as determined by spot plating (5 spots of 10 µl each) on TSA. Standardized cultures were diluted to obtain a final concentration of ~ 4 log colony forming unit (CFU)/cm^2 on the coupon. Ten µl of culture (2.5×10^5 CFU/ml) was spotted onto stainless steel (SS) coupons (314, type 4 finish, 0.5 × 0.5 cm size) and incubated at 15˚C and 100% RH. Six coupons were sampled at 0, 6, 24, and 48 hours. Coupons were rinsed with peptone saline (PS), placed in 1 ml of PS and sonicated at room temperature for 4 min (Elmasonic S120H sonicating bath, Fisher Scientific). The coupons were then vortexed vigorously for 60 s to release any remaining cells. The SS coupons were removed from the broth, and serial dilutions were made in PS. Appropriate dilutions were spot plated (5 spots of 10 µl each) onto TSA and plates were incubated for 48 hours at room temperature. Plate counts were converted to $Log_{10}(CFU/cm^2)$. After the sampling at 48 hours, all remaining coupons were incubated at 23% RH for the desiccation assay (section 3.2.5).

3.2.6 Desiccation

Following the protocol outlined by Piercey et al. (2016), coupons with preformed biofilms (section 3.2.4.) were incubated at 15˚C and 23% RH for seven days. Humidity was maintained using petri dishes containing saturated potassium acetate (Acros Organics, Fairlawn, NJ, USA), placed in the bottom of a SICCO Mini-1 Desiccator (SICCO, Bohlender, Grünsfeld, Germany) three days prior to desiccation. Data loggers were used to monitor temperature and RH throughout the experiment (Gemini Tinytag View 2, Interworld Electronics and Computer Industries Inc., Markham, ON, CA). Coupons were sampled after 1, 2, 3, 5, and 7 days following the protocol in section 3.2.4.

3.2.7 Benzalkonium Chloride Assays

For each of the 16 strains, 50 µl of thawed culture was transferred into 950 µl TSB-glu, incubated for 24 hours at room temperature, then standardized to an absorbance ($A_{450\ nm}$) of ~ 1.0 following protocol in section 3.2.6. Each of the benzalkonium chloride assays were completed according to protocols outlined by Piercey et al. (2016). For the minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) assays, standardized cultures were diluted and 10 µl was added to a well (n = 6) containing 190 µl of TSB-glu or TSB-glu with benzalkonium chloride (BAC, Acros Organics) to obtain a final concentration of ~10^7 CFU/cm^2. The concentrations of BAC tested were 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg/ml. To test the MIC, plates were incubated at 15˚C for 48 hours. Following incubation, 75 µl of the well contents were transferred to a new microtiter plate (Costar, #3370, Fisher Scientific) and the absorbance (A_{490}) was read (Biotek EL 808 Absorbance Microplate reader, Fisher Scientific).

The MBIC was assessed twice, once after 48 hours of incubation (15˚C), and once after 6 days. Following incubation, the spent media was discarded and replaced with 100 µl fresh TSB-glu and 10 µl 12 mmol MTT stain (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Fisher Scientific). The plates were incubated for 2 hours at 37˚C, then 85 µl of media was removed and 50 µl of dimethyl sulfoxide was added to the well. Following a 10-minute incubation (37˚C), the absorbance (A_{570}) was read.

For the minimum biofilm eradication concentration (MBEC) assay, standardized cultures were diluted and 10 µl was added to wells (n = 6) containing 190 µl of TSB-glu to obtain a final concentration of ~10^7 CFU/cm^2. Plates were incubated at 15˚C for 6 days, and then the spent media was removed and replaced by either TSB-glu or TSB-glu with BAC. The concentrations of BAC used were 40, 60, 80, 100, 120, and 140 µg/ml. The plates were incubated for 1 hour then stained with MTT following the same protocol as the MBIC assay. After the staining protocol, the absorbance (A_{570}) was read.

3.2.8 Statistical Analysis

The positive and negative predictive values of RAPID'*L.mono* agar were calculated using the following equations, where true positives and true negatives correspond to matching identification by sequencing and RAPID'*L.mono* agar, and false negatives and false positives are indicated by conflicting identification by RAPID'*L. mono* agar and 16S and/or *sigB* sequencing:

$$positive\ predictive\ value = \frac{\#\ of\ true\ positives}{(\#\ of\ true\ positives + \#\ of\ false\ positives)} \times 100$$

$$negative\ predictive\ value = \frac{\#\ of\ true\ negatives}{(\#\ of\ true\ negatives + \#\ of\ false\ negatives)} \times 100$$

Data from the biofilm, desiccation, and BAC assays was depicted in boxplots to identify outliers. Outliers were removed from the data sets prior to further analysis.

For the biofilm and desiccation data, ANOVA with a post-hoc Tukey's test was conducted in RStudio to assess if there were significant differences ($p<0.05$) between strains or a significant time effect.

The following equation was used to calculate the percent inhibition for the MIC, MBIC, or the percent reduction in survival for the MBEC:

$$\%\ inhibition = \frac{(positive\ control - blank) - (test - blank)}{(positive\ control - blank)} \times 100$$

For each of the BAC assays, the lowest concentration of BAC that resulted in a percent inhibition of $95\% \pm 5\%$ was considered to be the MIC, MBIC or MBEC.

3.3. Results

3.3.1 Genetic Identification of *Listeria* Species Isolated from Two Nova Scotia Watersheds

The relative frequency of the *Listeria* species found in each watershed during the first and second year of sampling is visualized in Figure 1. Greater species diversity was observed in the rural (MM) watershed than the urban (CP) watershed. Five species were isolated from the MM watershed (*L. monocytogenes* (Lm), *L. innocua* (Li), *L. seeligeri* (Ls), *L. welshimeri* (Lw), and *L. fleischmannii* (Lf)) and three from the CP watershed (*L. monocytogenes*, *L. innocua*, and *L. seeligeri*). During the first year of sampling the MM watershed, *L. innocua* (66%) was the dominant species, followed by *L. monocytogenes* (18%)*, L. seeligeri* (9%), *L. welshimeri* (5%), and *L. fleischmannii* (2%). In the second year, *L. fleischmannii* was not isolated and the relative frequencies of each species varied slightly from the previous year; however, the general trend remained the same. *L. innocua*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri* had a relative frequency of 59%, 28%, 8% and 5%, respectively (Figure 1). During the first year of sampling the CP watershed, *L. monocytogenes* was the most common species (37%), followed by *L. innocua* (32%), and *L. seeligeri* (30%). This order of species changed during the second year, where *L. seeligeri* (45%) was the most common species followed by *L. monocytogenes* (33%), and *L. innocua* (22%) (Figure 1).

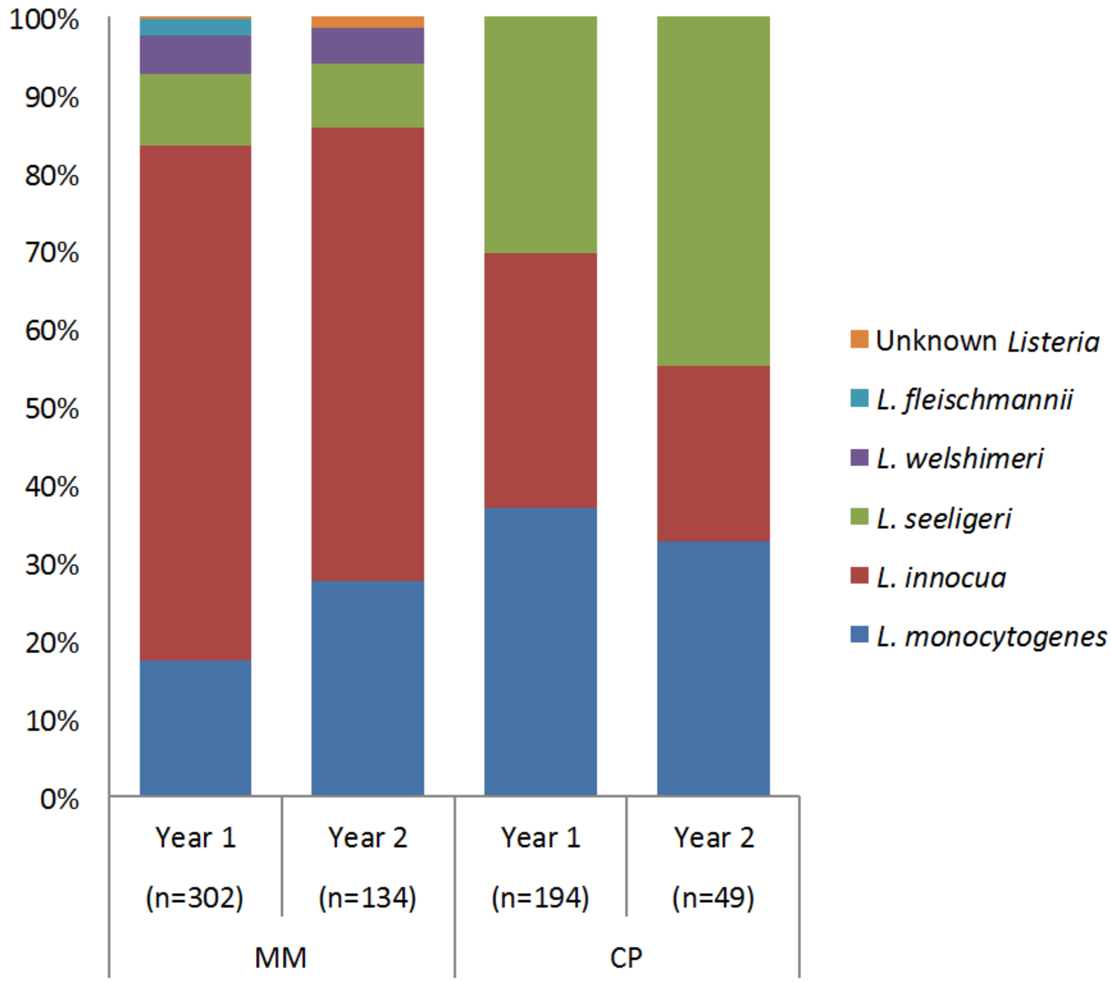

Figure 1. Annual proportion of *Listeria* species isolated from a rural (MM) and urban (CP) watershed during a two-year sampling period.

Three *Listeria* isolates were identified as one species from the 16S sequence and another species from the *sigB* sequence. These isolates are referred to as "unknown *Listeria*" in Figures 1 and 2, and their sequencing results are summarized in Table 3.

Table 3. *Listeria* isolates with an unclear identity based on phenotypic and genotypic identification.

Strain	RAPID'*L.mono* agar classification	16S rRNA sequence	*sigB* sequence
MM 2-14-2	*L. ivanovii*	*L. innocua*	*L. fleischmannii*
MM 4-25-8	*welshimeri* group	*L. monocytogenes*	*L. seeligeri*
MM 5-33-1	*welshimeri* group	*L. monocytogenes*	*L. seeligeri*

Strains were previously identified by RAPID'*L.mono* agar as belonging to one of four phenotypic categories. Sequencing of strains in the present study was completed to identify these strains at the species level. For most *Listeria* isolates, PCR confirmed their previous classification; however, there were some discrepancies between the sequencing results in this study and the RAPID'*L.mono* agar screening previously completed (Stea, 2013). Figure 2 shows the PCR identities of isolates previously classified by RAPID'*L.mono* agar as *L. monocytogenes*, *L. ivanovii*, *innocua* group, or *welshimeri* group. One strain, CP 1-27-7, experienced no growth on RAPID'*L.mono* agar. This strain was identified as *L. seeligeri* through sequencing of the 16S rRNA gene. Sequencing revealed that none of the 679 isolates were *L. ivanovii* even though culturing on RAPID'*L.mono* agar previously identified 18 of the isolates as *L. ivanovii*. *L. fleischmannii* was previously thought to be part of the *welshimeri* group; however, all of the strains identified as *L. fleischmannii* from 16S rRNAand *sigB* sequencing were previously classified as belonging to the *innocua* group (Stea, 2013). Table 4 contains the positive and negative predicative values of RAPID'*L.mono* agar. Positive and negative predictive values are the probabilities that a positive result is a true positive and a negative result is a true negative, respectively.

