Effect of Preservatives on Growth and Filamentation of *Listeria monocytogenes* on Ready-to-Eat Meats

by

Devon B. Willis

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Department of Agricultural, Food and Nutritional Science

University of Alberta

Abstract

Listeria monocytogenes is a foodborne pathogen known to contaminate RTE foods and grow at refrigeration temperatures. It is also known to form filaments when stressed, which may affect enumeration and compromise consumer safety. The objective of this study was to observe the growth and morphological changes in *L. monocytogenes* on RTE hams with multiple preservative treatments and stored at refrigeration temperatures.

Two strains were inoculated onto hams formulated with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w), chitosan (0.5% v/w added as a solution in 1% acetic acid), partially purified bacteriocins (25.6 activity units/g) from *Carnobacterium maltaromaticum* UAL307, and combinations of sodium lactate and sodium diacetate with chitosan or the partially purified bacteriocins. Inoculated hams were stored at 4°C for up to 120 d. Growth characteristics were determined by plating on PALCAM agar, while filamentation was assessed by imaging flow cytometry.

A combination of sodium lactate and sodium diacetate with bacteriocins caused a significant decrease in the growth of *L. monocytogenes* on the hams compared to the other antimicrobial treatments. Imaging flow cytometry found that the proportion of the bacterial population that filamented was significantly lower on hams formulated with chitosan, either with or without bacteriocins present.

These results show that different preservatives have different effects on the growth and cell morphology of *L. monocytogenes*, which may affect how these preservatives are applied and how this pathogen is regulated on RTE foods.

Preface

This thesis is an original work by Devon B. Willis. No part of this thesis has been previously published. Dr. Januana Teixeira and Dr. Danielle Balay from the University of Alberta provided the partially purified bacteriocins from *Carnobacterium maltaromaticum* UAL307.

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1. General Introduction and Literature Review

1.1 Introduction

Since its first characterization in rabbits by Murray et al. (1926), *Listeria monocytogenes* has been a pathogen of interest. *L. monocytogenes* is a Gram-positive, motile bacillus that is found ubiquitously in the environment. This combined with its ability to grow at common refrigeration temperatures (4°C) and a high mortality rate among those at-risk (Gellin et al., 1991; Schlech, 2000), makes *L. monocytogenes* an important bacterium to understand and control with regards to food safety. The importance of this is emphasized by recent foodborne outbreaks, resulting in numerous fatalities and millions of dollars lost.

In 2008, a large outbreak of listeriosis occurred in Canada occurred due to consumption of contaminated deli meats from Maple Leaf Foods, resulting in 23 deaths (Government of Canada, 2009; Howell and Miller, 2010). Another listeriosis outbreak occurred in South Africa in 2017, associated with "polony" (deli meat) and resulted in not only 204 fatalities, but millions of dollars lost due to mortality and hospitalization costs (Olanya et al., 2019). Both of these outbreaks, as well as many others, had a high mortality rate in at-risk populations, particularly the elderly, immunocompromised, and especially in pregnant women and fetuses/newborns (Buchanan et al., 2017; Conly and Johnston, 2008; McLauchlin et al., 2004; Ramaswamy et al., 2007; Schlech, 2000).

As a result of *L. monocytogenes* prevalence and risk, Health Canada has taken steps to monitor and control this pathogen. RTE foods are categorized based on the risk of growth of *L. monocytogenes*: Category 1 foods support the growth of *L. monocytogenes* growth, Category 2A

foods in which limited growth (less than 100 colony forming units (CFUs)/g over the stated shelf life) can occur, and Category 2B foods in which growth cannot occur (Health Canada, 2011; 2012). Methods in industry to control for *L. monocytogenes* growth include surveillance Hazard Analysis and Critical Control Point (HACCP) planning, physio-chemical treatments, and use of preservatives (Buchanan et al., 2017; McLauchlin et al., 2004; Health Canada, 2011). Bacteriocins, antimicrobial agents produced by bacteria, are one example of a preservative that are suggested as control methods (Zacharof and Lovitt, 2012).

The purpose of this research was to examine the effect of preservatives on the morphology of *L. monocytogenes* growing on RTE meats. Specifically, this means observing filamentation of *L. monocytogenes* when exposed to combinations of sodium lactate/diacetate, chitosan, and bacteriocins on packaged ham slices.

1.2 Regulatory Guidelines for *L. monocytogenes* in Canada

The Canada "Policy on *Listeria monocytogenes* in ready-to-eat foods" (2011) is the main regulatory guideline for food processing plants with regards to the presence and growth of *L. monocytogenes* on their products. This document, as well as Canada's current view on *L. monocytogenes* in food, was largely influenced by the listeriosis outbreak in 2008. This outbreak in deli meat from Maple Leaf Foods was a devastating reminder of the deadliness of *L. monocytogenes*, as well as the need to overhaul the food safety regulations.

The 2008 Maple Leaf Foods outbreak led to 57 confirmed cases and 23 fatalities over the course of approximately 8 months (Government of Canada, 2009; Health Canada, 2011). Additionally, the costs of this outbreak (including medical/nonmedical costs, productivity losses, plant and federal agency response costs) reached upwards of $242 million, with an approximate

$2.8 million per case cost (Thomas et al., 2015). While Maple Leaf Foods was able to keep customer trust through transparency and sincerity (Greenberg, 2009; Howell and Miller, 2010), the fact that such a major outbreak occurred displayed something lacking with the food safety guidelines of the time.

The most recent version of the "Policy on *Listeria monocytogenes* in ready-to-eat foods" (2011) recognizes different categories of RTE foods based on the ability of *L. monocytogenes* to can grow on products. Based on this, RTE foods are divided into different risk groups.

Category 1 includes RTE foods where *L. monocytogenes* can grow throughout the stated shelf life. These foods represent the highest risk to consumers and require strict oversight and testing. Any detection of *L. monocytogenes* within 5 x 25g analytical units results in a failed test. Foods in this category include deli-meats, soft cheeses, hot dogs, and pâté (Health Canada, 2011).

Category 2A includes RTE foods where there is potential limited growth of *L. monocytogenes* throughout the stated shelf life. Specifically, numbers of *Listeria* cannot exceed 100 CFUs/g in 5 x 10g analytical units. Foods in this category are mainly foodstuffs that lack a kill step or have a shelf life less than 5 days, including cold-smoked fish and fresh-cut produce (Health Canada, 2011).

Category 2B includes RTE foods where no growth of *L. monocytogenes* can occur, defined as a less than 0.5 log CFU/g increase throughout the stated shelf life. These foods may be subject to the 100 CFU/g guide the same as Category 2A foods, but are generally considered low priority/concern. Foods in this category include ice cream, hard cheeses, dry salami, and dry-salted fish (Health Canada, 2011).

However, recent evidence may bring current guidelines into question. In 2015, an outbreak of listeriosis in the United States was traced to ice cream used in milkshakes (Pouillot et al., 2016). The significance of this outbreak stems from the fact that it occurred in a traditionally considered low-risk RTE food, and the low levels of *L. monocytogenes* present (approximately 8 cells/g). Despite being widely distributed, the only patients severely affected by the contaminated product were highly susceptible hospital inpatients (Pouillot et al., 2016). Some may suggest, based on this information, that the current guidelines of acceptable threshold levels of *L. monocytogenes* in Category 2A and 2B RTE foods may need to be reconsidered, especially with regards to at-risk consumers.

1.3 Control of *L. monocytogenes* in Food

L. monocytogenes demonstrates an ability to live in a variety of environments and under differing conditions. In addition to surviving at low temperatures, it can also survive acidic and osmotic stress (Cole et al., 1990; Gandhi and Chikindas, 2007), and the ability to form biofilms affords the pathogen more resistance (Carpentier and Cerf, 2011; Gandhi and Chikindas, 2007). Due to this, many studies have been done to find effective measures to inhibit *Listeria* growth on foods.

The first step in controlling pathogens in food processing is developing a culture of safety among employers, employees, and customers. By taking time and resources to identify risks, evaluating practices, openly communicating with and empowering workers (Powell et al., 2011), food processing companies can begin to successfully implement effective control systems, starting with HACCP plans.

1.3.1 HACCP Plans

As *L. monocytogenes* is ubiquitous in the environment, there are many potential sources of contamination for manufacturing RTE processed foods. Because RTE food products do not always involve a heat-killing step prior to consumption, other measures are used to control pathogens such as *L. monocytogenes.* One such method is the implementation of a HACCP plan, in order to identify potential hazards, implement solutions, and verify the effectiveness of interventions.

A HACCP plan first requires prerequisite programs, which are the basic conditions within the plant to ensure safe food production. These can include for sanitation, recalls, equipment, training, and others. From these prerequisite programs, a HACCP plan is made to: 1) assess the hazard present, 2) identify critical control points, where a control measure can be introduced to reduce said hazard, 3) setting critical limits to distinguish safe and unsafe operations at the critical control point, 4) monitoring and measuring the control point, 5) establishing corrective actions if deviations are detected, 6) verification that corrective actions are working, and 7) record keeping. Implementation of and adherence to HACCP plans gives processing plants a foundation to assess risks before they result in contaminated foods being distributed to consumers.

While HACCP plans and prerequisite programs theoretically set a basis of control during food production, they do not prevent contamination in and of themselves. Physical and chemical interventions are still needed to reduce the risk of bacteria remaining and growing on produced foodstuffs.

1.3.2 Physio-chemical Control Methods

As stated above, *L. monocytogenes* is found in many environments and can survive under various stresses. *L. monocytogenes* is known for surviving and multiplying at refrigeration temperatures approaching 1°C (Borovic et al., 2014; Todd and Notermans, 2011). As well, it has the ability to survive wide pH differences (4.3-9.4), and slightly decreased water activity levels (a_W 0.92 and above) (Ingham et al., 2004; Lakicevic and Nastasijevic, 2017). These growth capabilities influence the types of controls established for *L. monocytogenes* on RTE foods.

According to the "Policy on *Listeria monocytogenes* in ready-to-eat foods" (2011), the definition of a RTE food where *L. monocytogenes* will not grow includes: a) pH < 4.4, regardless of a_W; b) a_W < 0.92, regardless of pH; c) combinations of factors (namely a_W and pH); or d) frozen. These parameters are set by the Codex Alimentarius Commission (CAC, 2009), and are taken as the limits of growth for *L. monocytogenes* on RTE foods. Foods that naturally have these parameters are classified as Category 2B (Health Canada, 2011), while others that fail to meet these criteria (Category 1 & 2A) must have methods to control the growth of *Listeria*.

As RTE foods do not always have a high temperature kill step prior to consumption, other methods of control need to be used. Many plants use sanitary practices, prerequisite programs, and HACCP plans to prevent contamination with *L. monocytogenes* in the first place. However, certain chemicals, specifically salt compounds, have proven useful for inhibition of *L. monocytogenes* in foodstuffs.

1.4 Additives/Processing Aids for Controlling *L. monocytogenes* in RTE Meats

Health Canada regulates the food additives permitted for use in foods produced within the country and those that are imported. Additives, by definition, are any substance that is added

to a foodstuff, becoming a constituent of that product or affecting its characteristics (CFIA, 2019). Under this definition are different classes of preservatives; additives specifically used to control microbial growth and spoilage on foods. Of these preservatives, Class 2 preservatives are specifically designated for use against bacterial foodborne pathogens. Currently, Health Canada recognizes potassium lactate, sodium acetate, sodium diacetate, sodium lactate, and the organism *Carnobacterium maltaromaticum* CB1 as additives that can be used as Class 2 preservatives to control the growth of *L. monocytogenes* on RTE foods within Canada (Health Canada, 2012).