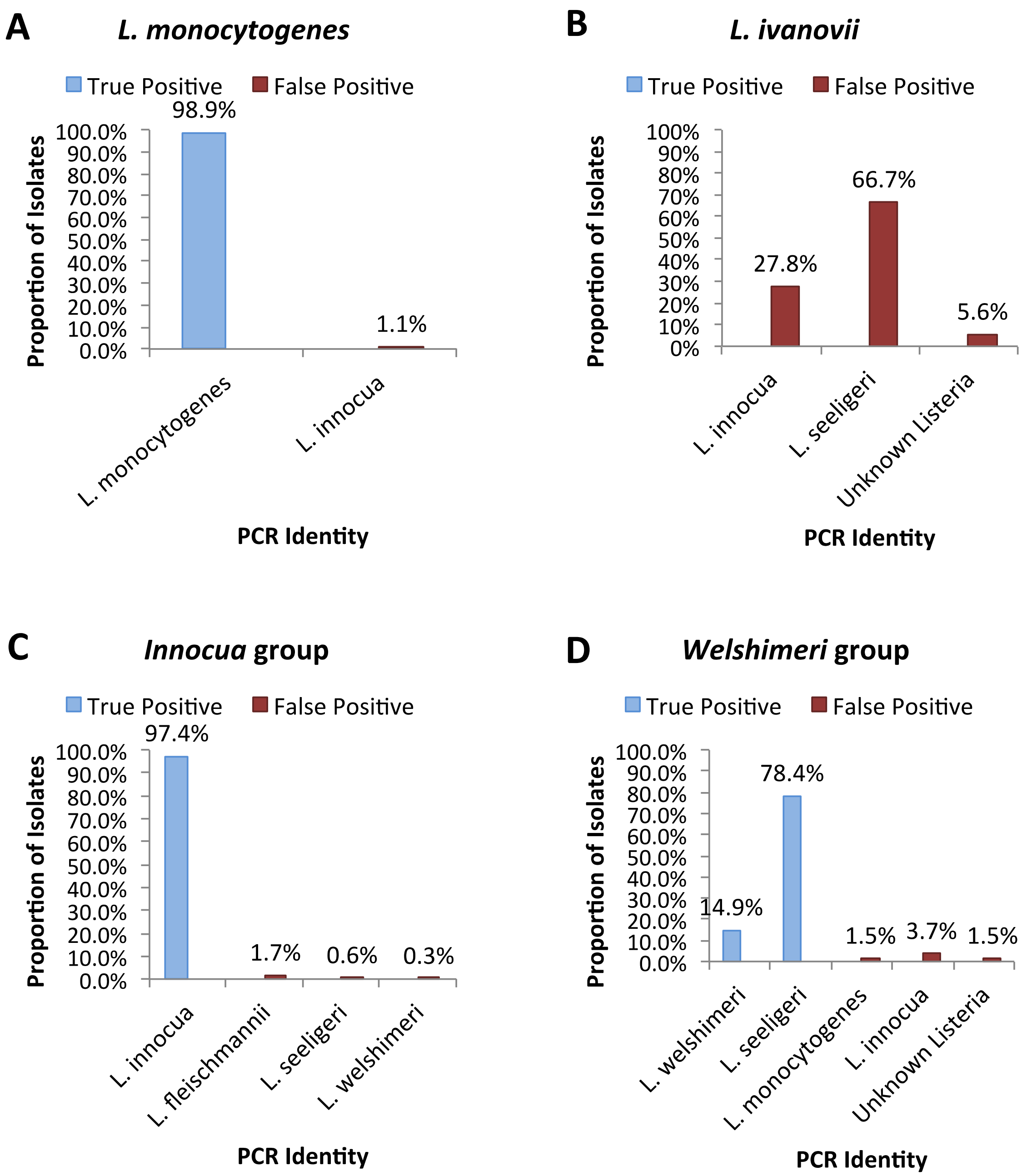

Figure 2. PCR identity (16S rRNA and/or *sigB* genes) of isolates versus the previously classification by RAPID'*L.mono* agar as *L. monocytogenes* (n=178) (A), *L. ivanovii* (n=18) (B), *innocua* group (n=349) (C) and *welshimeri* group (n=134) (D).

Table 4. Positive and negative predictive values of RAPID'*L.mono* agar.

RAPID'*L.mono* Category	**Positive Predictive Value**[a]	**Negative Predictive Value**[b]
L. monocytogenes (PIPLC+, xylose−)	98.9%	99.6%
L. ivanovii (PIPLC+, xylose+)	0.0%	100.0%
Innocua group (PIPLC−, xylose−)	97.4%	96.4%
Welshimeri group (PIPLC−, xylose+)	93.3%	96.1%

[a] positive predictive value is the probability that a positive result is a true positive and was calculated using the equation found in section 3.2.7.
[b] negative predictive value is the probability that a negative result is a true negative and was calculated using the equation found in section 3.2.7.

3.3.2 Motility of Watershed *Listeria* Species

Figure 3 contains images of the semi-solid agar plates after 72 hours of incubation. All strains demonstrated motility except for the two *L. fleischmannii* strains.

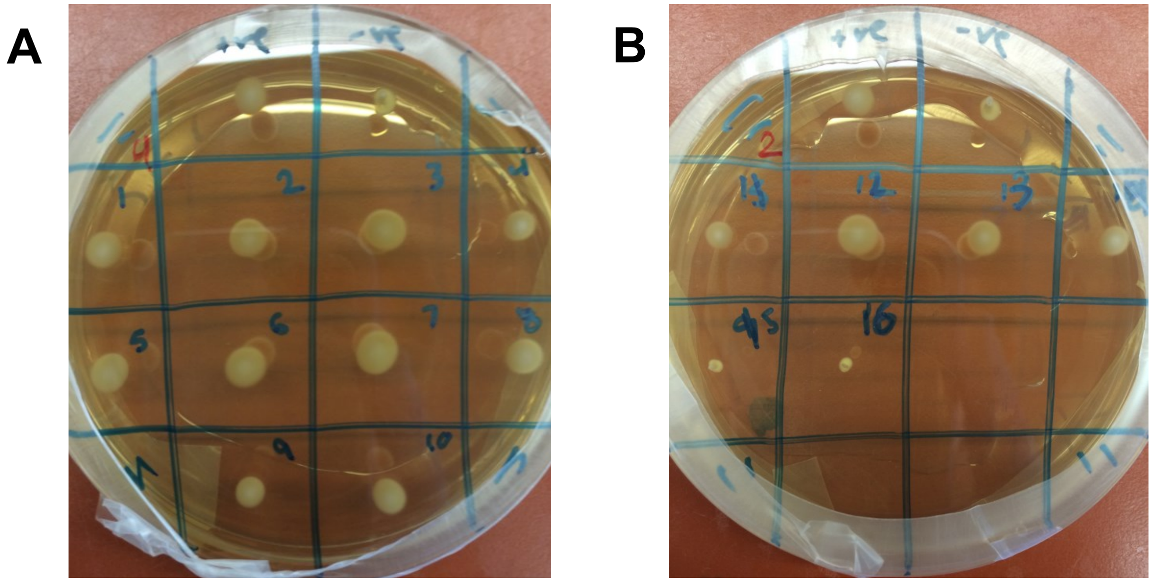

Figure 3. Growth and motility of *Listeria* isolates inoculated into semi-solid BHI agar and incubated for 72 hours at 15˚C. The positive (*L. monocytogenes* 568) and negative controls (*L. monocytogenes* 568 Δ*flaA*) are in the top left and top right squares, respectively, on each plate. On plate A, squares 1-10 are inoculated with Lm MM 4-17-5, Lm MM 1-13-7, Lm CP 4-5-1, Lm CP 5-2-3, Li MM 2-14-5, Li MM 1-14-8, Li CP 1-15-2, Li CP 4-14-3, Ls MM 1-2-4, and Ls MM 4-10-6, respectively. On plate B, squares 11-16 are inoculated with Ls CP 5-4-1, Ls CP 4-14-6, Lw MM 4-4-6, Lw, MM 2-8-1, Lf MM 3-14-3, and Lf MM 4-14-5, respectively.

3.3.3 Biofilm Formation of Watershed *Listeria* Species

The results of the biofilm assay are summarized in Table 5. A time effect was observed, with all strains experiencing a significant ($p<0.05$) increase in log CFU/cm^2 at each subsequent sampling time (Figure B1, Appendix B). At 6 hours, all strains experienced a decrease in log CFU/cm^2 from their initial inoculation levels. Ls CP 4-14-6 had significantly ($p<0.05$) greater log loss than Ls MM 1-2-4 and Lf MM 3-14-3 (Table 5). All other strains were not significantly different from each other after 6 hours. At 24 hours, all strains experienced a population increase; however, none of the strains differed significantly ($p>0.05$). The greatest difference in biofilm formation between strains was observed after 48 hours. In general, *L. monocytogenes*, *L. welshimeri* and *L. seeligeri* exhibited significantly (p<0.05) more biofilm formation than *L. innocua* and *L. fleischmannii* (Figure 4)

Table 5. Biofilm formation on stainless steel coupons by different *Listeria* spp. isolated from fresh water samples. Shown are the average log increase (CFU/cm^2) of *Listeria* strains (n=4-6) forming biofilms at 15˚C and 100% RH over a 48 hour period.

Strain	6 hours	24 hours	48 hours
Lm MM 4-17-5	-0.65 ab*, C**	0.92 a, B	3.06 ab, A
Lm MM 1-13-7	-0.31 ab, C	0.98 a, B	3.14 ab, A
Lm CP 4-5-1	-0.54 ab, C	1.18 a, B	2.96 bcd, A
Lm CP 5-2-3	-0.52 ab, C	1.15 a, B	3.34 a, A
Li MM 2-14-5	-0.61 ab, C	0.90 a, B	2.71 cd, A
Li MM 1-14-8	-0.44 ab, C	1.11 a, B	3.03 abc, A
Li CP 1-15-2	-0.64 ab, C	1.07 a, B	2.64 d, A
Li CP 4-14-3	-0.57 ab, C	0.77 a, B	2.70 cd, A
Ls MM 1-2-4	-0.27 a, C	0.89 a, B	3.12 ab, A
Ls MM 4-10-6	-0.63 ab, C	0.95 a, B	2.97 bcd, A
Ls CP 5-4-1	-0.53 ab, C	1.03 a, B	3.04 abc, A
Ls CP 4-14-6	-0.70 b, C	0.54 a, B	2.92 bcd, A
Lw MM 4-4-6	-0.30 ab, C	0.62 a, B	3.06 abc, A
Lw MM 2-8-1	-0.38 ab, C	1.09 a, B	3.08 ab, A
Lf MM 3-14-3	-0.22 a, C	0.72 a, B	2.65 d, A
Lf MM 4-14-5	-0.41 ab, C	0.63 a, B	2.95 bcd, A

*= Grouping of the log change (log time – log start) of different strains at a given time period, where log change values sharing a letter are not significantly different (p > 0.05)
** = Grouping of the log change (log time – log start) at different sampling times for a given strain, where log change values sharing a letter are not significantly different (p > 0.05)