Multiple studies have shown the effectiveness of these preservatives in the inhibition of *L. monocytogenes* in foods. Salts such as sodium lactate and diacetate have inhibitory effects against the growth of *L. monocytogenes* (Barmpalia et al., 2005; Glass et al., 2002; Shelef and Yang, 1991). The compound chitosan, from crustacean shells, has also been shown to be effective in limiting *Listeria* growth (No et al., 2002; Ye et al., 2008). Finally, there is interest in bacteriocins as treatment against bacteria in foods, in particular against *L. monocytogenes* (Davies et al., 1997; Lakicevic and Nastasijevic, 2017; Liu et al., 2014a; Martin-Visscher et al., 2008; Schillinger et al., 1991). To maximize effectiveness, these preservatives are often used in combination with each other and other control methods, known as a hurdle approach (Benabbou et al., 2009; Lakicevic and Nastasijevic, 2017; Mbandi and Shelef, 2002).

1.4.1 Sodium Lactate and Sodium Diacetate

Both sodium lactate and sodium diacetate are approved preservatives for the control of *L. monocytogenes* on RTE foods in Canada (Health Canada, 2012). They are salts of lactic acid and acetic acid, respectively. These acids are both weak acids, meaning they are partially dissociated in solution, which is postulated to be the reason for their antimicrobial activity. These weak acids cross the lipid barrier of the bacterial cell membrane, where they can alter the pH homeostasis of

the cell, disrupting important metabolic functions (Salmond et al., 1984). Others have suggested that the acids, once inside the cells, inhibit cellular uptake of important substrates, such as amino acids and phosphates (Freese et al., 1973). Regardless of the true mechanism of action, the effect these compounds have on *L. monocytogenes* growth is well documented.

Synergistic effects of using both lactate and diacetate salts have been reported in the literature. Multiple studies have shown that using combinations of sodium lactate with sodium diacetate significantly enhances their effects on *L. monocytogenes* growth (Barmpalia et al., 2005; Glass et al., 2002; Mbandi and Shelef, 2002, 2001; Schlyter et al., 1993; Shelef and Yang, 1991). This synergistic effect also extends to other preservatives, such as the bacteriocin nisin when used with sodium diacetate on smoked trout (Nykänen et al., 2000). However, other factors such as temperature, moisture content, and pH also play a role in the efficacy of these preservatives (McDonnell et al., 2013). Regardless, sodium lactate and diacetate continue to be used as preservatives in RTE foods due to their efficacy when used together, and their limited effect on food quality.

1.4.2 Chitosan

Chitosan is a glucosamine polymer, formed by the deacetylation of chitin, the structural polymer found in shellfish exoskeletons. It is also produced by several fungi species. While not included in Health Canada's list of approved preservatives for RTE foods for control of *L. monocytogenes*, it has gained attention for its antimicrobial, nontoxic, and biodegradable properties. However, the mechanism of action against bacteria is still poorly understood. A common theme in the mode of action is the positive charge of the chitosan molecules and their interaction with the negatively charged cell membrane (Chen et al., 1998; Rabea et al., 2003). Activity is also influenced inversely by pH, and degree of oligomerization also affects the mode

of action (Benabbou et al., 2009; No et al., 2002). While some believe this interaction simply disrupts membrane stability and causes leakage, evidence suggest multiple complex interactions within the cell, requiring further investigation (Kong et al., 2010).

Due to chitosan's solubility and increased effectiveness at lower pH, use with organic acids such as lactic and acetic acid can increase the antimicrobial properties of the compound (No et al., 2002; Ye et al., 2008). Bacteriocins also show synergistic effects with chitosan, regardless of the amount of oligomerization (Benabbou et al., 2009). These synergistic effects make chitosan ideal to be used with other preservatives for controlling *L. monocytogenes* on RTE foods. This, in addition to its biodegradability and nontoxicity, make chitosan a potentially powerful antilisterial agent in RTE foods.

1.4.3 Bacteriocins

Bacteriocins are low molecular weight peptides, produced by bacteria and have a more selective antimicrobial activity spectrum compared to antibiotics. In terms of food safety, bacteriocins are attractive options for their high specificity and their adherence to the demand for "natural" food ingredients. Bacteriocins are grouped into multiple classes: Class I, Class II, and Class III, (Zacharof and Lovitt, 2012). Pediocin-like bacteriocins, such as pediocin and leucocin A, are heat stable peptides less than 10 kDa, and do not require enzymatic modification after production to be effective (Alvarez-Sieiro et al., 2016). They are noted for antimicrobial effect against *Listeria*, but evidence suggests resistance to these bacteriocins may develop (Gravesen et al., 2002).

Multiple studies have shown the effectiveness of bacteriocins against *L. monocytogenes* in particular across a wide variety of foods. Nisin from *Lactococcus lactis* subsp. *lactis* and Pediocin from *Pediococcus acidilactici* demonstrate effective growth inhibition in meat products

(Ming et al., 1997; Schillinger et al., 1991). Cheeses are also suitable for use of bacteriocins against *L. monocytogenes*, with high retention times within the food matrix (Davies et al., 1997). Recently, it has also been suggested that the use of bacteriocin-producing *Lactobacillus* may help control *L. monocytogenes* biofilm formation in industrial settings (Pérez-Ibarreche et al., 2016). Despite strains specificity and high production costs, bacteriocins are effective control methods for foodborne pathogens, especially *L. monocytogenes*.

1.5 Bacterial Filamentation

Bacteria have many responses to adverse growing conditions and external stresses. These responses lead to altered metabolic and morphological states to allow survival of the cells against the stress. In *L. monocytogenes*, a well-documented response to the presence of preservatives is filamentation.

Broadly speaking, filamentation is a response by cells exposed to sub-lethal stress, causing an interruption of cell division and cell elongation. With cells unable to fully divide, long chains of connected cells are generated. These filaments vary in size based on type of bacterial cell, and the percentage of the bacterial population that filaments varies based on the type and severity of stress (Gill et al., 2007; Jones et al., 2003). Filaments are of significance to the food industry due to the fact that filamentation is reversible, potentially resulting in filaments breaking apart into individual cells on the foodstuffs and an underestimation of bacterial numbers (Giotis et al., 2007).

To understand the importance of the filamentation of *L. monocytogenes* on RTE foods, an understanding of the mechanisms and reasons behind bacterial filamentation must be ascertained.

1.5.1 Mechanism of Filamentation

Bacterial cell division, or binary fission or cytokinesis, is the process which a single bacteria splits into two daughter cells. Briefly, the process involves the replication of genetic material, and the movement of the original and replicated molecules to opposite polar sides of the cell in an energy-dependent manner (Rokney et al., 2009). The lengthened cell then constricts at the equatorial region, until the plasma membrane is cut off and forms two fully enclosed, identical cells. A group of highly regulated and coordinated proteins are responsible for the constriction that eventually separates the two daughter cells.

The mechanism of filamentation has been reviewed in detail extensively in the past by researchers including Adams and Errington (2009), Erickson et al. (2010), Huang et al. (2013), and Jones et al. (2013). Simply, FtsZ is an ancient cytoskeletal protein integral to bacterial cell division. A homolog of tubulin, FtsZ polymerizes within the cell upon binding of GTP to form a structure of short, continuous or discontinuous pieces known as the Z ring (Adams and Errington, 2009; Huang et al., 2013). Z ring localization is in part determined by the Min system to prevent polar septation (Marston et al., 1998), and the ring itself is bound to the membrane by accessory proteins FtsA and ZipA (Pichoff and Lutkenhaus, 2002).

A multitude of other proteins work in concert with this Z ring to constrict the cell membrane and peptidoglycan layers and facilitate septation (Hu et al., 1999), and lack of these proteins can lead to the inhibition of cell division and the formation of long chains of undivided cells and nucleoids, or filaments (Boer, 2010). Therefore, any conditions which disturb the conformation or concentration of these proteins, or those which affect the polymerization of FtsZ or the formation of septal peptidoglycan, can induce filamentation in bacterial cells (Jones et al., 2013).

1.5.2 Conditions of Filamentation

There are many circumstances, specifically those found in foods, under which bacteria can form filaments. When essentials for growth are limited, the stringent response has been shown to divert cellular activities from growth to survival, resulting in arrest of cell division and filament formation (Magnusson et al., 2005; Wainwright et al., 1999). Both acidic and alkaline environments have also demonstrated the ability to induce bacterial filamentation (Giotis et al., 2007; Vail et al., 2012), and high-pressure treatment can interfere with expression of *FtsZ*, inhibiting cell division (Malone et al., 2002). Evidence also shows that salt concentration (McMahon et al., 2007; Pratt et al., 2012), cold temperatures (Jones et al., 2003), and elevated CO_2 levels (Jydegaard-Axelsen et al., 2005; Li et al., 2003) can all lead to filamentation in bacteria.

While adverse environmental conditions have shown to be able to alter bacterial growth and morphology, antimicrobials have demonstrated similar effects. Jones et al. (2013) reviewed the effects of antimicrobial use in foods on filament formation in associated bacteria. Specifically, their review found that while chitosan has many applications in food preservation, filamentation of *Staphylococcus aureus* exposed to chitosan has been seen (Díaz-Visurraga et al., 2010). Another antimicrobial group of interest in terms of filamentation is bacteriocins. While the mechanism may not be known, multiple researchers have demonstrated that foodborne pathogens growth in the presence of bacteriocins can induce filamentation in at least a portion of the population (Martínez et al., 2000; Ratti et al., 2010; Salomon and Farias, 1992).

1.5.3 Filamentation in *L. monocytogenes*

Foodborne pathogens demonstrate the ability to filament under stressful conditions, such as *Escherichia coli*, *Salmonella enterica* subsp. *enterica*, *S. aureus*, and *L. monocytogenes*. In

particular, recent interest has been given to the specific circumstances that elicit this morphological change in *L. monocytogenes*.

Listeria spp. are small Gram-positive bacteria, 0.5-4 μm in diameter and 0.5-2 μm in length (Giotis et al., 2007; Jamshidi and Zeinali, 2019). Therefore, a *Listeria* filament should be longer than a typical cell, due to incomplete replication. However, there is no agreed upon definition for what counts as an "atypical cell", namely a filament. While some posit that cells twice the mean cell length are considered filamented (Jürgens and Sala, 2000), others suggest that cells more than 4-6x the mean cell length are elongated (Hahn et al., 1999). Additionally, while one can assume that filamented bacteria would be longer than a traditional cell, the extent of filamentation and the proportion of the population that forms filaments vary between conditions and over time. These considerations must be taken into account when looking at filamentation in bacteria, including foodborne pathogens such as *L. monocytogenes*.

Giotis et al. (2007) examined *L. monocytogenes* filamentation induced by sublethal alkaline stress. They found that at a pH greater than or equal to 9.0, *Listeria* formed long filamentous chains longer than normal cells. As many cleaning and sanitizing agents used in food processing facilities are alkaline substances, these results are relevant to food safety and sanitation procedures. Bacteriocins also have the ability to induce filamentation in *L. monocytogenes*. When co-cultured with a bacteriocin-producing *Leuconostoc mesenteroides*, filaments exceeding 6.6 μm were observed (Ratti et al., 2010). This demonstrates that more research needs to be done to examine the effects bacteriocins have on the morphology of their target. Liu et al. (2014) observed the effects of NaCl in vacuum-packaged ham on the genetic regulation of *L. monocytogenes* cell division. From their experiments, important genes linked to cell division (e.g. *ftsX*, *murZ*, *gnd*) were down-regulated on hams formulated with a high salt

concentration, and this resulted in filament formation. Salt is a frequently used as an integral preservative in the production of RTE meats, and as such the effect of salt concentration on *Listeria* growth is integral to understanding food safety and regulation.

Combined stressors also disrupt *L. monocytogenes* growth, inducing filamentation. While it has been shown that CO_2 upshift increases rates of filamentation on meat (Jydegaard-Axelsen et al., 2005), combining CO_2 with refrigeration temperatures can also elongate cells of *L. monocytogenes*, observable through both fluorescent microscopy and increased ATP generation (Li et al., 2003). Vail et al. (2012) also demonstrated that filaments of *L. monocytogenes* form when cultures are grown at refrigeration temperatures combined with the addition of stressors such as salt or pH extremes. While filamentation was observed with both stressors, their study showed that the mechanism may vary based on the type of stress despite the extent of filamentation remaining relatively the same.

Filamentation in *L. monocytogenes* is important with regard to food safety namely for what happens when the stressor that caused the morphological change is removed. Studies have validated that when filaments of *L. monocytogenes* are subjected to conditions closer to the optimum of growth, filaments will septate, finish cell division, and separate into multiple individual cells. (Jørgensen et al., 1995; Jydegaard-Axelsen et al., 2005). This is significant with regards to the food safety standards in Canada, specifically the Category 2A of RTE foods, which cannot contain more than 100 CFU/g of *L. monocytogenes* (Health Canada, 2011). As these standards are determined by plate count methods, and filamentous *Listeria* would present as a single colony on agar plates, the actual numbers of *L. monocytogenes* on RTE foods may be underestimated once the filaments septate into single cells. This problem necessitates better

methods of determining accurate numbers of bacterial cells on foodstuffs, and the ability to identify and distinguish filaments in a population of typical cells.

1.6 Use of Microscopy and Flow Cytometry to Measure Filamentation

Efficient and accurate identification and quantification of bacteria is paramount in the food industry. Methodologies for microbiological testing in RTE foods include enrichment for high risk Category 1 products and enumeration for Category 2 products (Health Canada, 2011; CAC, 2009). As stated above, plate count methods lack the ability to differentiate filamented cells from single cells, leading to potential underestimation of total bacterial loads. This necessitates other means of examining food samples for microbiological presence, namely ones able to visually identify filaments. Two such technologies that fulfill this need include fluorescent-based microscopy and flow cytometry.

1.6.1 Microscopy

Microscopy is the field of science involving the use of microscopes to view objects that cannot be seen with an unaided eye. The use of lenses to magnify images has existed for centuries, but through increasing technological sophistication, higher magnifications and better resolutions are becoming possible. This can allow for not only viewing microscopic cells, but also their constituents with increased fidelity, allowing for better visualization of these normally imperceptible structures.

Microscopy generally falls into three categories: optical, electron, and scanning probe. Optical microscopy, which involves using light passing through the specimen or reflected off of it to view it, is a common technique in microbiological studies despite the magnification not being as strong as electron or scanning probe microscopy. However, problems with contrast can

occur when viewing cells via an optical microscope. As cells and their constituents are colorless or transparent, it can be difficult to discern them from the background. This necessitates the use of a stain to help identify the cells and make them more visible to the observer.

Fluorescent probes are commonly used to stain specimens to be viewed by microscopy. Fluorescent microscopy has many advantages over basic brightfield microscopy. By filtering out all light but the wavelengths of interest, fluorescent microscopy can identify a single fluorescent molecule in a non-fluorescent background, providing superior sensitivity. Fluorophores, the molecules that are able to fluoresce, can be designed with many physiological sensors and markers, allowing for the staining of specific organelles or even to identify metabolic processes taking place within the cell (Lichtman and Conchello, 2005). Additionally, multiple fluorophores with different excitation/emission wavelengths can be used, allowing for detailed information about different subcellular structures and processes to be gathered simultaneously (Hamilton, 2009).

However, fluorescent microscopy-based methods for quantifying bacteria in food samples face several issues. Primarily, microscopy is labour intensive and generally low throughput, requiring researchers to manually observe each stained sample individually. Additionally, there are many factors that can reduce fluorescent image quality. These include improper choice or concentration fluorophore, proper light source choice and angles, proper filters, background fluorescence, and photobleaching of the fluorophore (Lichtman and Conchello, 2005).

Altogether, fluorescent microscopy is a powerful tool when observing cells, and benefits from the increased contrast to allow for more detailed observations. For applications in the food industry, fluorescent microscopy has the ability to help detect viable but nonculturable bacteria

as well as show filamentation in bacteria such as *Salmonella* spp. and *Listeria* spp. (Fakruddin et al., 2013; Hazeleger et al., 2006). However, the need for faster and user-friendly methods of assessing the morphology of foodborne pathogens requires the use of more advanced methodologies.

1.6.2 Flow Cytometry

Flow cytometry is a powerful tool to analyze cell features in mixed sample populations, and has been described in detail in previous studies (Cossarizza et al., 2017; Picot et al., 2012; Veal et al., 2000). Briefly, the basics of flow cytometry will be discussed. Cell suspensions first pass through narrow tubing, which have fast flowing sheath fluid running through them. Through hydrodynamic focusing, the fast moving sheath fluid passing through the narrow tubing focuses the sample core to the point where only single cells can pass through, allowing for the viewing of individual cells, or "events". Once the flow has been established, the stream of single cells then passes through an area where a laser intersects the stream at a 90° angle. As the cells pass through this laser, detectors can infer information from the cells based on the scatter properties and potential fluorescence. These scatter properties include the forward scatter, which correlates to the relative size of cells, side scatter, which correlates to relative cellular granularity, and fluorescence emitted by any stains or dyes. Many flow cytometers have cell sorting potential, allowing them to break the sample stream into droplets containing individual events, and through analysis of scatter properties, assign these droplets to categories defined by the user. The sorter then charges the droplets either positively or negatively, and uses charged deflection plates to push the droplets into multiple designated collection tubes.

Comparisons between fluorescent microscopy and flow cytometry are often made, as both can make use of fluorescent probes to help enumerate cells. Previous studies have shown

that when compared to fluorescent microscopy, flow cytometry results display exceptional agreement (Andersson et al., 1988; Zipf et al., 1984). However, it should be noted that these studies looked at enumeration of human cells and not microbial ones, and that research into comparisons between fluorescent microscopy and flow cytometry in terms of bacterial cells is lacking. Nevertheless, it can be seen from these studies that while the results are comparable between the two techniques, flow cytometry notably has the advantages of standardized controls, less labour intensive, and being observer independent (Andersson et al., 1988; Zipf et al., 1984). These benefits make flow cytometry a favourable option when observing morphological and physiological aspects of bacterial samples.

Flow cytometry has many applications in industrial microbiology, specifically in the food industry. The combination light-scattering and fluorescence measurements allow for information to be gathered on a wide range of cellular parameters, including membrane integrity, enzyme activity, respiratory activity, intracellular pH, and pathogen detection (Comas-Riu and Rius, 2009). In turn, data gathered on these parameters allows flow cytometry to be successfully applied to determining cell numbers and viability, validating starter cultures, probiotics, and preservation technologies, and identifying spoilage or pathogen contamination (Comas-Riu and Rius, 2009). Specifically, flow cytometry has been used to observe morphological changes of bacteria under stress, including those found in foodstuffs. Wickens et al. (2000) successfully used flow cytometry to observe filamentation of *Escherichia coli* after ciprofloxacin exposure, while Jones et al. (2003) used the technology to quantify cell elongation in *E. coli* near the temperature minimum for growth. Flow cytometry was also utilized to examine the filamentation of *L. monocytogenes* both after exposure the sodium lactate and sodium diacetate and growth on

vacuum-packaged ham with varying NaCl concentrations (Liu et al., 2017, 2014b). Altogether, flow cytometry has proven to be a useful tool in the realm of food microbiology.

Despite its benefits over microscopy, flow cytometry requires indirect methods like light-scattering and fluorescence to examine cell populations in samples. Imaging flow cytometry works to combine both the imaging capabilities of microscopy and the speed and ease of use of flow cytometry to allow a broader range of applications (Cossarizza et al., 2017). Among imaging flow cytometers, Amnis® ImageStream® Mk II allows for 12 images of each event to be captured, 10 of which can be fluorescent with different fluorochromes. With images up to 60x magnification combined with the sheer throughput capable with flow cytometry, vast amounts of data can be generated per sample. With analysis by the proper software, such as IDEAS, the data can produce informative plots in addition to high-resolution fluorescent images. The use of combined fluorescent imaging with flow cytometry analysis can prove useful for examining microbiological samples, specifically looking at morphological changes as a result of stress induced by growth on foodstuffs containing preservatives or antimicrobials.

1.7 Objectives

Based on the literature, it is my hypothesis that the combined use of preservatives, either sodium lactate/diacetate or chitosan, with bacteriocins will increase the amount of detectable filamentation of *L. monocytogenes* on RTE meats.

The objectives of this study were to: 1) determine the growth of *L. monocytogenes* on hams treated with different combination of preservatives, and 2) to use microscopy and flow cytometry to observe filamentation of *L. monocytogenes* on the treated hams.

2. Materials and Methods

2.1 Bacterial Strains and Media

The strains used in this study were *Listeria monocytogenes*, strains FS12 (serotype 1/2b, (Bohaychuk et al., 2006)) and 08-5923 (serotype 1/2a, (Gilmour et al., 2010)). These strains were chosen based on previous studies indicating their ability to form filaments on meat products (Liu et al., 2017), and *L. monocytogenes* 08-5923 involvement in the 2008 Canada listeriosis outbreak (Gilmour et al., 2010). Cultures of these strains were kept as frozen stocks in 30% glycerol in distilled H_2O at -80°C. Strains were cultured from frozen stocks into Tryptic Soy Broth (TSB, BD-Canada) and incubated overnight at 37°C. To adapt the strains to lower temperatures prior to use in experiments, strains were inoculated into fresh TSB and incubated at 22°C for 10-11 h.

To determine counts of bacteria, three media were used. Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol *Listeria* Selective Agar with selective supplement (PALCAM, Oxoid-Canada) was used for enumeration of *L. monocytogenes* collected from ham samples. All Purpose Tween agar (APT, BD-Canada) was used for enumeration of lactic acid bacteria and Plate Count Agar (PCA, BD-Canada) was used for a total enumeration from ham samples. Violet Red Bile Agar (VRBA, BD-Canada) was used to ensure ham samples were free of coliform contamination. PALCAM plates were made fresh before sampling days and kept no longer than a week, while APT and PCA plates were made in bulk. All plates were stored in the dark at 4°C until use.