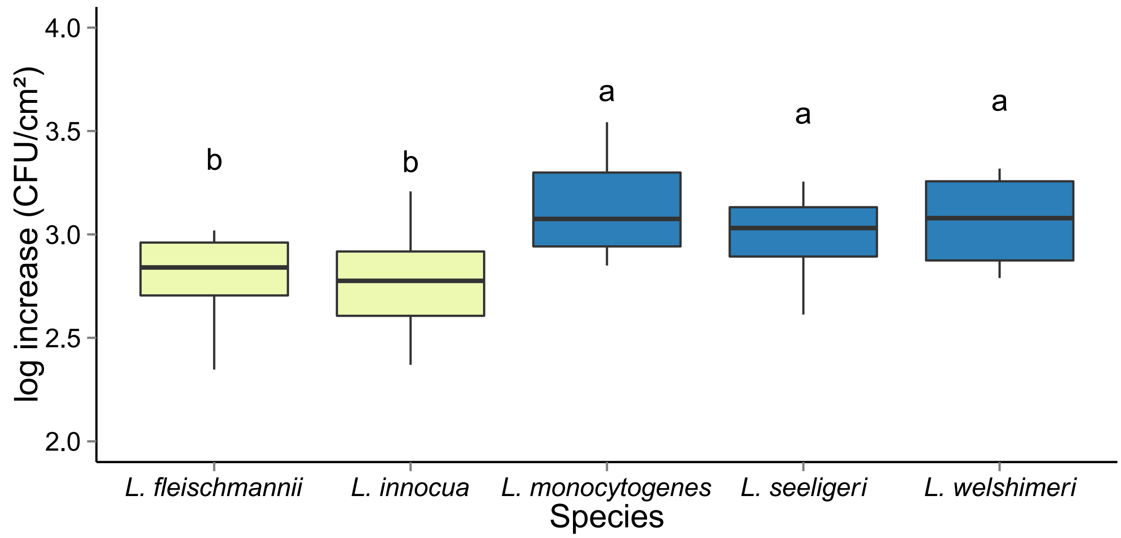

Figure 4. Biofilm formation (log increase CFU/cm^2) of *Listeria* species incubated at 15˚C and 100% RH for 48 hours on stainless steel coupons. The central line in the box plot represents the median (50^{th} percentile), while the lower and upper edges of the box represent the 25^{th} and 75^{th} percentiles, respectively. The whiskers represent the 10^{th} and 90^{th} percentiles. Letters above box plots represent Tukey post hoc test groupings, where treatments sharing the same letter are not significantly different ($p>0.05$).

3.3.4 Desiccation Tolerance of Watershed *Listeria* Species

The results of the desiccation assay are summarized in (Table 6). The log change values in the desiccation assay were more variable than those observed for the biofilm assay in regards to time and strain effects. The log losses between different sampling days were not significantly different ($p>0.05$) for four strains: Li MM 1-14-8, Li CP 1-15-2, Li CP 4-14-3 and Lw MM 2-8-1, meaning that the number of surviving cells in the biofilms, following an initial drop in survivors after the first 24 hours, remained unaltered during the 7-day desiccation (Figure B2, Appendix B). For all other strains, there was a time effect on the log loss. For example, strains Lw MM 4-4-6 and Lf MM 4-14-5 exhibited significantly ($p<0.05$) higher log loss after 7 days of desiccation than after 24 hours. At each sampling day, there were significant strain differences, as evident by the groups of strains at a given sampling day in Table 6.

Table 6. Desiccation survival on stainless steel coupons following biofilm formation of different *Listeria* spp. isolated from fresh water samples. Shown are the average log loss (CFU/cm^2) of *Listeria* strains (n=4-6) subjected to desiccation at 15˚C and 23% RH over a 7 day period.

Strain	Day 1	Day 2	Day 3	Day 5	Day 7
Lm MM 4-17-5	abc*, BC** 0.79	ab, C 0.73	bcd, B 1.44	cde, BC 1.02	a, A 2.46
Lm MM 1-13-7	abcd, C 0.69	abc, C 0.69	b, AB 2.11	abc, BC 1.51	a, A 2.41
Lm CP 4-5-1	e, C -0.28	cd, B -0.01	gh, BC -0.03	gh, B 0.03	fg, A 0.30
Lm CP 5-2-3	e, B -0.28	bcd, A 0.27	h, B -0.16	h, B -0.14	g, AB -0.03
Li MM 2-14-5	a, B 1.30	a, B 0.99	b, A 2.15	bcd, B 1.14	a, A 2.31
Li MM 1-14-8	cde, A 0.33	d, A -0.07	fgh, A 0.20	fgh, A 0.31	efg, A 0.33
Li CP 1-15-2	abcd, A 0.72	abcd A 0.31	fgh, A 0.30	fgh, A 0.26	efg, A 0.33
Li CP 4-14-3	cde, A 0.14	abcd, A 0.37	efgh, A 0.46	efg, A 0.52	cdefg, A 0.61
Ls MM 1-2-4	bcde, C 0.40	abcd, C 0.34	a, A 3.21	ab, B 1.65	bcd, B 1.24
Ls MM 4-10-6	bcde, B 0.41	abcd, B 0.42	cdefg, B 0.82	a, A 2.04	a, A 2.28
Ls CP 5-4-1	ab, AB 1.23	ab, B 0.76	bc, A 1.67	a, A 1.85	ab, AB 1.62
Ls CP 4-14-6	cde, C 0.30	abcd, C 0.39	cd, B 1.23	a, A 1.97	bc, B 1.36
Lw MM 4-4-6	e, B -0.17	bcd, AB 0.01	efgh, AB 0.33	fgh, AB 0.31	defg, A 0.42
Lw MM 2-8-1	abc, A 0.93	a, A 1.02	cdef, A 1.00	bcd, A 1.15	bcdef, A 1.00
Lf MM 3-14-3	de, C -0.05	bcd, BC 0.16	defgh, AB 0.62	fgh, BC 0.19	bcdef, A 0.93
Lf MM 4-14-5	cde, B 0.34	abcd, AB 0.67	defgh, AB 0.63	def, AB 0.71	bcde, A 1.18

*= Grouping of the log change (log start – log time) of different strains at a given time period, where log change values sharing a letter are not significantly different (p > 0.05)
** = Grouping of the log change (log start – log time) at different sampling times for a given strain, where log change values sharing a letter are not significantly different (p > 0.05)

The source of the strains appeared to affect the desiccation tolerance of some species. *L. monocytogenes* and *L. innocua* strains from the urban watershed (CP) showed a significantly ($p<0.05$) lower log reduction after 7 days than strains from the rural watershed (MM) (Figure 5). In contrast, there was no significant difference in log loss between *L. seeligeri* strains from different watersheds (Figure 5). *L. welshimeri* and *L. monocytogenes* were the most and least desiccation-tolerant species, respectively, isolated from the MM watershed (Figure 6). For strains isolated from the CP watershed, *L. monocytogenes* and *L. seeligeri* were the most and least desiccation-tolerant species, respectively (Figure 7).

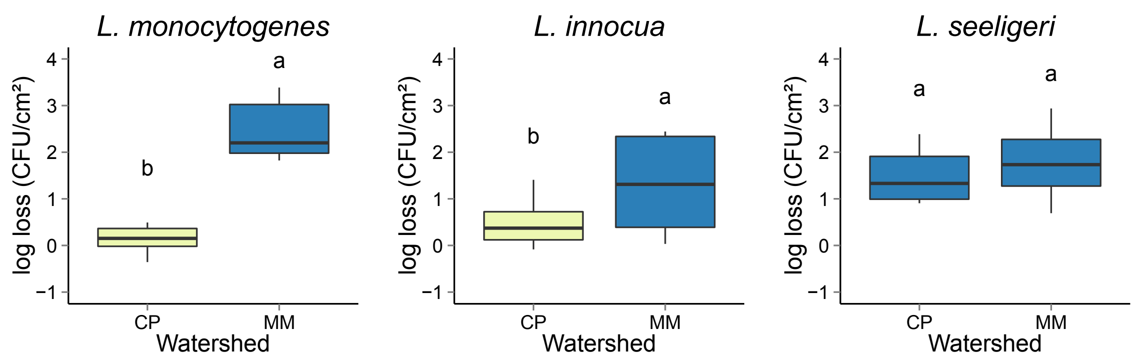

Figure 5. Average log loss of *L. monocytogenes*, *L. innocua*, and *L. seeligeri* strains (n = 8-12) isolated from CP and MM watersheds after 7 days incubation at 15˚C and 23% RH. The central line in the box plot represents the median (50th percentile), while the lower and upper edges of the box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles. Letters above box plots represent Tukey post hoc test groupings, where treatments sharing the same letter are not significantly different ($p > 0.05$).

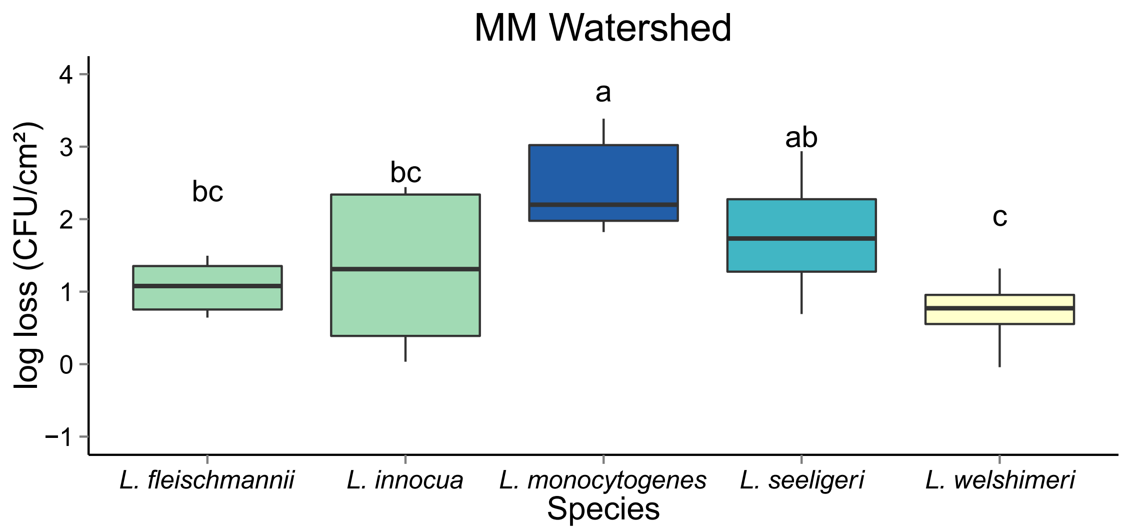

Figure 6. Average log loss of *Listeria* species isolated from the MM watershed after 7 days incubation at 15˚C and 23% RH. The central line in the box plot represents the median (50th percentile), while the lower and upper edges of the box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles. Letters above box plots represent Tukey post hoc test groupings, where treatments sharing the same letter are not significantly different (p>0.05).