2.2 Ham Production

To test the effects of different combinations of common preservatives and antimicrobials on the formation of filaments by *L. monocytogenes* on ready-to-eat meats, six formulations of

chopped-and-formed hams were made. All hams were made at Agri Food Discovery Place (AFDP) on South Campus, University of Alberta according to the protocol of Liu et al., 2014.

Raw materials included pork leg meat (from a federally inspected meat processing facility), sodium chloride, sodium erythorbate, dextrose monohydrate, sodium tripolyphosphate (TSPP), and Prague powder (94% NaCl, 6% $NaNO_2$), all of which were obtained from Griffith Laboratories™, Canada. For forming, 75 mm diameter Nylon casings were obtained from UniPac, Alberta, Canada. The antimicrobials used included sodium lactate/sodium diacetate (Griffith Foods, Toronto, ON), partially purified bacteriocins from *Carnobacterium maltaromaticum* UAL 307 (purified by J.S. Teixeira and D.R. Balay) and practical grade chitosan from shrimp shells (190-375 kDa; Sigma-Aldrich #417963; CAS# 9012-76-4).The total sodium content for all treatments was 3% (w/w).

Treatments included: 1. control ham with no added antimicrobials other than nitrite and salt; 2. Ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w); 3. Ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g; activity units were determined with *L. monocytogenes* as the indicator organism); 4. Ham with chitosan (0.5% v/w added as a solution in 1% acetic acid); 5. Ham with chitosan and partially purified bacteriocins as above; and 6. Ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent at 0.6%.

2.3 Inoculation and Packaging

Prior to inoculation, both strains *of L. monocytogenes* were streaked onto TSA and incubated overnight at 37°C. One colony was inoculated into 5 mL TSB and incubated overnight

at 37°C. A 1% subculture made for each strain by adding 50 µL of the overnight culture to 5 mL fresh TSB, which was incubated at 22°C for 10-11 h. Measurements of cell density at OD_{600} was used to ensure both strains were at same level of growth before use in applied experiments.

Hams were taken from 4°C storage to be sliced. One slicer was used for the Control, Sodium Lactate/Diacetate, and Sodium Lactate/Diacetate + bacteriocin treated hams, another clean slicer was used for the HMW Chitosan and HMW Chitosan + bacteriocin treated hams, and another clean one was used for the bacteriocin control hams (Meat slicer SL-220, Haisland Machinery Co., Ltd., China). Each ham was sterilized with 70% ethanol spray, and was then opened with a flamed scalpel. Slicing was done at 2 mm thickness, and sterile forceps were used to arrange slices on an aluminum foil-lined tray sterilized via autoclaving. Sixteen ham slices of one treatment per tray (both strains, six sample days plus two extra) were then covered with aluminum foil and stored at 4°C until ready for inoculation.

Once strains were at an OD_{600} of approximately 0.50, the cultures were centrifuged at 3210 x *g* for 10 min. The supernatant was removed and the pellet was resuspended in 5 mL 0.85% NaCl. A 1/10 dilution of resuspended cells in 4.5 mL 0.85% NaCl was made, and this was used for inoculation of the hams. The diluted inoculum was diluted in 0.85% NaCl and plated onto TSA plates to determine the cell population.

Each ham slice was inoculated with 78 µL of inoculum to achieve approximately 10^4 CFU/cm^2 per ~78 cm^2 ham slice. The cell suspension was spread over the surface of the ham with sterile plastic spreaders. After inoculation, the ham slices were individually placed in pre-labeled vacuum package bags using sterile forceps. The bags were vacuum packaged (Multivac C200; Pemberton and Associates, ON, Canada) and stored in a plastic bin at 4°C until they were analyzed for cell density and filamentation.

Sampling

Samples were analyzed for cell counts and filamentation after 2, 15, 30, 60, 90, and 120 d of storage. Packages were opened aseptically with sterile scissors, and 7.8 mL of cold 0.85% NaCl was added to the bag. The bag was gently massaged for 15 seconds on each side. To preserve samples for determination of filamentation, an aliquot of 5.5 mL of diluent was transferred to a labelled 15 mL tube and 1.375 mL of 10% (v/v) formaldehyde (Ricca Chemical-USA) was added to the samples. The fixed cells were held at 4°C until further analysis (microscopy and flow cytometry).

From the bags, 1 mL of diluent was used to determine cell counts. A dilution series was prepared with 1/10 dilutions. To determine cell counts, 100 µL of each dilution was plated on PALCAM, PCA, and APT plates, and spread with plastic spreaders. PALCAM plates were incubated for 24 h at 37°C, PCA plates were incubated for 48 h at 22°C, and APT plates were incubated for 48 h at 22°C in anaerobic jars (GasPak EZ, BD-Canada).

Additionally on Day 2, selective counts were done to determine coliform counts as an indication of sanitary practice. Cell suspensions (1 mL) from each sample were added to sterile petri plates. Molten VRBA (~45°C) was poured into the plate, and the plate was swirled to mix. After the plates cooled, they were incubated for 24 h at 37°C prior to enumeration.

2.4 Sample Preparation

To help remove ham residue and fat from the fixed samples, a modified protocol by Fukushima et al. (2007) was utilized. This protocol uses a buoyant density step gradient of Percoll to separate cells from food matrix residue via density centrifugation. Percoll is a colloidal

solution of silica particles coated in polyvinylpyrrolidone, and is useful based on its low viscosity and osmolarity, and nontoxicity towards cells.

Two Percoll solutions of differing density were made. A 1.123 g/mL solution (designated Standard Isotonic Percoll [SIP]) was made by mixing stock Percoll solution with 1.5 M NaCl (9:1). A 1.030 g/mL solution was made by mixing SIP solution with 0.15 M NaCl (215:785). These solutions and the stock Percoll were kept at 6°C until needed.

In 1.5 mL tubes, 1 mL of fixed sample solution was centrifuged and pelleted at 14500xg for 5 minutes. The pellets were resuspended and washed twice with 1 mL 0.15 M NaCl. After the final wash, the pellets were resuspened in 0.5 mL 0.15 NaCl, and transferred to another 1.5 mL tube, which contained 0.5 mL each of the SIP and 1.030 g/mL Percoll solutions layered one on top of the other. After the washed samples were carefully pipetted on top of the Percoll solutions, the tube was centrifuged at 14500 x *g* for 5 minutes. Afterwards, a layer of residue was usually present above the bottom of the tube. The centrifuged sample (1 mL) was taken from both above and below the residue layer, with care taken to acquire the sample as close to the residue layer without taking any of the residue layer itself. Once retrieved, the cleaned samples were transferred to a clean 1.5 mL tube and placed at 4°C until flow cytometry the next day.

2.5 Flow Cytometry

Prior to flow cytometry analysis, filamented controls of both *L. monocytogenes* FS-12 and 08-5923 were made. Colonies from TSA plates were incubated in 5 mL 10% NaCl TSB for 48 hr at 30°C with agitation. These controls were then washed twice with 0.15 M NaCl similar to the samples as described above, and transferred to 1.5 mL tubes. Both controls and samples were stained with 0.5 µL SYTO® BC green fluorescent nucleic acid stain (Life Technologies, Thermo

Fisher Scientific) at least 30 min before cytometry analysis. During addition of stain, stained samples and the stain itself were kept on ice and out of direct light as much as possible.

Flow cytometry was done using the Amnis® ImageStream®X Mark II imaging flow cytometer (Luminex Corporation, Texas) located at the Heritage Medical Research Center (HMRC), North Campus, University of Alberta. Prior to use, the machine was tested and calibrated according to the recommended instructions by a trained technician. The settings used for the flow cytometer were as follows. The 488 nm filter was used at either 120 or 50 mW, depending on the intensity of stain in the sample. The side scatter (SSC) filter was set to 2.66 mW for all samples. Magnification was set to 60X, and fluidics was set to low speed and high sensitivity. The events count gate was set to either 10000 or 5000 events, depending on the cell concentration of the sample.

Once a sample was run through the machine, a plot of all events was generated displaying Aspect Ratio vs. Area. A gate was manually created to separate cells from the calibration beads. A separate plot was generated of all events displaying Normalized Frequency vs. Intensity of the selected channel. A gate was manually created to select events with an intensity roughly between 1e4 and 1e7. From this gated population, another plot was generated displaying Normalized Frequency vs. Intensity of the selected channel. Another gate was manually created to select the events roughly between 1e4 and 1e7. These selected events were exported and saved for data analysis.

2.6 Flow Data Analysis

Data analysis was done at HMRC, using IDEAS® software package (Luminex Corporation, Texas). Exported files from the flow cytometry computer were uploaded, and the

filamented control files were used to set the gates that would be applied to the experimental sample files.

A plot was generated of all events displaying Intensity of Channel 2 (SYTO® BC fluorescence) vs. Intensity of Channel 6 (calibration bead fluorescence), and a gate designated "R1" was manually set around the population of cells, excluding the calibration beads (Figure 2.1, A). The calibration beads were a small group localized at Channel 2 intensity of about 1e3 to 1e4, and Channel 6 intensity of approximately 1e5. Another plot was generated of all events displaying Area (the size of the picture taken, representing cell size) vs. Intensity of Channel 2, and a gate designated "fil" was manually set around the population of filamented cells (Figure 2.1, B). A final plot was generated of only events from the "R1" gated population, displaying Area vs. Intensity of Channel 2 (Figure 2.1, C). The "fil" gate from the previous plot was placed on this plot to identify the filamented population within the events designated as cells. This process was applied to all samples analyzed by the imaging flow cytometer.

Individual events were observed to see if the "fil" gate encompassed all filamented cells. The gate was manually adjusted along the y-axis (Channel 2 intensity) to include events found to be filamented cells (Figure 2.1, B and C). This adjustment was done with samples of cells harvested from strain FS12, control treatment, day 30, and strain 08-5923, control treatment, day 30, and this new gate was applied to all other treatments. Day 30 samples were chosen for adjusting the gate to ensure cells were filamentated, and allow the adjusted gates to include as many filamented events as possible. Individual events were chosen to represent examples of filamented cells, and the brightfield and fluorescent images of the chosen events were extracted for viewing. Finally, tables were generated to show the total event count and relative percent of each population in each plot.