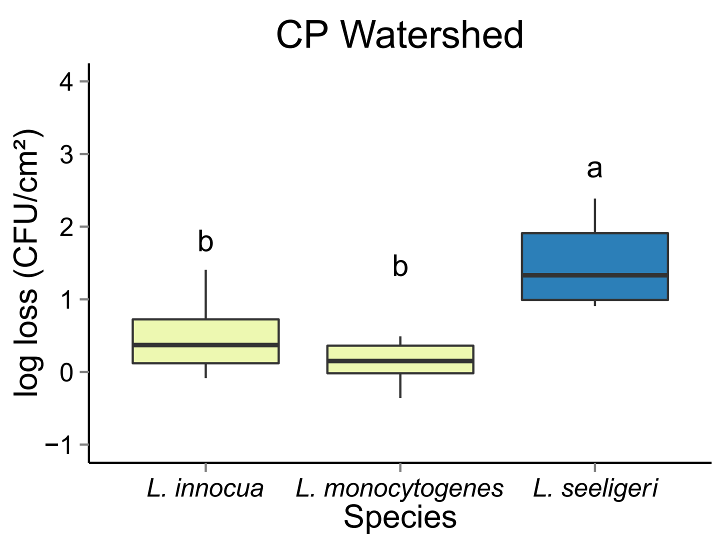

Figure 7. Average log loss of *Listeria* species isolated from the CP watershed after 7 days incubation at 15˚C and 23% RH. The central line in the box plot represents the median (50th percentile), while the lower and upper edges of the box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles. Letters above box plots represent Tukey post hoc test groupings, where treatments sharing the same letter are not significantly different (p>0.05).

3.3.5 Benzalkonium Chloride Resistance of Watershed *Listeria* Species

The MIC, MBIC, and MBEC value of each species is illustrated in Figure 8. Most strains exhibited an MIC of 2.5 µg/ml (Figure 8A). Two *L. innocua* strains and one *L. welshimeri* had an MIC of 5 µg/ml. After biofilm formation for 48 hours, *L. innocua* and *L. welshimeri* strains were the most BAC resistant with an MBIC of 5 µg/ml (Figure 8B). *L monocytogenes, L. seeligeri*, and one of the *L. fleischmannii* strains had an MBIC of 2.5 µg/ml. The other *L. fleischmannii* strain was the most susceptible to BAC with an MBIC of 1.25 µg/ml. After 6 days of biofilm formation in the presence of BAC, two strains of *L. monocytogenes* and three strains of *L. innocua* exhibited an MBIC of 10 µg/ml (Figure 8C). *L. welshimeri*, one strain of *L. innocua*, two strains of *L. monocytogenes*, and three strains of *L. seeligeri* had an MBIC of 5 µg/ml. *L. fleischmannii* and one strain of *L. seeligeri* had an MBIC of 2.5 µg/ml. For the MBEC assay, where mature biofilms (6 days) were subsequently treated with BAC, three strains of *L. monocytogenes* and two strains of *L. innocua* were the most resistant to BAC with MBEC values above 140 µg/ml (Figure 8D). One strain from each of the following species demonstrated MBECs of 140 µg/ml: *L. monocytogenes*, *L. seeligeri, and L. welshimeri.* One strain of *L. innocua* and one *L. seeligeri* have an MBEC of 120 µg/ml**.** Two *L. seeligeri*, one *L. welshimeri* and one *L. fleischmannii* showed MBECs of 100 µg/ml. One *L. innocua* and one *L. fleischmannii* strain had the lowest MBEC, 80 µg/ml. In the MIC and 48-hour MBIC assays, *L. innocua* and *L. welshimeri* were overall more resistant to BAC than the other species. In the 6-day MBIC and MBEC assays, *L. innocua* and *L. monocytogenes* were more resistant to BAC than the other species. *L. fleischmannii* was the most susceptible to BAC in the MBEC and the 48-hour and 6 day MBIC assays. The MIC, MBIC and MBEC values of strains isolated from the MM and the CP watersheds were similar.

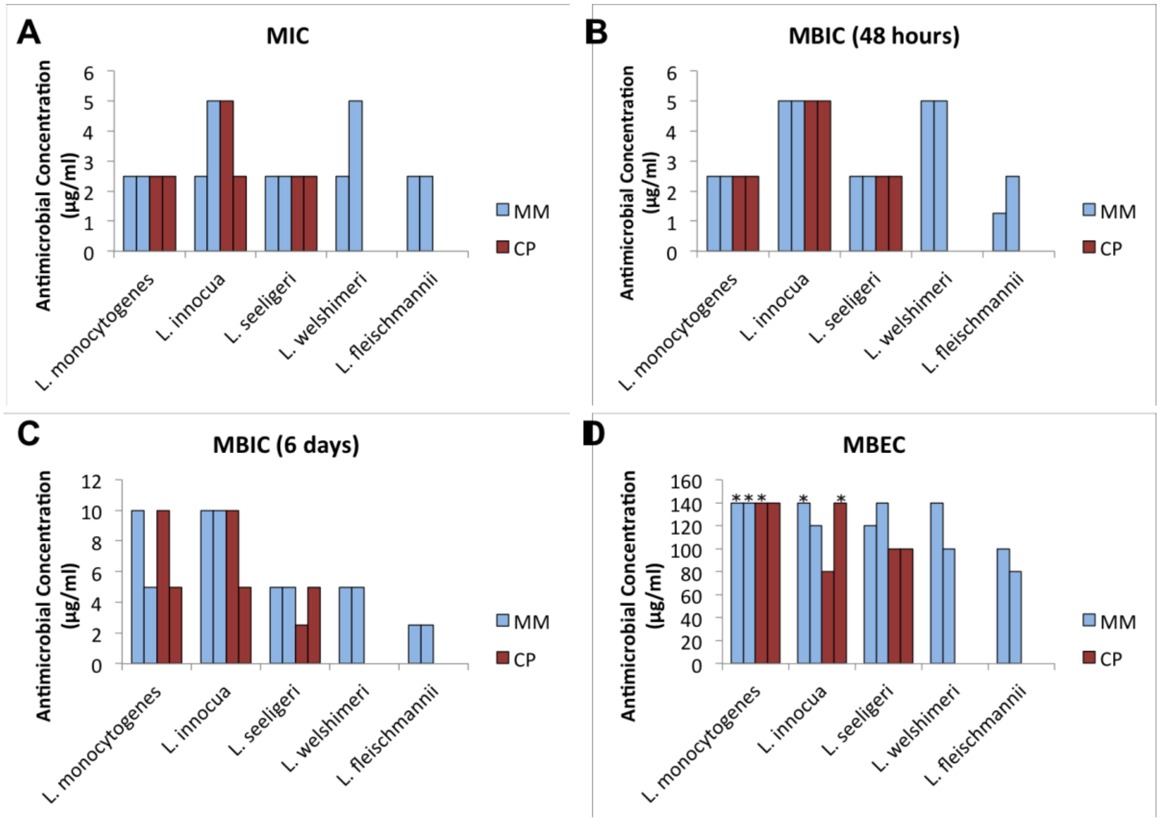

Figure 8. Comparison of the MIC (A), 48 hour MBIC (B), 6 day MBIC (C) and the MBEC (D) of *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. fleischmannii* strains isolated from a rural (MM) and urban (CP) watershed. * means that the metabolic activity in mature biofilms was not inhibited by the highest BAC concentration applied in the MBEC assay.

3.4 Discussion

3.4.1 Diversity of *Listeria* Species in an Urban and a Rural Nova Scotia Watershed

There was a difference in the diversity of species among the urban and rural watersheds, both in regards to the number of different species and the relative prevalence of each species in the watersheds, with the rural watershed showing greater diversity. Differences in *Listeria* diversity between different environments have been observed before. Sauders et al. (2012) observed that *L. innocua* and *L. monocytogenes* were more often associated with urban environments, while *L. seeligeri* and *L. welshimeri* were significantly associated with natural environments. The association between *L. welshimeri* and rural, natural environments was also observed in the present study; however, the trends among other species were not. For example, *L. seeligeri* and *L. innocua* were more frequently isolated from the urban and rural environments, respectively, while the number of *L. monocytogenes* isolates from each watershed was similar. *L. innocua* was the most prevalent species, representing nearly 52% of the *Listeria* isolated from the two watersheds. Multiple studies on the diversity of *Listeria* species in surface waters have also found *L. innocua* to be a dominant species (Frances et al., 1991; Linke et al., 2014). Similar to findings by Sauders et al. (2012), *L. ivanovii* was not isolated from either the MM or CP watersheds. In contrast, an Austrian study of *Listeria* diversity in surface waters found that *L. ivanovii* was one of the dominant species, especially in regions with wildlife or domestic ruminants (Linke et al., 2014). Finding trends in data from different studies on *Listeria* diversity in the environment can be challenging due to the limited number of studies, differences in climate, the type of environmental sample use (e.g. soil, water), the properties of the sample (e.g. pH, nutrients), methods used to isolate *Listeria*, and other variables associated with experimental design. In addition, some studies of *Listeria* in surface waters or other environments only targeted *L. monocytogenes* (Lyautey et al., 2007).

Of the 679 sequenced isolates, only 6 were identified as *L. fleischmannii*, with all coming from two different water samples obtained from the same watershed on the same sampling day. This suggests that *L. fleischmannii* may not be an important part of the microbiota of the watersheds. Nevertheless, the presence of *L. fleischmannii* is a significant finding, since this species has so far only been isolated in the US and Europe (Bertsch et al., 2013; den Bakker et al., 2013; Chiara et al., 2015).

The diversity and prevalence of species isolated from the two watersheds may have been affected by the techniques used to isolate *Listeria* spp. from the water samples. One of the first steps in the isolation process was enrichment in LEB, followed by Fraser broth. When multiple *Listeria* spp. are present, certain enrichment media can favour the growth of one species over another. For example, Egelhardt et al (2016) observed growth in Fraser broth favoured *L. innocua* over *L. monocytogenes*. Similarly, *L. innocua* and *L. welshimeri* have been found to outcompete *L.monocytogenes* in buffered LEB (Keys et al., 2013; Dailey et al., 2015). The next step in isolating *Listeria* spp. from the watershed samples was streaking Fraser broth cultures onto PALCAM agar, and selecting colonies that produced a black colour (Stea et al., 2015). *Listeria* hydrolyses aesculin, forming a black halo around colonies. The agar also contains mannitol, which can be fermented by some contaminants such as enterococci and staphylococci and consequently cause the agar to turn yellow (Oxoid, n.d.). *Listeria monocytogenes* and many other *Listeria* spp. do not ferment mannitol. However, some *Listeria* spp. including *L. grayi*, *L. rocourtiae*, *L. weihenstephanensis*, *L. booriae* and *L. newyorkensis* can ferment mannitol; therefore, only selecting colonies that produce a black colour on PALCAM agar might exclude mannitol-fermenting *Listeria* spp. (Weller et al., 2015). Such species may therefore have been overlooked in the original isolation work by Stea et al. (2015).

Three strains were found to have conflicting identities based on their *sigB* and 16S rRNA sequences (Table 3). Two separate colonies from the same broth culture were used for 16S rRNA and *sigB* PCR; therefore, if broth cultures were contaminated with two types of *Listeria* this could have resulted in different 16S rRNA and *sigB* identities. Alternatively, an unclear PCR identity could reflect a novel *Listeria* species. Further phenotypic tests or sequencing of part or all of the genome could provide greater insight into the identity of these three *Listeria* strains.

RAPID'*L.mono* agar classifies *Listeria* spp. based on PI-PLC activity and xylose fermentation into four categories: *L. monocytogenes*, *L. ivanovii*, *innocua* group (*L. innocua*, *L. marthii*, and *L. grayi*) or *welshimeri* group (*L. welshimeri*, *L. seeligeri*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis*, *L. riparia*, *L. booriae*, and *L. newyorkensis*). This medium is often used to identify pathogenic *Listeria* species: *L. monocytogenes* and *L. ivanovii*. RAPID'*L.mono* agar was previously used to classify the *Listeria* isolates (Stea et al., 2015), of which 52% were sequenced in the present study. All 18 strains previously identified as *L. ivanovii* were identified as either *L. innocua*, *L. seeligeri* or unknown Listeria through 16S and *sigB* sequencing. For a *Listeria* isolate to appear as *L. ivanovii* on RAPID'*L.mono* agar (blue colony surrounded by yellow halo) it must have PI-PLC activity and ferment xylose. *L. innocua* typically does not fit either of these criteria, while *L. seeligeri* ferments xylose but is negative for PI-PLC (Weller et al., 2015). This result could indicate horizontal gene transfer between *Listeria* species. The possibility of horizontal gene transfer can be investigating by PCR targeting the PI-PLC and xylose PTS genes. Based on the present findings, the use of RAPID*'L.mono* agar alone may not be adequate for the correct identification of *L. ivanovii*.