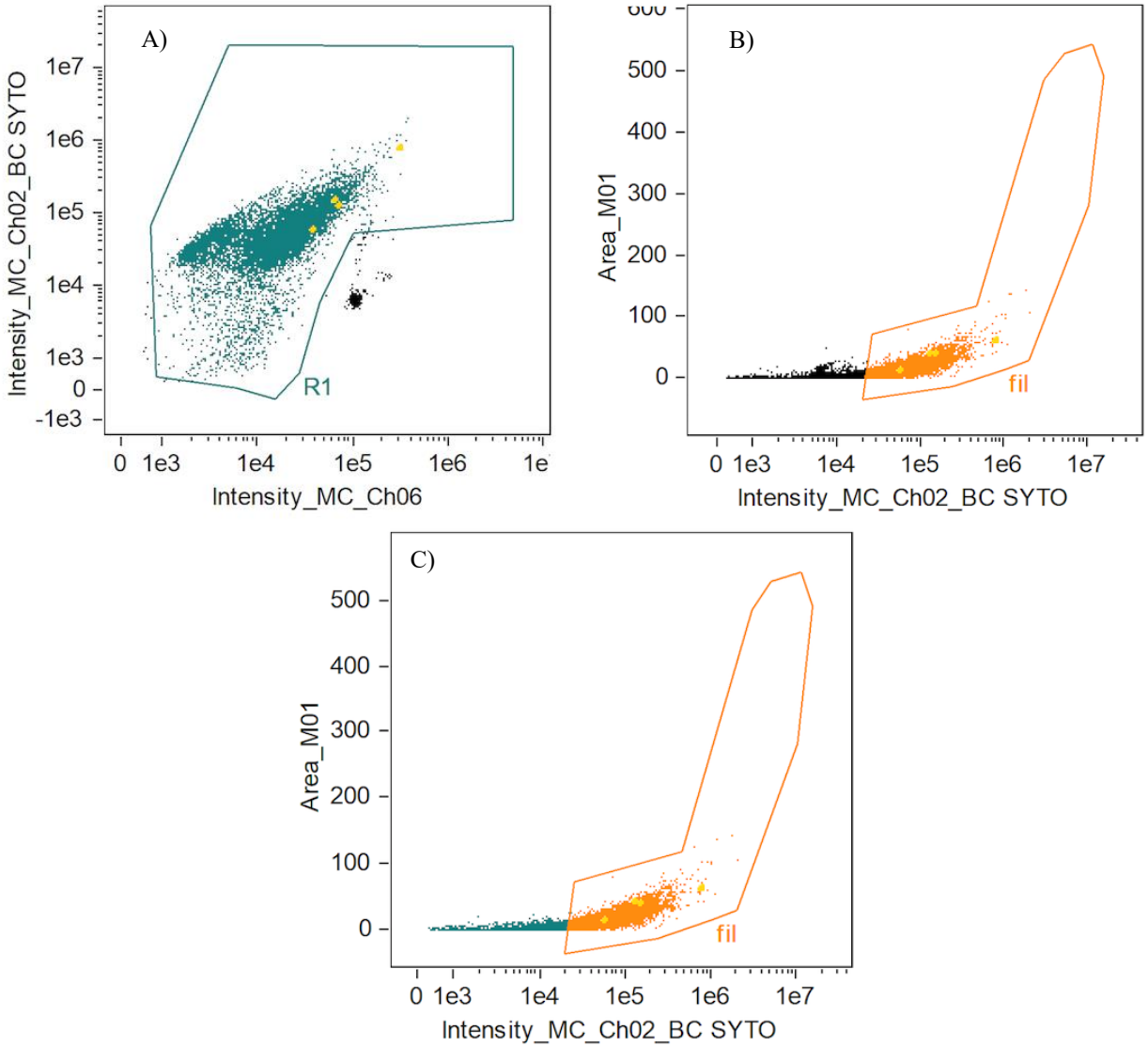

Figure 2.1. Flow cytometry plots generated for data analysis of *Listeria monocytogenes* samples harvested from hams. Images were taken via Amnis® ImageStream® MkII and analyzed using IDEAS® software package. The sample randomly chosen as an example control ham inoculated with *L. monocytogenes* FS12 and stored for 60 d. A) Plot of all events displaying Intensity of Channel 6 (calibration bead fluorescence) vs. Intensity of Channel 2 (SYTO® BC fluorescence). The gate "R1" was manually placed around all events representing cells and excluding events representing calibration beads. B) Plot of all events displaying Intensity of Channel 2 vs. Area (the size of the picture taken, representing cell size). The gate "fil" was generated using filamented control samples, and manually adjusted to include filamented cells that were shorter than the filamented control, but larger than typical cells. C) Plot of events selected by "R1" gate displaying Intensity of Channel 2 vs. Area. The "fil" gate generated in the previous plot was applied to this plot, capturing all filamented cells within the population of cells captured in the first plot. This process was repeated for all samples, and the "fil" gate was applied to all plots.

2.7 Statistical Analysis

All experiments were conducted in triplicate independent experiments. Data were subjected to analysis for normality using the PROC UNIVARIATE procedure of the University Edition of SAS (SAS Institute Inc., Cary, NC and were considered to be normally distributed if $P>0.05$. All data were normally distributed. Data were subjected to Analysis of Variance using the PROC GLM procedure of SAS. Tukey's posthoc test was used to determine differences among means. The significance value was $P<0.05$.

3. Results

3.1 Microbiological Analyses

Initial counts for the ham before inoculation determined on TSA plates were below the limit of detection. In addition, counts on VRBA plates after 2 d of storage of all of the inoculated hams were below the detection limit, indicating that there was no contamination with coliforms (data not shown).

Plate counts were used to determine the growth of *L. monocytogenes* samples harvested from the treated hams after incubation at 4°C. Generally, hams treated with the combination of sodium lactate and sodium diacetate with partially purified bacteriocins from *C. maltaromaticum* UAL 307 had lower counts of *L. monocytogenes* when compared to hams with no antimicrobials or those treated with other antimicrobials (Figures 3.1 - 3.3).

PCA was used to determine the total aerobic counts for the hams (Figure 3.1). For strain FS12 (Figure 3.1, A), there were no significant differences among treatments at days 2 and 120 of storage. For *L. monocytogenes* FS12, after 15 d of storage the mean log count for the ham with sodium lactate/sodium diacetate and bacteriocin was significantly lower ($P<0.05$) than the mean log counts for the other treatments. This difference continued at 30 and 60 d of storage, with the sodium lactate/sodium diacetate and bacteriocin treatment being significantly lower than the control, the bacteriocin only, and chitosan or sodium lactate/sodium diacetate treatments, respectively. By day 90, the mean log count for the sodium lactate/sodium diacetate and bacteriocin treatment was only significantly lower than that for the control. For strain 08-5923 (Figure 3.1, B), the mean log counts for the sodium lactate/sodium diacetate and bacteriocin treatment were significantly lower ($P<0.05$) than other treatments at 30 d of storage. For samples

inoculated with *L. monocytogenes* 08-5923, after two days of storage samples formulated with chitosan or chitosan with bacteriocins had significantly ($P<0.05$) lower counts on PCA agar (Figure 3.1, B).

Counts on APT agar (Figure 3.2) were similar to the PCA counts. The counts for strain FS12 showed that the combination of sodium lactate/sodium diacetate with bacteriocin caused a significant reduction in mean log counts on days 15 through 90 (Figure 3.2, A). However, at 30 and 60 d of storage, the sodium lactate/sodium diacetate with bacteriocin treatment was only significantly different from the control, bacteriocin only, and chitosan with bacteriocin or sodium lactate/sodium diacetate treatments, respectively, and only significantly different from the control by day 90. Mean log counts for *L. monocytogenes* 08-5923 were similar to mean log counts determined on PCA (Figure 3.2, B). At 30 d of storage, the mean log counts for the sodium lactate/sodium diacetate with bacteriocin treatment was significantly lower than the control, bacteriocin, sodium lactate/sodium diacetate, and chitosan treatments, and by day 60 it was only significantly different from the control.

PALCAM agar was used to determine counts of *Listeria* (Figure 3.3). For hams inoculated with *L. monocytogenes* FS12 (Figure 3.3, A), the sodium lactate/sodium diacetate with bacteriocin treatment had significantly lower mean log counts from 15 through 60 d of storage. For both days 15 and 60, the sodium lactate/sodium diacetate with bacteriocin treatment had significantly lower mean log counts of *L. monocytogenes* than all other treatments, while at 30 d of storage, the sodium lactate/sodium diacetate with bacteriocin treatment was only significantly lower than the control and both chitosan and chitosan with bacteriocin treatments. For hams inoculated with *L. monocytogenes* 08-5923, only at day 30 was the sodium lactate/sodium diacetate with bacteriocin treatment significantly lower than all of the other

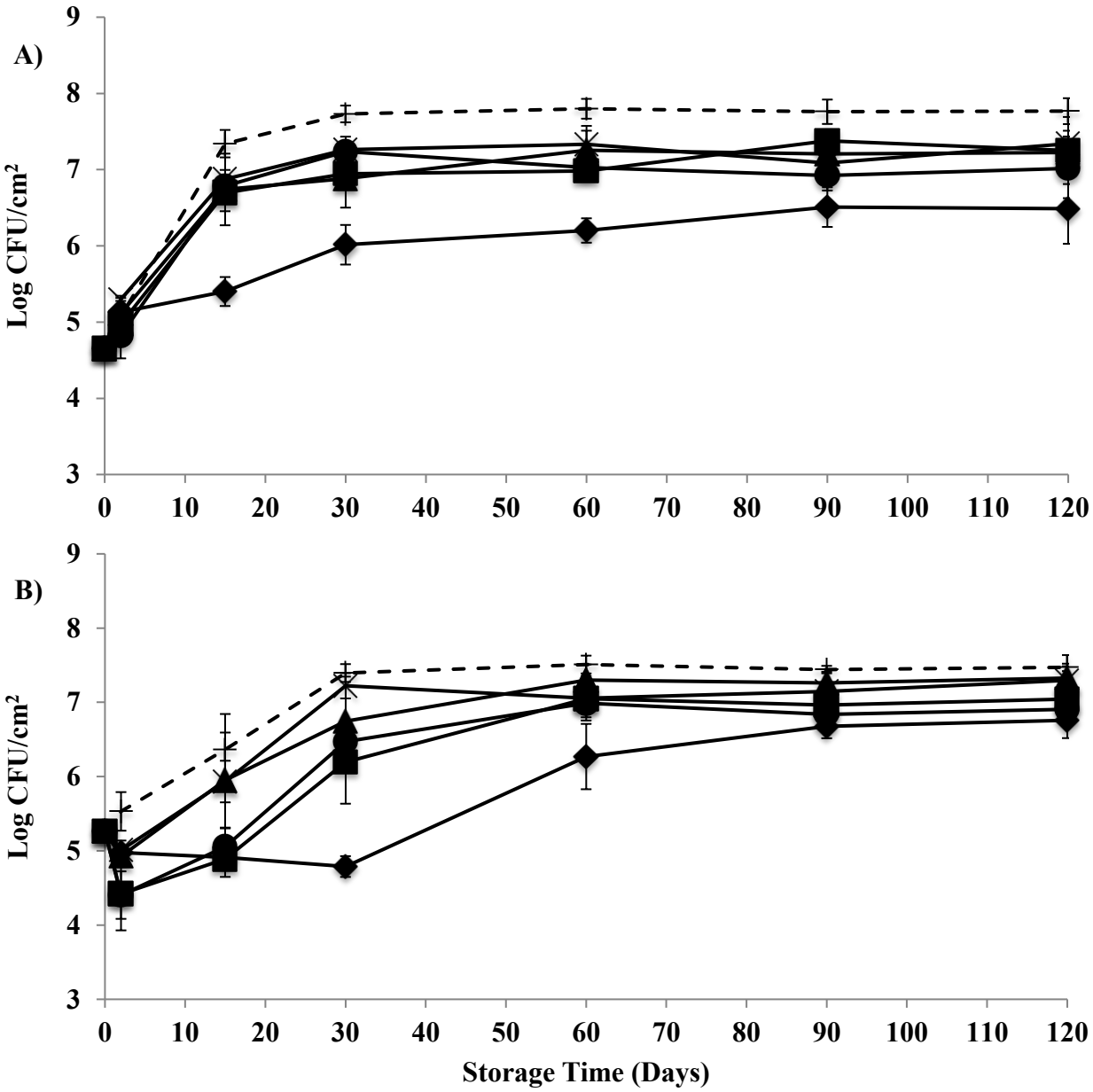

Figure 3.1. Mean log total aerobic cell counts (log CFU/cm^2) of vacuum packaged ham samples stored at 4°C for 120 d and inoculated with two strains of *Listeria monocytogenes*. Counts were determined on PCA media incubated at 20°C for 48 h. Data for *L. monocytogenes* FS-12 are in plot A; data for *L. monocytogenes* 08-5923 are in plot B. Treatments included: + with dashed lines indicates control ham with no added antimicrobials other than nitrite and salt; ▲ Ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w); ◆ Ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g); ● Ham with chitosan (0.5% v/w added as a solution in 1% acetic acid); ■ Ham with chitosan and partially purified bacteriocins as above; and X Ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent. n=3 independent replicates. Error bars indicate standard error.