L. fleischmannii was expected to be part of the *welshimeri* group because of previous reports that it ferments xylose (Weller et al., 2015). However, all strains identified as *L. fleischmannii* by 16S rRNA and *sigB* sequencing were previous classified as belonging to the *innocua* group, indicating that the *L. fleischmannii* isolated from the MM watershed do not ferment xylose.

3.4.2 Motility of *Listeria* Species Isolated from Nova Scotia Watersheds

In agreement with previous findings, *L. monocytogenes*, *L. innocua*, *L. seeligeri*, and *L. welshimeri* strains were all motile, and *L. fleischmannii* strains were immotile as reported in the past (Bertsch et al., 2013). It has been suggested that motility can affect the biofilm formation and desiccation tolerance of *Listeria* spp. (Lemon et al., 2007, Hingston et al., 2015). This purpose of this assay was to confirm the motility of the 16 strains, since their motility could be relevant when interpreting results from the biofilm, desiccation, and BAC assays.

3.4.3 Biofilm Formation of *Listeria* Species Isolated from Nova Scotia Watersheds

Biofilms allow microorganisms to resist desiccation, ultraviolet light, and antimicrobials; therefore, the biofilm forming ability of bacteria is a concern in the food industry. On average, *L. monocytogenes* strains exhibited the most biofilm formation, although it was not significantly ($p>0.05$) higher than that of the *L. welshimeri* and *L. seeligeri* strains tested. The biofilm formation of most *Listeria* spp. is not well characterized; however, the ability of *L. monocytogenes* to form biofilms is not a novel concept, and has been linked to persistence in food processing plants as well as specific outbreaks like the 2008 outbreak caused by Maple Leaf brand deli meats (Weatherhill, 2009; Nakamura et al., 2013). *L. innocua* and *L. fleischmannii* formed significantly less biofilm after 48 hours than the other species. The weaker biofilm formation of *L. fleischmannii* may be in part due to its immotility. Several studies have found immotile *L. monocytogenes* mutants to have decreased biofilm production compared to the motile wild-type (Lemon et al., 2007; Chang et al., 2012). Conversely, Piercey et al. (2016)

found than an immotile mutant, *Lm* 568 Δ *flaA*, formed more biofilm than the wild-type. One significant difference between these previous studies on motility and biofilm formation and the present study is the type of assay used. Instead of SS coupons and spot plate enumeration, biofilms were formed in wells of polystyrene microtiter plates and biofilm mass was evaluate by staining. Spot plate enumeration measures viable cells, whereas staining with crystal violet targets live and dead cells, as well as non-cellular biofilm components such as EPS. The material used in a biofilm assay is important since previous work has observed that *L. monocytogenes* forms varied amounts of biofilm depending on the surface material (Mafu et al., 1990; Blackman and Frank, 1996). SS coupons were selected for this experiment because it is a commonly used material in food processing facilities.

3.3.4 Desiccation Tolerance of *Listeria* Species Isolated from Nova Scotia Watersheds

Desiccation tolerance of some species was affected by the location from which the strains were isolated. For *L. innocua* and *L. monocytogenes* the decrease in viable cells of MM isolates was significantly greater ($p<0.05$) than that observed for CP isolates after 7 days of desiccation. This trend was particularly evident when looking at *L. monocytogenes*, whose MM Lm strains experienced the greatest decreases of all 16 strains, while CP Lm strains were the least affected, with one strain showing a slight gain in log CFU/cm^2 over the 7 day period. The significant difference in desiccation tolerance between strains from different watersheds could be the result of different selective pressures in urban and rural environments. For example, in a drier environment, species with greater resistance to desiccation are more likely to survive, reproduce, and consequently pass on their resistance to future generations. Overtime, the selective pressure of the dry environment can result in a bacterial population with greater desiccation tolerance than a population in a wetter environment (Alpert, 2005).

3.3.5 Benzalkonium Chloride Resistance of *Listeria* Species Isolated from Nova Scotia Watersheds

For the BAC assays involving biofilms, MBIC and MBEC, *L. fleischmannii* was the most susceptible to BAC. Biofilms have been linked to increased antimicrobial resistance; therefore, the BAC susceptibility of *L. fleischmannii* may be the result of decreased biofilm production (Table 5) (Nakamura et al., 2013). For all strains, the MBIC at 6 days was equal or greater to the MBIC at 48 hours, indicating that frequency of sanitation is an important consideration when evaluating what concentration of benzalkonium chloride is appropriate for inhibiting the biofilm formation of *Listeria* species in food processing environments. The importance of frequent sanitation is further supported by the observation that *L. monocytogenes*, a human pathogen and major food safety concern, was one of the most BAC-resistant species in the 6 day MBIC and MBEC assays. The highest concentration of BAC tested in the MBEC assay, 140 μg/ml, was unable to reduce the survival of some strains by 95% ± 5%; however, this concentration of BAC is lower than what is recommended for the disinfection of hard surfaces. Health Canada (2015) recommends that in commercial areas, ≥450 μg/ml BAC is applied to hard surfaces for a minimum of 10 min. Further testing is needed to ascertain if the MBEC of these strains is below the recommended concentration.

CHAPTER 4 EFFECT OF OSMOLYTES ON THE DESICCATION TOLERANCE OF *LISTERIA MONOCYTOGENES*

4.1 Introduction

L. monocytogenes is a foodborne pathogen that has been isolated from many types of environments including soil, water, vegetation, sewage, farm environments, animal feeds, and food processing plants (Sauders and Wiedmann, 2007). Although *L. monocytogenes* is ubiquitous in the environment, foods typically become carriers through cross-contamination within processing plants. *L. monocytogenes* is able to colonize surfaces in a food processing plant, and survive sanitation procedures and other unfavourable conditions such as desiccation. Certain strains of *L. monocytogenes* have been found to persist for years, including 7 years in ice cream and cheese processing plants, and 8 years in a facility producing chilled pizza, pasta, and other RTE meals (Unnerstad et al., 1996; Miettinen et al., 1999; Keto-Timonen et al., 2007). This long-term persistence of *Listeria* is in part attributed to its stress tolerance. *L. monocytogenes* can tolerate a wide range of environmental conditions including high salt concentrations, low pH, refrigerator temperatures, and desiccation (Lado and Yousef, 2007).

Under environmental stress conditions such as high osmolarity, low temperature and desiccation, bacteria can produce and/or accumulate large amounts of osmolytes called compatible solutes. The accumulation of compatible solutes help bacteria survive environmental stresses by balancing osmolarity, stabilizing enzyme function, and helping to maintain membrane integrity (Sleator and Hill, 2001). Many types of compounds can act as compatible solutes including sugars, amino acids and their derivatives, and polyols (da Costa et al., 1998).

Several compounds have been identified as compatible solutes for *L. monocytogenes* including glycine betaine, carnitine, acetylcarnitine, proline, proline betaine, γ-butyrobetaine, and 3-dimethylsulphoniopropionate (Bayles and Wilkinson, 2000; Huang et al., 2015). Acetylcarnitine, proline betaine, γ-butyrobetaine, and 3-dimethylsulphoniopropionate act as osmo- and cryoprotectants for *L. monocytogenes* (Bayles and Wilkinson, 2000). However, their effects during desiccation have yet to be studied. Glycine betaine, carnitine and proline have been found to improve the desiccation tolerance of *L. monocytogenes*, but most studies have so far only tested one concentration of compatible solute on a single strain of *L. monocytogenes*. More research is needed to ascertain whether the protective effects of these compatible solutes are concentration- or strain-dependent. Sucrose, lactose, trehalose and other osmolytes have been found to act as compatible solutes for certain bacteria, but their effects on the stress tolerance of *L. monocytogenes* remain largely unknown.

The general aim of this study was to evaluate the impact of select osmolytes on the desiccation tolerance of *L. monocytogenes*. In order to determine whether the effects of the osmolytes are concentration- or strain-dependent, three concentrations of each osmolyte were tested on three strains of *L.* monocytogenes, each isolated from a different environmental source. The results of this experiment will provide greater insight into whether naturally occurring osmolytes found in food soils contribute to *L. monocytogenes* persistence.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Culture Conditions

Listeria monocytogenes strain 568, a food processing plant isolate, strain 085578 (Lm 08), a clinical isolate (blood), and strain CP 4-5-1, a watershed isolate, were used in this study (Kalmokoff et al., 2001; Gilmour et al., 2010; Stea et al., 2015). Bacterial strains were stored at -80˚C in Tryptic Soy Broth (TSB) (Bacto, BD Canada, Oakville, ON) supplemented with 20% sterile glycerol (Fisher Scientific, Whitby, ON, Canada).

4.2.2 Preculturing *L. monocytogenes*

Ten ml of TSB was inoculated with Lm 568, Lm CP 4-5-1, or Lm 08 (from colonies) and incubated at 15˚C for 48 h. Cells were harvested by centrifugation (10,000 × *g*, 10 min) and re-suspended in minimal media (Premaratne et al., 1991) to achieve an absorbance ($A_{450\ nm}$) of 1.0 and a final cell concentration of approximately 10^9 CFU/ml as determined by spot plating (5 spots of 10 µl each) on Tryptic Soy Agar (TSA). For each strain, 0.1 ml of standardized bacterial culture was added to 0.9 ml minimal media containing either 0 mM (control), 1 mM, 5 mM or 25 mM osmolyte solution. The osmolytes tested were choline, glycine betaine (GB), carnitine, proline, inositol, sucrose, trehalose and lactose (Acros Organics, Fairlawn, NJ, USA). The precultures without or with the specified concentrations of osmolytes were incubated at 15˚C for 3 days.

4.2.3 Desiccation of *L. monocytogenes*

Saturated solutions of potassium acetate (Acros Organics) were placed in the desiccation chamber (SICCO, Bohlender, Grünsfeld, Germany) three days prior to desiccation to achieve a relative humidity (RH) of 23% at 15˚C. Data loggers were used to monitor temperature and RH throughout the experiment (Gemini Tinytag View 2, Interworld Electronics and Computer Industries Inc., Markham, ON, CA).

For each preculture, cells were harvested by centrifugation (10,000 × *g*, 10 min) and then re-suspended in the same media used in the initial preculture step (minimal media with 0 (control), 1 mM, 5 mM, or 25 mM osmolyte) to achieve an absorbance ($A_{450\ nm}$) of 1.0 and a final cell concentration of approximately 10^9 CFU/ml as determined by spot plating (5 spots of 10 µl each) on TSA. Following protocol by Hingston (2013), ten µl of each sample was spotted in the bottom of triplicate wells of a 96 well plate (Costar, #3370, Fisher Scientific). Sterile minimal media without and with the osmolyte solutions were spotted in triplicate as a control. The 96 well plates were incubated at 15˚C and 23% RH for 5 days. After the desiccation period, 150 µl fresh TSB was added to each well and the plates were incubated at 15°C. The regrowth of the desiccation survivors was monitored by absorbance measurements ($A_{490\ nm}$) every 3 hours until stationary phase was reached after approximately 48 hours. Absorbance values were used to create a growth curve, which in turn was used to determine when station phase was achieved.