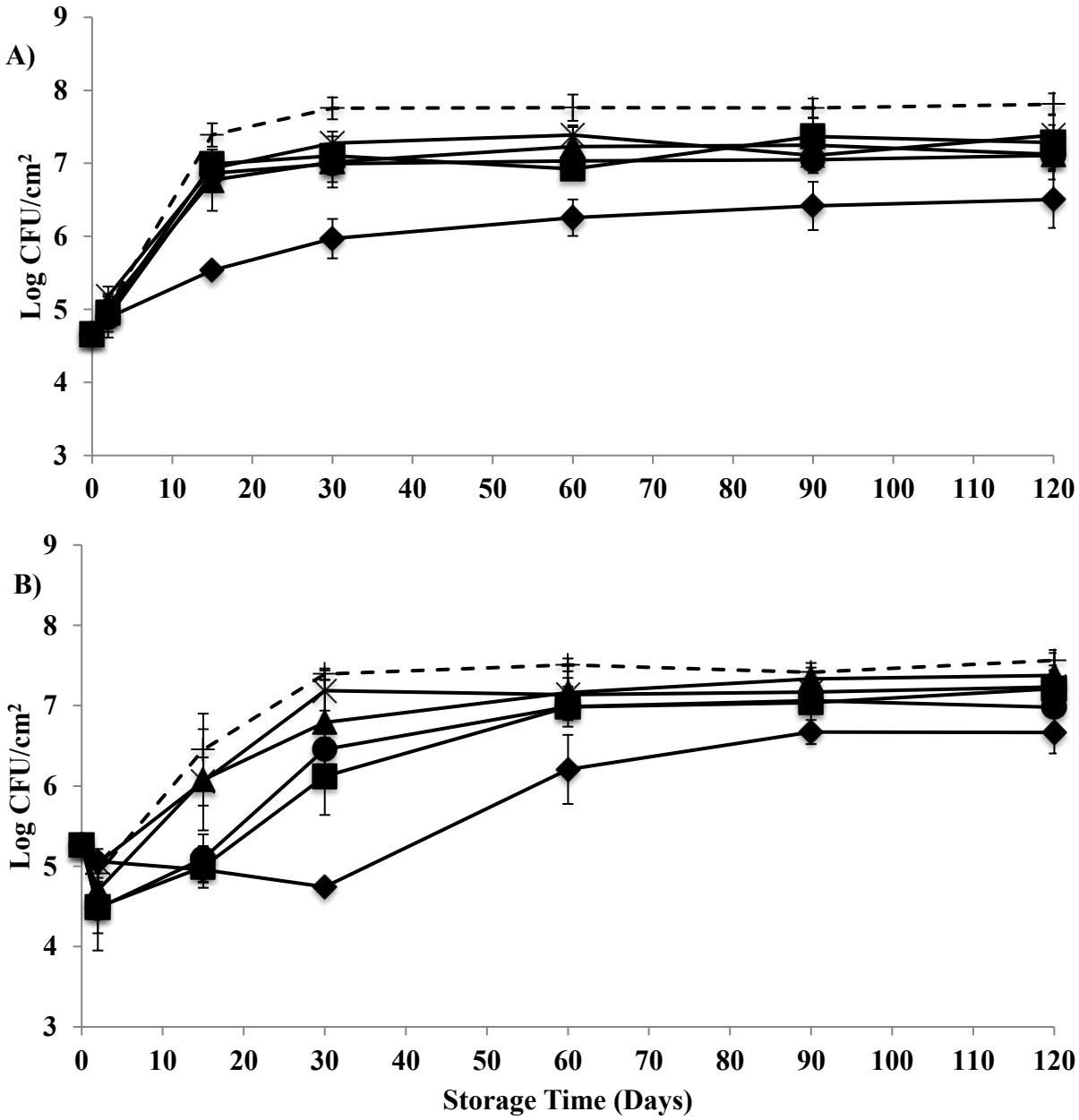

Figure 3.2. Mean log cell counts (log CFU/cm^2) of vacuum packaged ham samples stored at 4°C for 120 d and inoculated with two strains of *Listeria monocytogenes*. Counts were determined on APT media incubated at 20°C for 48 h. Data for *L. monocytogenes* FS-12 are in plot A; data for *L. monocytogenes* 08-5923 are in plot B. Treatments included: + with dashed lines indicates control ham with no added antimicrobials other than nitrite and salt; ▲ Ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w); ◆ Ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g); ● Ham with chitosan (0.5% v/w added as a solution in 1% acetic acid); ■ Ham with chitosan and partially purified bacteriocins as above; and X Ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent. n=3 independent replicates. Error bars indicate standard error.

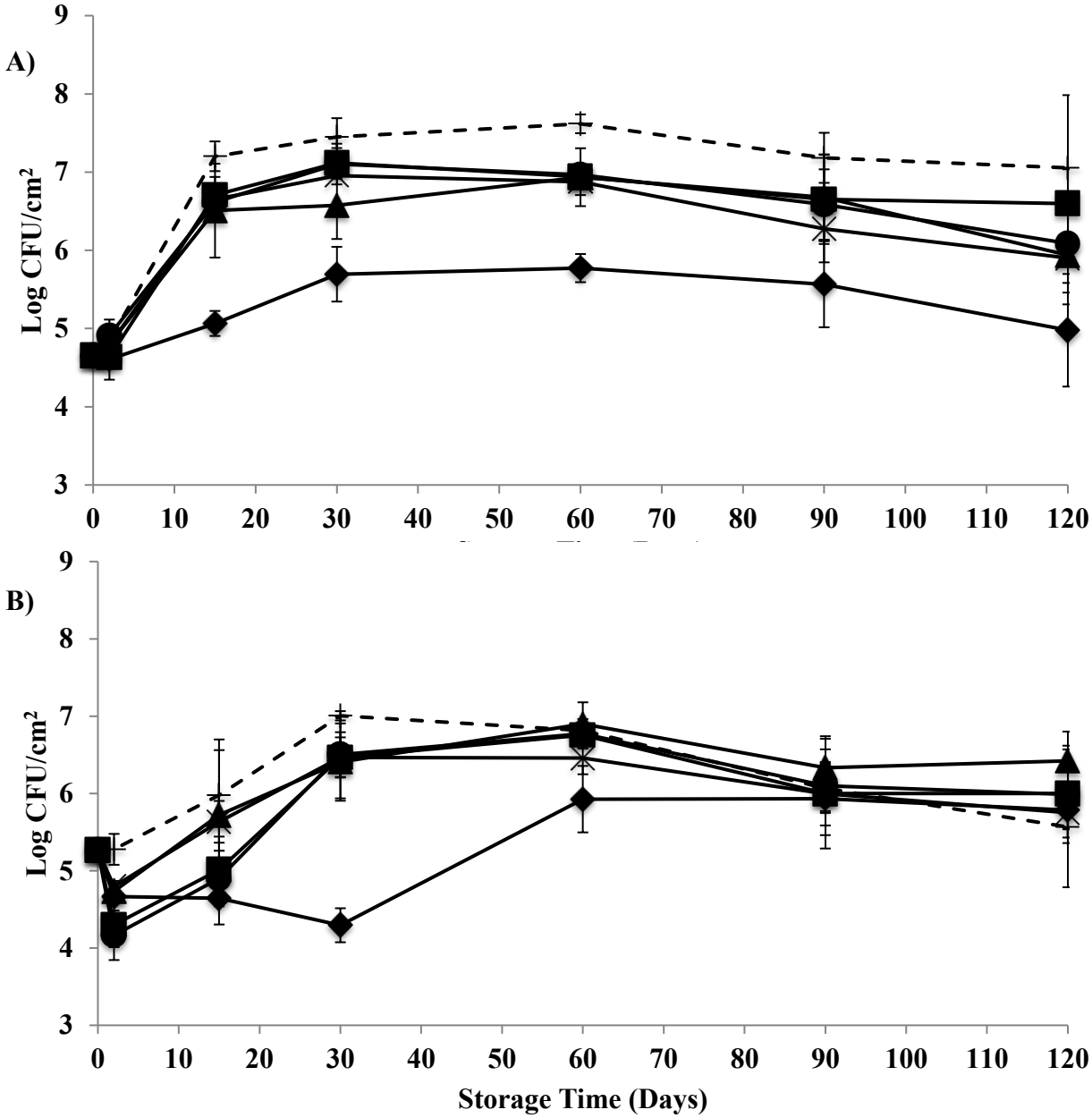

Figure 3.3. Mean log cell counts (log CFU/cm^2) of vacuum packaged ham samples stored at 4°C for 120 d and inoculated with two strains of *Listeria monocytogenes*. Counts were determined on PALCAM media incubated at 37°C for 24 h. Data for *L. monocytogenes* FS-12 are in plot A; data for *L. monocytogenes* 08-5923 are in plot B. Treatments included: + with dashed lines indicates control ham with no added antimicrobials other than nitrite and salt; ▲ Ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w); ◆ Ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g); ● Ham with chitosan (0.5% v/w added as a solution in 1% acetic acid); ■ Ham with chitosan and partially purified bacteriocins as above; and X Ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent. n=3 independent replicates. Error bars indicate standard error.

treatments, and was not different than the other treatments at any other time during storage (Figure 3.3, B). For samples inoculated with *L. monocytogenes* 08-5923, after two days of storage samples formulated with chitosan or chitosan with bacteriocins had significantly ($P<0.05$) lower counts on PALCAM agar (Figure 3.3, B) but this difference was not maintained during storage.

Also, at 2 d of storage, the treatments involving chitosan (both with sodium lactate/sodium diacetate and with partially purified bacteriocins) with *L. monocytogenes* 08-5923 had drops in cell counts compared to the FS-12 strain (Figures 3.1-3.3). This initial drop was only significant in the PCA and PALCAM counts and only when compared to the control. The lowered counts were not maintained after 2 d of storage, and counts became comparable to the control treatment.

3.2 Confirmation of Filamentation of *L. monocytogenes* by Fluorescent Imaging

Fluorescent images were taken simultaneously during flow cytometry with the Amnis® ImageStream® MkII, with an image taken for every event detected by the cytometer.