4.2.4 Statistical Analysis

Analysis was based on the concept that increased and decreased desiccation survival would result in changes to the 'time to regrowth' as compared to the control (Hingston, 2013). ANOVA with a post-hoc Tukey's test was conducted in RStudio to assess if there was a significant difference ($p<0.05$) in the amount of time to reach A_{490} = 0.3 between the control and osmolyte treatments. An absorbance of 0.3 was selected as it is the approximate midpoint of the exponential phase, and would therefore ensure that cells were no longer in lag phase and had not yet reached the stationary phase.

4.3 Results

Figures 9 and 10 illustrate the change in ‘time to regrowth’ (treatment – control) required for each culture to reach an absorbance (A_{490}) of 0.3. These results are also summarized in Table 7. Three osmolytes, proline, inositol and trehalose, had no significant ($p>0.05$) effect on desiccation survival. The remaining five osmolytes had either a positive or negative effect on desiccation tolerance, and these effects were often strain- and concentration-dependent.

All three concentrations of choline significantly ($p<0.05$) improved the desiccation survival of Lm 568, while the other two strains were not significantly impacted by the presence of choline. Carnitine also exhibited positive effects on desiccation survival. Lm CP 4-5-1 and Lm 08 exhibited increased desiccation resistance when treated with 1 mM and 5 mM carnitine.

GB, sucrose, and lactose, all had a negative impact on desiccation survival. Desiccation tolerance was only affected by the highest concentration of osmolyte tested. Twenty five mM sucrose significantly ($p<0.05$) decreased the desiccation survival of all three strains. The desiccation survival of Lm 568 was also negatively affected by 25 mM GB or lactose.

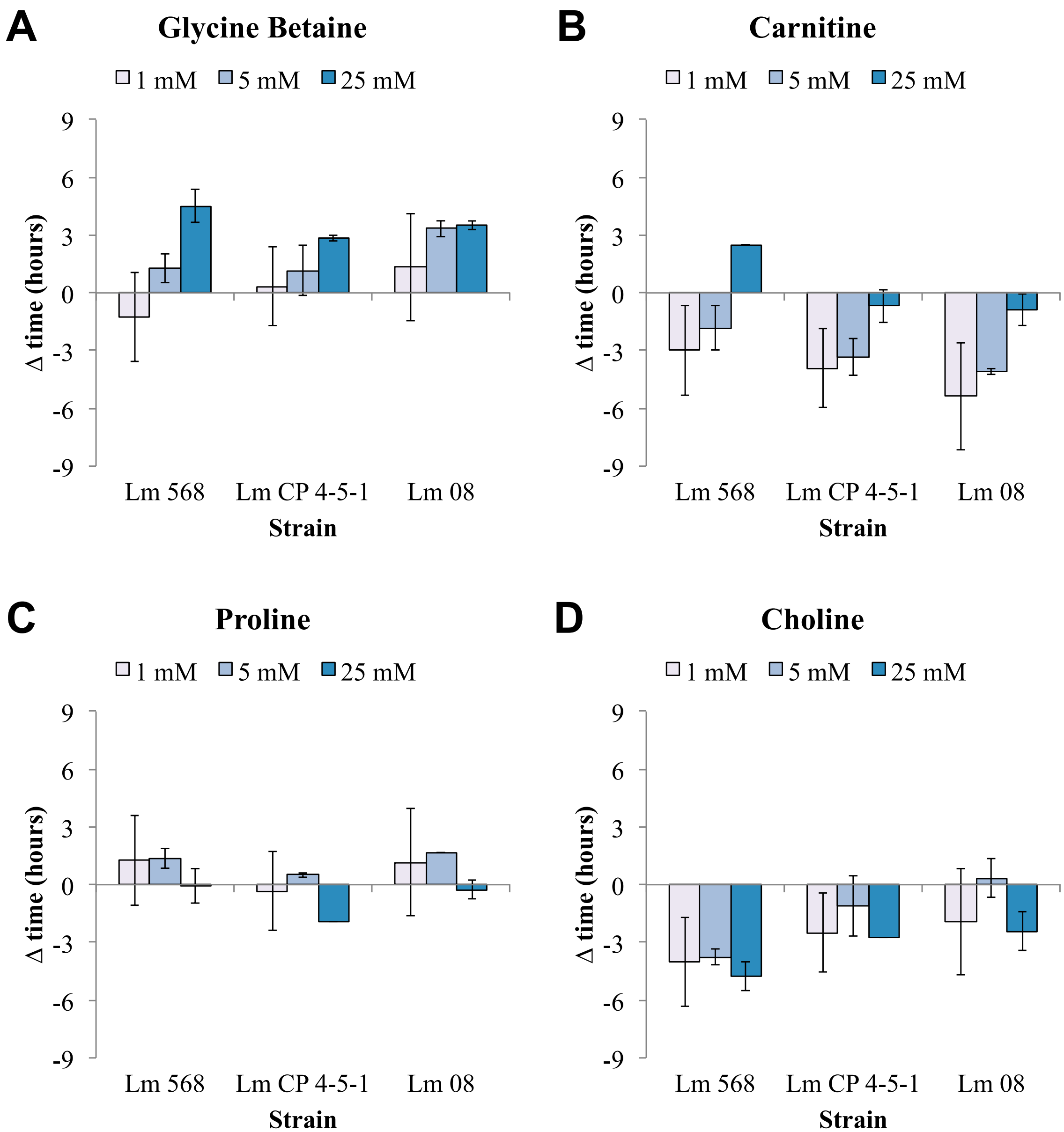

Figure 9. Change in 'time to regrowth' (treatment – control) for Lm 568, Lm CP 4-5-1 and Lm 085578 cells to reach an A_{490} of 0.3 following preculture and desiccation (7 days at 23% RH and 15°C) with 1 mM, 5 mM and 25 mM GB (A), carnitine (B), proline (C), choline (D). Negative Δtime values indicate a protective effect of the osmolyte. Error bars indicate the standard deviation.

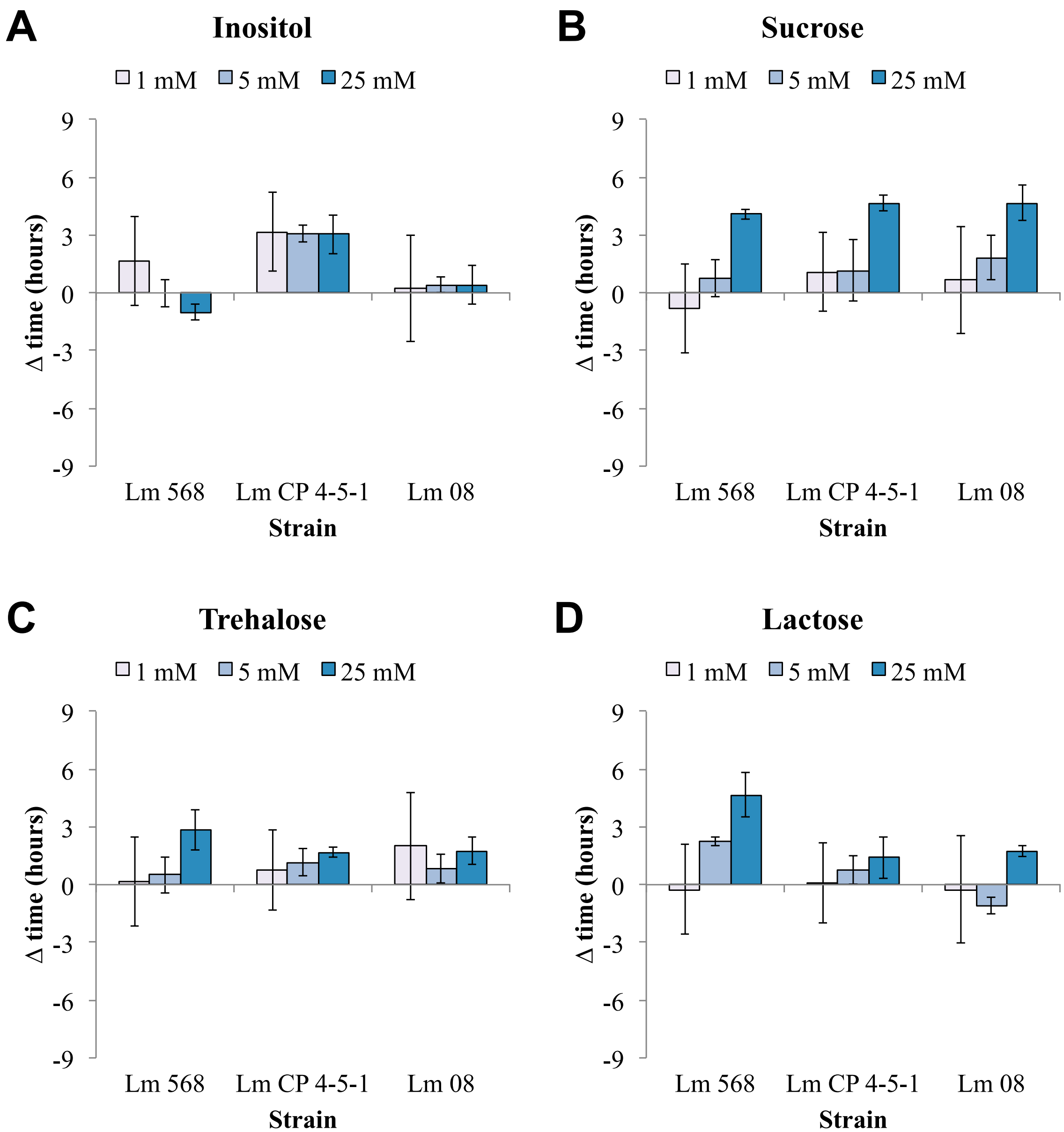

Figure 10. Change in 'time to regrowth' (treatment – control) for Lm 568, Lm CP 4-5-1 and Lm 085578 cells to reach an A_{490} of 0.3 following preculture and desiccation (7 days at 23% RH and 15°C) with 1 mM, 5 mM and 25 mM inositol (A), sucrose (B), trehalose (C) and lactose (D). Negative Δtime values indicate a protective effect of the osmolyte Error bars indicate the standard deviation.

Table 7. Effect of different concentrations of osmolytes on the desiccation survival of three strains of *L. monocytogenes*: Lm 568, Lm CP 4-5-1, and Lm 085578. Symbols show whether there was no significant (at the 5% level) difference (·), an increase (+) or a decrease (−) in desiccation survival compared to the control. Desiccation survival was measured as the 'time to regrowth' to achieve A_{490} = 0.3 following desiccation.

Osmolyte	Lm 568			Lm CP 4-5-1			Lm 085578		
	1 mM	5 mM	25 mM	1 mM	5 mM	25 mM	1 mM	5 mM	25 mM
GB[a]	·	·	−	·	·	·	·	·	·
Carnitine	·	·	·	+	+	·	+	+	·
Proline	·	·	·	·	·	·	·	·	·
Inositol	·	·	·	·	·	·	·	·	·
Choline	+	+	+	·	·	·	·	·	·
Sucrose	·	·	−	·	·	−	·	·	−
Trehalose	·	·	·	·	·	·	·	·	·
Lactose	·	·	−	·	·	·	·	·	·

[a] – GB – glycine betaine

4.4 Discussion

The current study demonstrated that osmolytes can significantly impact the desiccation survival of *L. monocytogenes*; however, this depends on the osmolyte, its concentration, and the *Listeria* strain in question. Proline, inositol and trehalose had no significant ($p>0.05$) effect on desiccation survival. Little is known about inositol as a compatible solute for bacteria. Although inositol has not been investigated as a compatible solute for *L. monocytogenes*, it has been shown that the intracellular concentration of inositol is 2.1-fold greater at 8˚C than 37˚C, suggesting that the cell may accumulate inositol as a response to cold stress (Singh et al., 2011). The same study observed that the intracellular concentration of trehalose was 1.4-fold greater at the lower temperature. Previous research has found that proline can have protective effects on *L. monocytogenes* survival during high osmolarity, and desiccation, but to a lesser extent than glycine betaine and carnitine (Beumer et al., 1994; Huang et al., 2015). Contrary to results in the present study, Huang et al. (2015) observed that 1 mM proline was sufficient to significantly improve the desiccation of tolerance of *L. monocytogenes*. Another study observed that this concentration of proline had no significant effect on *L. monocytogenes* survival at low temperature or high osmolarity (Bayles and Wilkinson, 2000). Increasing the concentration of proline to 10 mM was found to significantly improve the osmotolerance of *L. monocytogenes* (Beumer et al., 1994). Although previous studies found 1 mM or 10 mM proline to be sufficient for improved survival under environmental stresses, the present study found that 25 mM proline was insufficient to significantly improve *L. monocytogenes* desiccation survival. This may indicate low sensitivity of the microtiter assay used.

Choline and carnitine were both found to improve the desiccation survival of *Listeria monocytogenes*. Previous studies found that 1 mM carnitine acts as an osmo- and cryoprotectant for *L. monocytogenes*, as well as improve survival under desiccation (Bayles and Wilkinson, 2000; Huang et al., 2015). Little is known about choline as a compatible solute for *L. monocytogenes* or its effects of on the desiccation tolerance of bacteria in general. Choline has been found to have osmoprotective capacity for *Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa,* as well as thermoprotective effects in *Bacillus subtilis* (Boch et al., 1994; Hoffmann and Bremer, 2011; Lamark et al., 1996; Fitzsimmons et al., 2012). Interestingly, choline is a precursor to glycine betaine, a known compatible solute for *L. monocytogenes*, and it is believed that the protective effect of choline is dependent on its conversion to glycine betaine (Wood et al., 2001). Le Rudulier et al. (1984), found that *E. coli* mutants unable to convert choline to glycine betaine were not protected against osmotic stress by choline unlike the wild-type.

Glycine betaine is widely accepted as a compatible solute for *L. monocytogenes* under a variety of stress conditions including high osmolarity, low temperature, and desiccation (Ko et al., 1994; Bayles and Wilkinson, 2000; Angelidis and Smith, 2003; Dreux et al., 2008; Huang et al., 2015). Previous findings do not align with the results from this study. Desiccation of *L. monocytogenes* in 1 mM and 5 mM GB had no significant effect on desiccation tolerance, while at 25 mM GB, Lm 568 displayed a significant increase in sensitivity to desiccation. In contrast, previous research found that 1 mM GB improved the survival of *L. monocytogenes* desiccated on stainless steel (SS) better than carnitine and proline (Huang et al., 2015). In another study, it was observed that 1, 2.5, 5, 25, and 250 mM GB significantly improved the desiccation survival of *L. monocytogenes* in microtiter plate wells (Dreux et al., 2008). The discrepancy between the GB results from the present study and Dreux et al (2008) may be in part due to differences in experimental conditions such as strain, RH, desiccation medium, and method of bacterial enumeration. The non-significant effect of GB observed for many of the treatments may indicate low sensitivity of the microtiter assay used in the present study.

Sucrose and lactose both had a negative impact on desiccation tolerance of at least one of the tested strains of *L. monocytogenes*. Previous research on these two osmolytes as compatible solutes is limited. Singh et al. (2011) observed that the intracellular concentration of sucrose in *L. monocytogenes* was 7.2-fold greater at 8˚C than 37˚C, suggesting that sucrose may act as a thermoprotectant. The effect of sucrose on *L. monocytogenes* desiccation survival was previously unknown; however, sucrose was found to improve the desiccation survival of *S. aureus* and *Salmonella enterica* (Chaibenjawong and Foster, 2011; Gruzdev et al., 2012). There has been some work investigating lactose as a compatible solute for *L. monocytogenes.* El Kest and Marth (1991) observed that lactose could act as a cryoprotectant for *L. monocytogenes*.

For many of the osmolytes tested, their effect on desiccation was not consistent for all three strains of *L. monocytogenes.* Previous studies comparing osmolyte activity for two or three *L. monocytogenes* strains observed similar results across all strains (Beumer et al., 1994; Bayles and Wilkinson, 2000). The three strains used in the present study were isolated from different sources: a food processing plant, human blood, and a watershed. Bacteria in different environments are exposed to different selective forces and may therefore evolve to better adapt to their environment. The difference in osmolyte effect between strains may be the result of adaptation to different environments.

The assay used in the present study was designed as an initial screening to gauge the overall impact of osmolytes on *L. monocytogenes*. Osmolytes that showed significant differences in desiccation tolerance in the microtiter plate assay would be selected for a desiccation assay using SS coupons to better mimic a food-processing environment. In the microtiter plate assay, the only new, previously unstudied osmolyte to show a protective capacity was choline. The low sensitivity of the microtiter plate assay had some influence on the decision not to proceed with the SS coupon assay; however, the main factor was the lack of new osmolytes identified in the first assay.

CHAPTER 5 CONCLUSION

5.1 Project Summary

The diversity of *Listeria* species in an urban and a rural Nova Scotia watershed was evaluated. Presumptive *Listeria* isolates (n=679) from the two watersheds were identified through sequencing of the 16S rRNA and/or *sigB* genes. Greater species diversity was observed in the rural watershed. *L. monocytogenes*, *L. innocua*, and *L. seeligeri* were found in both environments. *L. welshimeri* and *L. fleischmannii* were only isolated from the rural Middle Musquodoboit (MM) watershed. The isolation of *L. fleischmannii* in Canada has not yet been reported. Some isolates were previously classified as *L. ivanovii* through culturing on RAPID'*L.mono* agar; however, DNA sequencing of 16S rRNA and *sigB* genes did not identify any isolates as *L. ivanovii*. The high false positive rate observed in this study raises serious concern about the specificity of RAPID'*L.mono* agar for *L. ivanovii*.

Fitness assays compared the motility, biofilm formation, desiccation tolerance and antimicrobial resistance of the five species isolated from the watersheds. *L. fleischmannii*, the only immotile species evaluated, was one of the poorest biofilm formers and was generally the most susceptible to BAC, suggesting that motility might improve biofilm formation and further supporting the concept that biofilm formation can improve antimicrobial resistance. *L. monocytogenes* strains isolated from the MM watershed were the most desiccation sensitive, while *L. monocytogenes* strains isolated from the CP watershed were the most desiccation tolerant. This difference in desiccation tolerance between strains from different watersheds highlights that the selective pressures in a particular environment can have an effect on the on the fitness of *Listeria* and consequently its persistence in food processing plants.

The presence and accumulation of compatible solutes is known to improve bacterial survival under environmental stresses such as high osmolarity, low temperature, and desiccation. Eight osmolytes (glycine betaine (GB), carnitine, proline, choline, inositol, sucrose, trehalose and lactose) were assessed as compatible solutes for *L. monocytogenes* at 23% RH. Three strains of *L. monocytogenes* and three concentrations of osmolyte were used to ascertain if the protective effects of the osmolytes were strain or concentration dependent. Only carnitine and choline improved the desiccation tolerance of *L. monocytogenes*, and these effects were concentration and strain dependent. Prior to this study, the impact of choline on the desiccation tolerance of *L. monocytogenes* was unknown.

The results of this study provide greater insight into natural reservoirs of pathogenic *Listeria*. Studying the diversity of *Listeria* species in the two watersheds gives insight into natural aqueous reservoirs of pathogenic *Listeria* species and possible transmission pathways of *L. monocytogenes*. Evaluation of biofilm formation, desiccation tolerance, and antimicrobial resistance, provides further understanding of factors that contribute to *L. monocytogenes* persistence in food processing environments. An improved knowledge of the transmission pathways and factors affecting persistence can contribute to the development of more effective sanitation programs thereby reducing the contamination of food products and resulting illness.

5.2 Future Directions

1. Develop improved methods of isolating *Listeria* spp. from environmental samples that do not prevent the selection of certain *Listeria* spp. (e.g. mannitol fermenting species).
2. Conduct further phenotypic and/or genotypic testing to identify the three unknown *Listeria* isolates.
3. Further evaluate the ability of RAPID'*L.mono* agar to correctly identify *L. ivanovii* by sequencing more *Listeria* isolates that were previously identified as *L. ivanovii* from culturing on RAPID'*L.mono* agar.
4. Provide a more thorough comparison of biofilm formation and desiccation tolerance, by sampling coupons over a longer period of time and for the desiccation assay repeat assay at multiple RHs.
5. Further investigate the difference in desiccation survival between *L. monocytogenes* strains from different environments by repeating desiccation assay protocol with more *L. monocytogenes* isolates from each watershed.
6. Test higher concentrations of BAC for the strains whose biofilms were not completely eradicated by 140 μg/ml BAC.
7. Evaluate the resistance of the *Listeria* strains to other commonly used disinfectants.
8. Develop a desiccation assay with greater sensitivity to identify new compatible solutes for *L. monocytogenes*.

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APPENDIX A MAPS OF MM AND CP WATERSHEDS

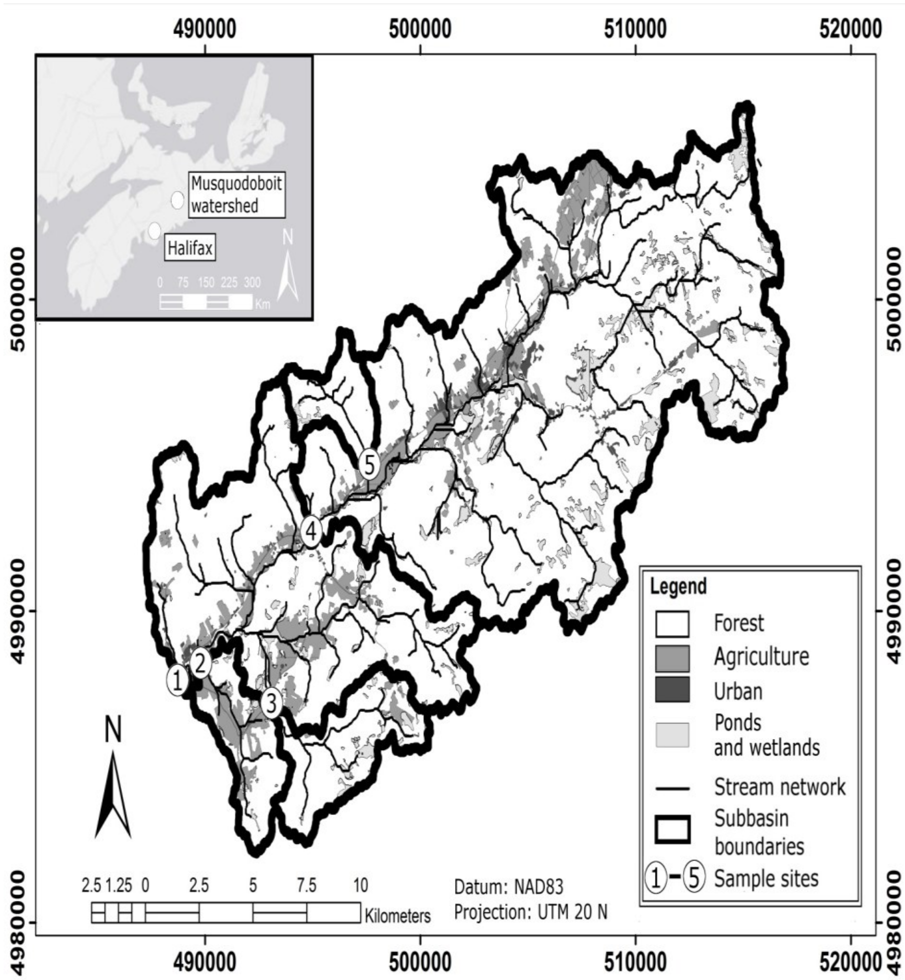

Figure A1. Map of Middle Musquodoboit watershed and sampling locations (Stea et al., 2015).