To both confirm that these strains of *L. monocytogenes* form filaments and to provide a control group to set the initial gates during flow cytometry, filamentation was induced in *L. monocytogenes* samples via salt stress. Both strains of *L. monocytogenes* were grown for 48 hr at 25°C in TSB 10% NaCl, and were analyzed with the Amnis® ImageStream® MkII alongside experimental samples harvested from the hams. From Figure 3.4, it was observed that both strains were capable of forming very long filaments, with some reaching lengths upwards of 60 µm, compared to unfilamented or "typical" cells. This both confirmed that the strains of *Listeria*

used can form filaments under salt stress, and provided a control group to form the base gate in further flow analysis, explained in more detail below.

Figure 3.5 displays selected images taken from samples for all ham treatments, comparing unfilamented and filamented cells. Unfilamented cells were approximately 1 to 2 μm long, which within the range of typical *L. monocytogenes* cells (Giotis et al., 2007; Jamshidi and Zeinali, 2019). Filamented cells were noticeably longer, typically longer than 7 μm than than unfilamented cells. The morphology of *Listeria* filaments harvested from hams did not differ among treatments or between strains. While longer than non-filamented cells when compared to cells of *L. monocytogenes* grown in TSB 10% NaCl for 48 hr, the filaments harvested from ham samples were smaller that those observed in the population exposed to 10% NaCl in TSB (Figure 3.4 vs 3.5).

3.3 Percentage of *L. monocytogenes* Population Filamented Determined by Flow Cytometry

Flow cytometry data was acquired with the Amnis® ImageStream® MkII, and gating was applied to the data and modified using filamented controls and fluorescent microscopy images. Throughout storage of hams inoculated with either of the strains of *L. monocytogenes*, it was observed that the strains inoculated onto hams treated with chitosan or chitosan with bacteriocins had a lower proportion of filamented cells within the sample population (Figures 3.6 and 3.7)

The percentage of the population of filamented *L. monocytogenes* FS12 grown on hams with chitosan was consistent across all sampling times, having a significantly lower percent of the population within the filament gate (Figure 3.6). Additionally, the cells harvested from ham with chitosan with bacteriocin also had significantly lower percent filamentation on days 15, 60, and 90. Conversely, there were much higher levels of filamentation in the population of cells

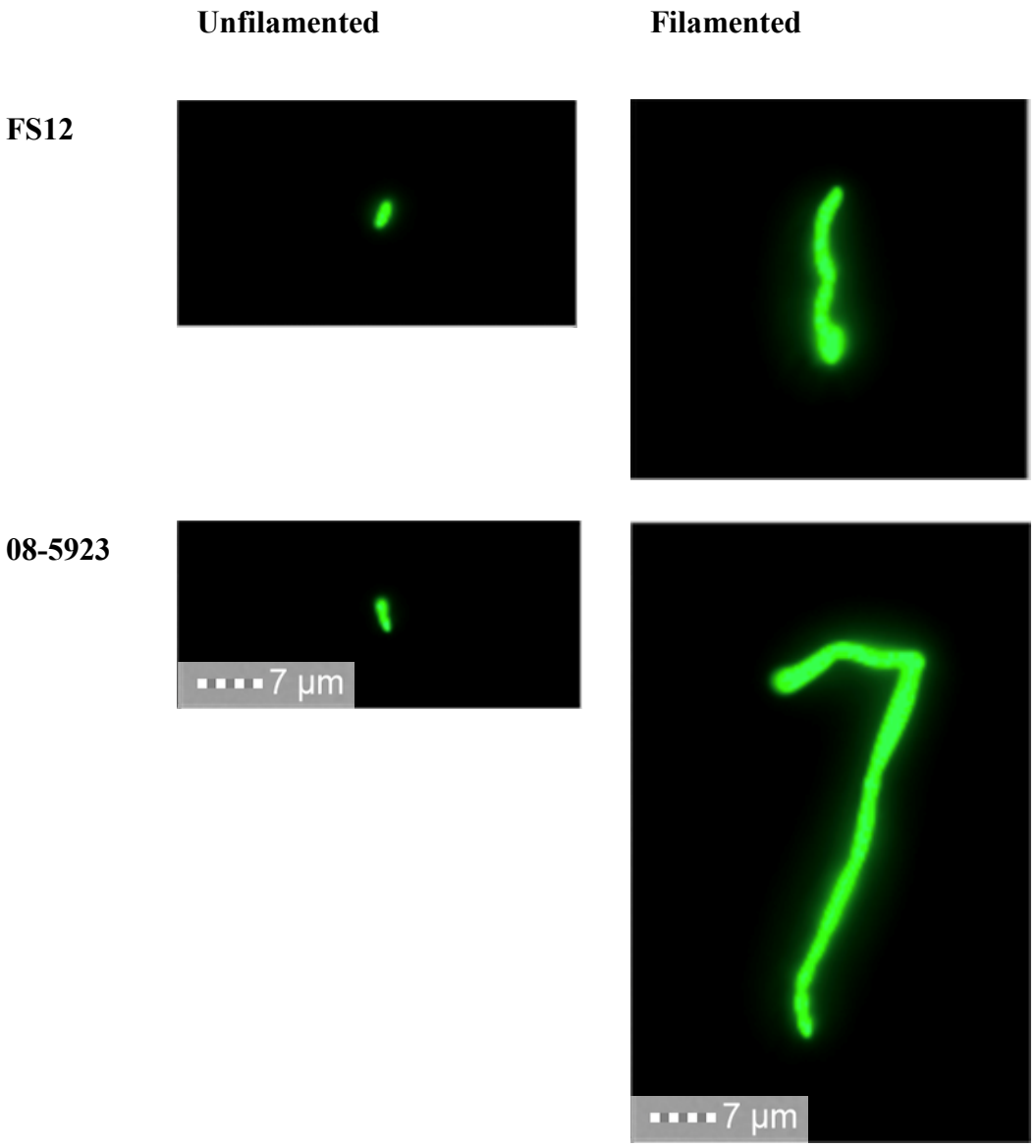

Figure 3.4. Fluorescent images of two strains of *Listeria monocytogenes* for use as filamented cell controls for gating of flow cytometry data. Images were taken via Amnis® ImageStream® MkII. Cells were stained with SYTO BC fluorescent nucleic acid stain, and images were taken at 60x magnification. Filaments were generated by growing *L. monocytogenes* in TSB with 10% NaCl for 48 hr at 25°C. Images were chosen randomly from control samples used during analysis of ham samples.

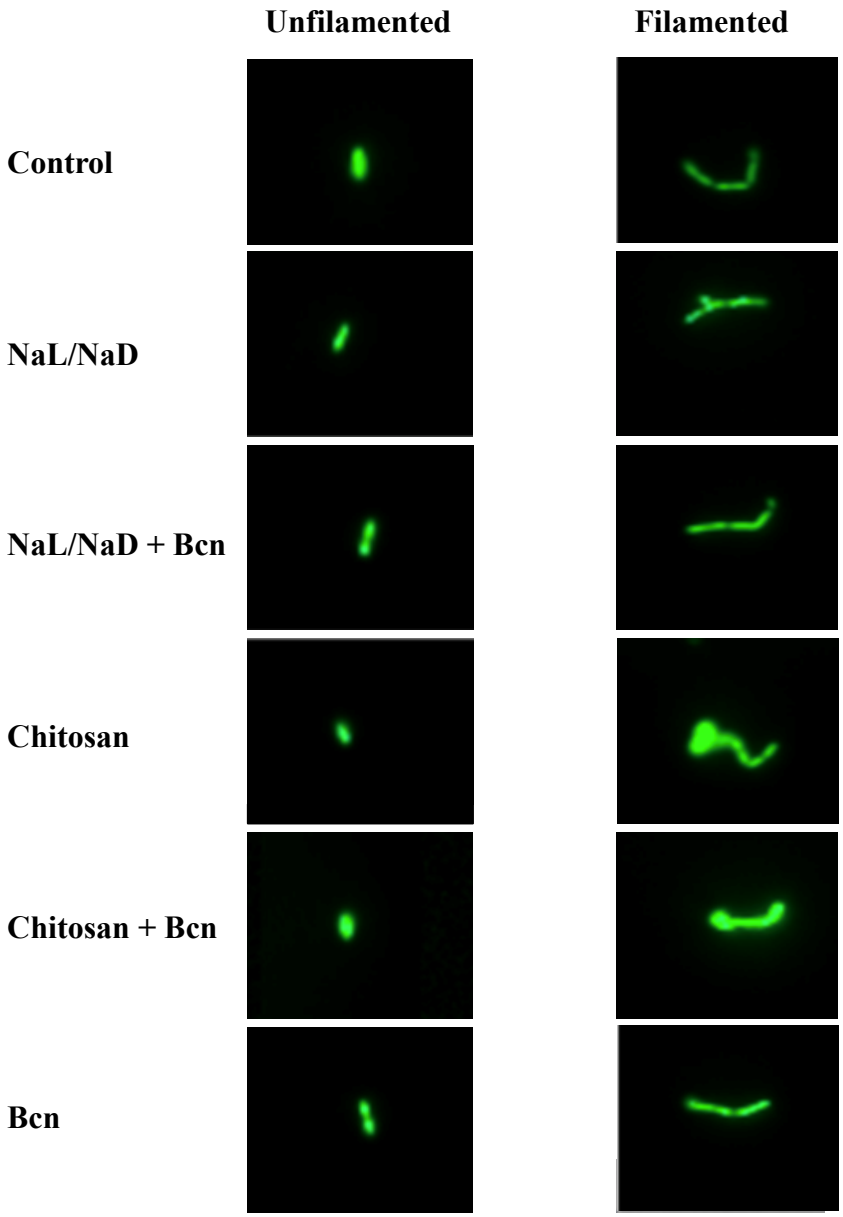

Figure 3.5. Fluorescent images of two strains of *Listeria monocytogenes* on vacuum packaged ham stored at 4°C for 120 d. Images were taken via Amnis® ImageStream® MkII. Cells were stained with SYTO BC fluorescent nucleic acid stain, and images were taken at 60x magnification. Treatments included: Control indicates control ham with no added antimicrobials other than nitrite and salt; NaL/NaD ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w); NaL/NaD + Bcn ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g); Chitosan ham with chitosan (0.5% v/w added as a solution in 1% acetic acid); Chitosan + Bcn ham with chitosan and partially purified bacteriocins as above; and Bcn ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent. Images were chosen randomly between the two strains and across all treatment and sampling times.

harvested from the control, sodium lactate/sodium diacetate with and without bacteriocin, and bacteriocin only treatments, ranging between approximately 60 and 90% of the tested population after 15 d of storage.

The percentage of filamented cells of *L. monocytogenes* 08-5923 displayed a similar pattern, with the cells harvested from the chitosan and chitosan with bacteriocin treatment having a significantly lower percent of the population filamented (Figure 3.7). At 15, 60, 90, and 120 days of storage, these treatments had significantly lower amounts of filaments in their sample populations compared to the other treatments. While slightly lower than the percentage of filamentation observed with *L. monocytogenes* FS12, the control, sodium lactate/sodium diacetate with and without bacteriocin, and bacteriocin only treatments had filamented populations at approximately 60 to 80% after 30 days of storage.