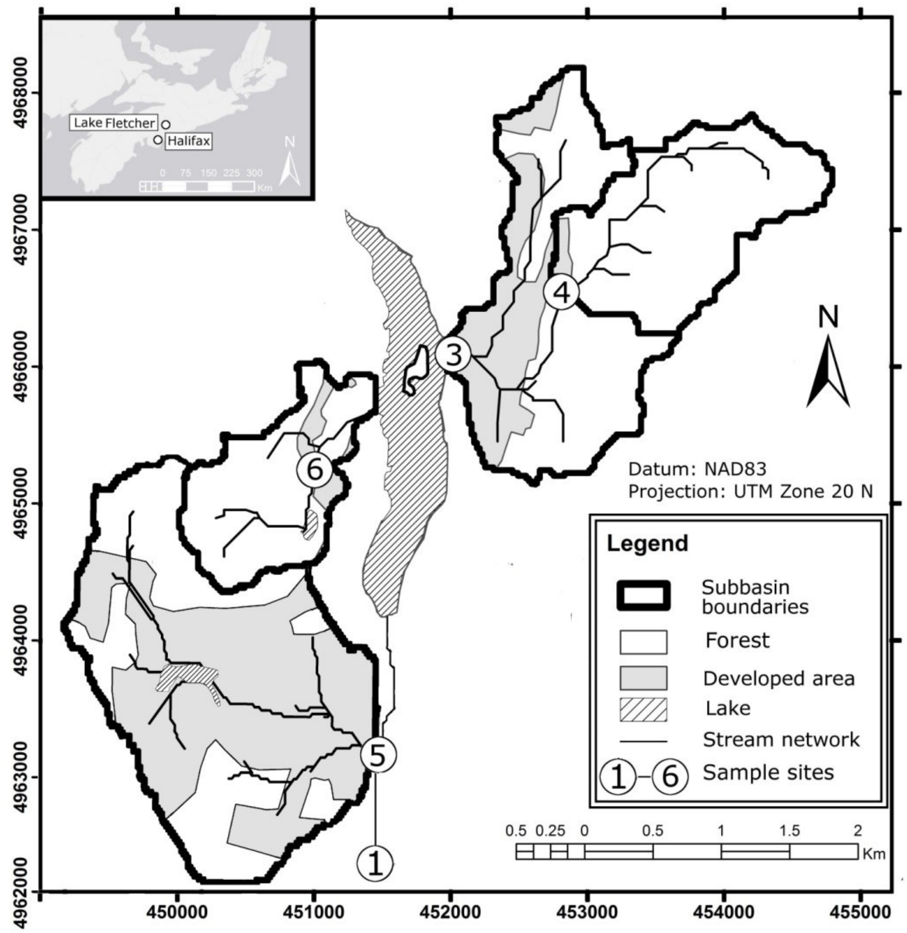

Figure A2. Map of Collin's Park watershed and sampling locations (Stea et al., 2015).

APPENDIX B SUPPLEMENTARY DATA

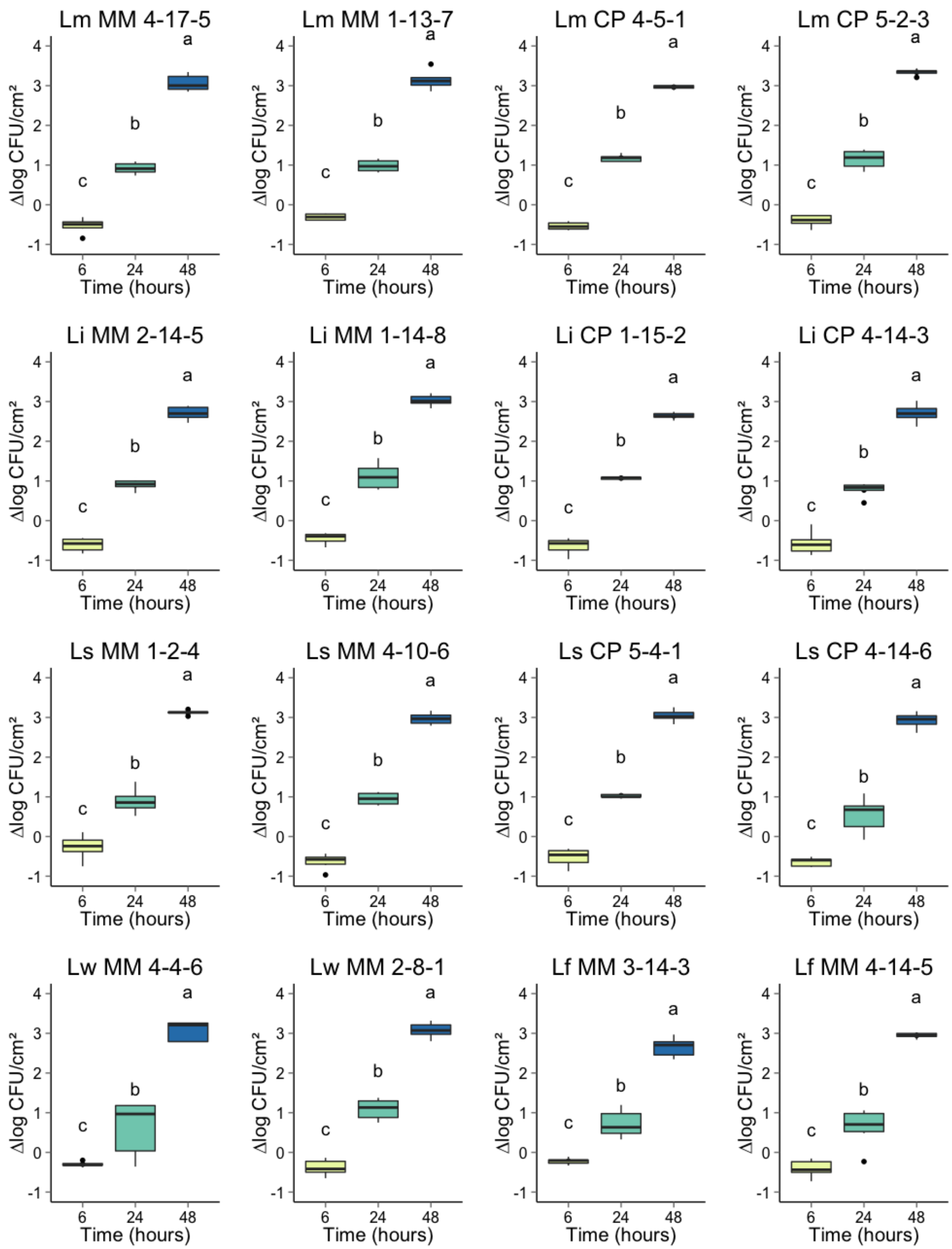

Figure B1. Change in log CFU/cm^2 of *Listeria* strains on SS coupons incubated at 15˚C and 100% RH for 6, 24 and 48 hours. The central line in the box plot represents the median (50th percentile), while the lower and upper edges of the box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles, and outliers are shown as black circles. Letters above box plots represent Tukey post hoc test groupings, where treatments sharing the same letter are not significantly different ($p>0.05$).

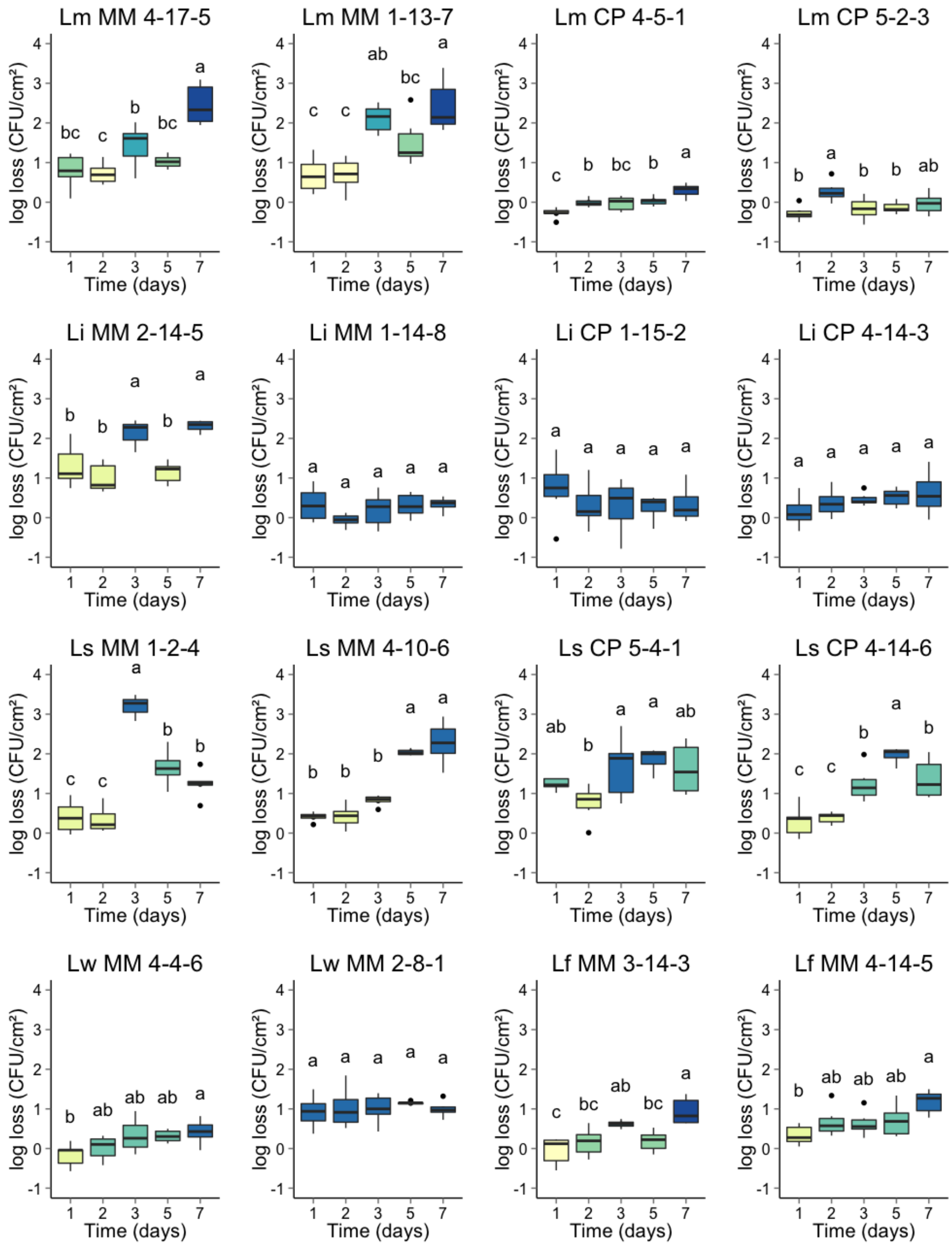

Figure B2. Log loss (CFU/cm^2) of *Listeria* strains on SS coupons incubated at 15˚C and 23% RH for 1, 2, 3, 5, and 7 days. The central line in the box plot represents the median (50th percentile), while the lower and upper edges of the box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles, and outliers are shown as black circles. Letters above box plots represent Tukey post hoc test groupings, where treatments sharing the same letter are not significantly different (p>0.05).

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Title: Comparison of the Prevalences and Diversities of Listeria Species and Listeria monocytogenes in an Urban and a Rural Agricultural Watershed

Author: Emma C. Stea, Laura M. Purdue, Rob C. Jamieson et al.

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