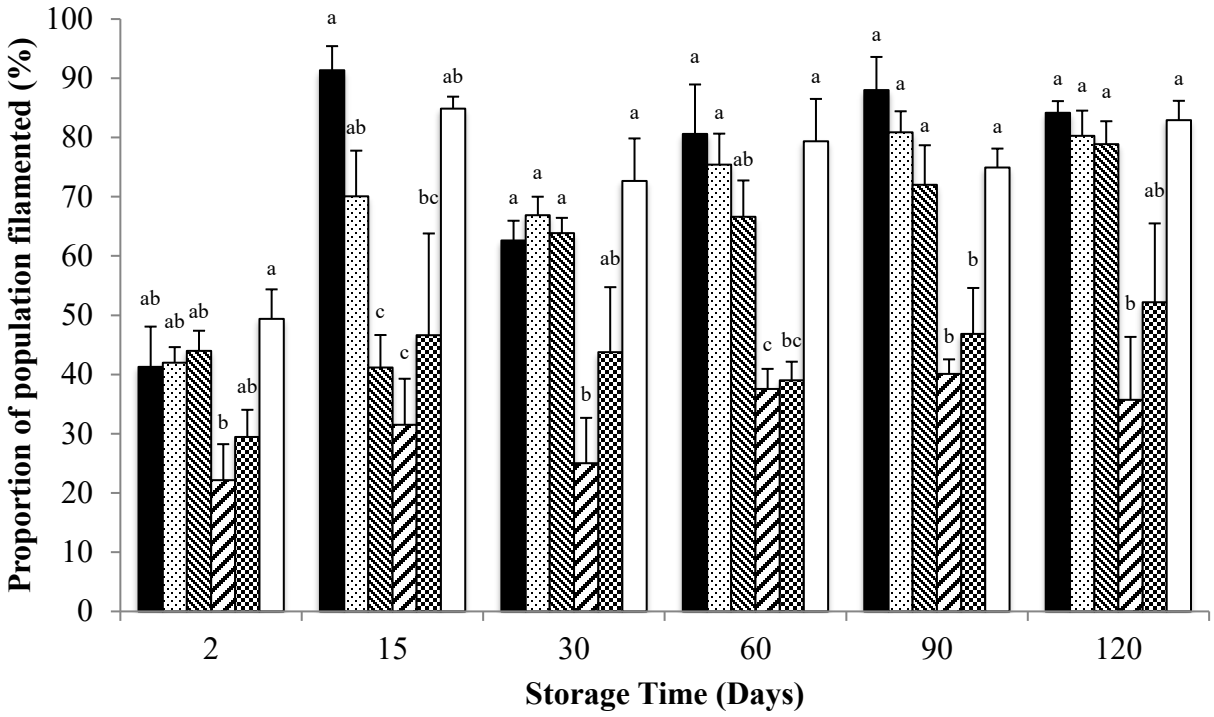

Figure 3.6. Mean percentage of filaments in the population of *Listeria monocytogenes* FS12 on vacuum packaged ham stored at 4°C for 120 d. Data was collected via flow cytometry with Amnis® ImageStream® MkII. Treatments included■ indicates control ham with no added antimicrobials other than nitrite and salt; ⬚ Ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w); ▧ Ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g); ▨ Ham with chitosan (0.5% v/w added as a solution in 1% acetic acid); ▩ Ham with chitosan and partially purified bacteriocins as above; and □ Ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent. n=3 independent replicates. Letters indicate statistical differences among treatments within sampling days. Error bars indicate standard error.

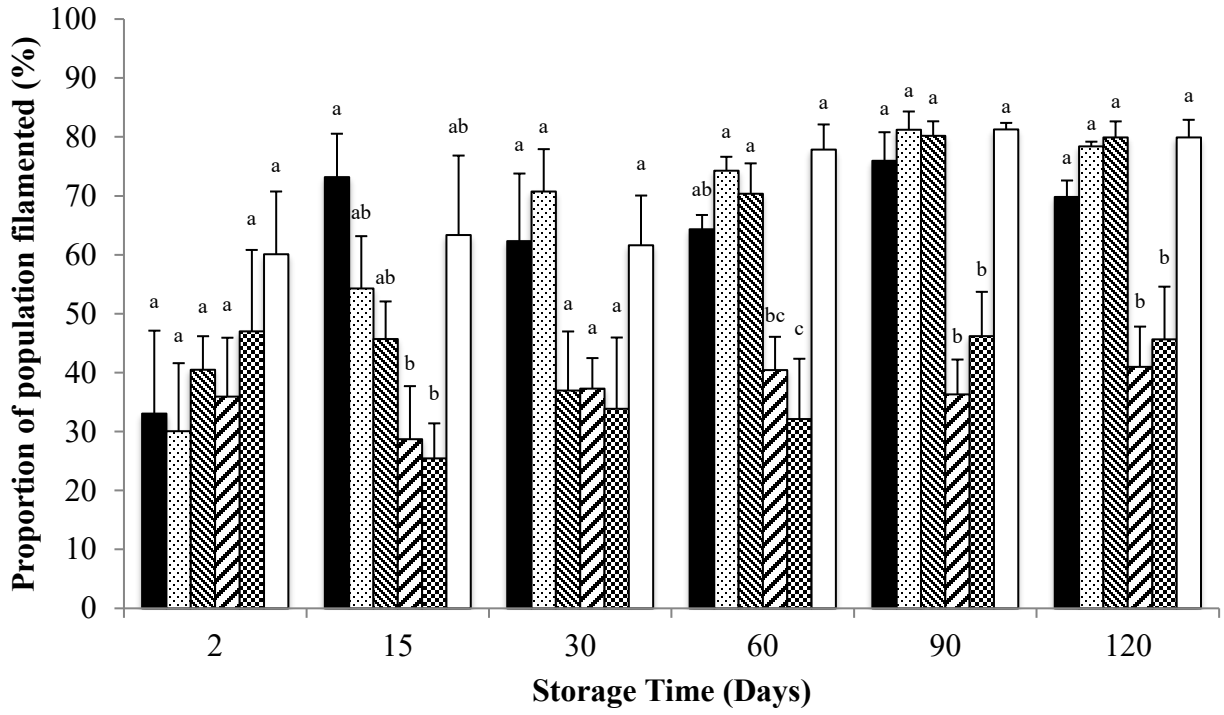

Figure 3.7. Mean percentage of filaments in the population of *Listeria monocytogenes* 08-5923 on vacuum packaged ham stored at 4°C for 120 d. Data was collected via flow cytometry with Amnis® ImageStream® MkII. Treatments included■ indicates control ham with no added antimicrobials other than nitrite and salt;⬚ Ham with sodium lactate (1.4% w/w) and sodium diacetate (0.1% w/w);▧ Ham with sodium lactate/sodium diacetate and partially purified bacteriocins (25.6 activity units/g);▨ Ham with chitosan (0.5% v/w added as a solution in 1% acetic acid);▩ Ham with chitosan and partially purified bacteriocins as above; and□ Ham with partially purified bacteriocins as above. In all cases, if sodium was added as part of an antimicrobial treatment, the amount of sodium chloride added was adjusted to keep the sodium concentration consistent. n=3 independent replicates. Letters indicate statistical differences among treatments within sampling days. Error bars indicate standard error.

4. Discussion and Conclusions

Listeria monocytogenes is a well-known food associated pathogen, capable of growing at multitude of different conditions, and is known to contaminate ready-to-eat foods. In terms of food safety, preservatives such as sodium lactate and sodium diacetate, chitosan, and bacteriocins all demonstrate the ability to induce stress and inhibit growth of *Listeria* spp. (Alvarez-Sieiro et al., 2016; Glass et al., 2002; Health Canada, 2012; Ye et al., 2008; Zacharof and Lovitt, 2012). However, evidence shows that under stressful conditions, *L. monocytogenes* can form filaments of multinucleated cells (Liu et al., 2014a; Ratti et al., 2010). This poses a problem to microbiological testing of RTE products, with filamentation potentially resulting in an underestimation of actual counts of *L. monocytogenes*, and potentially increasing risk to susceptible consumers.

The research reported in this thesis sought to add to the previous work to examine the stress response of *L. monocytogenes* by observing the morphological changes of the bacteria when exposed to different preservatives on vacuum-packaged RTE hams at refrigeration temperatures. While there are many studies examining the effects of preservatives on bacterial growth, few specifically assess filamentation as a result of exposure to preservatives in meat, and fewer still that specifically examine the morphology of *L. monocytogenes* on meat.

In this study, the growth of bacteria on vacuum packaged ham with different antimicrobials was monitored on three different microbiological media. For all samples, regardless of strain or antimicrobial treatment, the cell counts were similar on all three media (PCA, APT and PALCAM; Figures 3.1 to 3.3). Initial counts on the meat determined prior to use

in experiments demonstrated that the meat used in this study had little contamination as counts were below detectable limits. The initial concentration of *L. monocytogenes* put onto the ham slices was around 10^5 CFU/cm^2. It is important to note that an aseptic control was not included in this study. While this would have been useful to determine whether plate counts were affected by any potential contamination, the fact that that initial counts of hams before inoculation on TSA plates were below detection limits coupled with the high inoculation level of *L. monocytogenes* made it likely that no other bacteria grew on the ham slices. Based on this knowledge, it is likely that the changes in cell counts over time determined on PCA and APT media are due to the growth of *L. monocytogenes*.

In terms of effective preservatives used to control *L. monocytogenes*, much work has been done examining the effects of sodium lactate and sodium diacetate on *Listeria* spp. growth. Traditionally, these organic acid salts are used in meat products as humectants, for pH control, and meat flavour enhancement (Shelef and Yang, 1991). Sodium lactate and diacetate also display antibacterial properties, making them ideal choices for preservatives. Both of these chemicals are approved additives to be used as Class 2 preservatives (antibacterial) under Health Canada regulations (Health Canada, 2012). This approval is based on multiple studies, which confirm the effectiveness of these chemicals to inhibit the growth of *L. monocytogenes*, specifically in the context of meat matrices. While some research suggests that sodium diacetate alone can delay *L. monocytogenes* growth in meat, it is commonly seen that a synergistic effect is displayed when sodium lactate and sodium diacetate are used together, increasing their listericidal effect. Combinations of 2.5-1.8% sodium lactate with 0.5-0.2% sodium diacetate were deemed sufficient to not only inhibit *L. monocytogenes* growth, but also the growth of *Salmonella enterica* serovar Enteritidis, and spoilage lactic acid bacteria on meat products at

refrigeration temperatures (Barmpalia et al., 2005; Glass et al., 2002; Mbandi and Shelef, 2001; Schlyter et al., 1993). These antibacterial properties, as well as their other uses in meat product formulation, make sodium lactate and sodium diacetate attractive preservatives.

While the effectiveness of these organic acid salt preservatives has been demonstrated in literature, this was not observed in the current study. Counts of *L. monocytogenes* harvested from hams treated with sodium lactate and sodium diacetate were not significantly different from counts on control hams for either of the strains tested (Figure 3.3). This lack of inhibitory effect could possibly be due to lower concentrations of both chemicals in the hams used in this study, with 1.4% w/w sodium lactate and 0.1% w/w sodium diacetate. The hams in this study were made using formulations from a previous study (Liu et al., 2014b), which were made in accordance to Canada's sodium reduction strategy (Health Canada, 2010). Due to concerns of high salt levels in consumer diets, there is a push for producers to reduce sodium levels as much as possible in processed foods. In trying to reduce sodium content in the hams, the lower concentration of sodium lactate and sodium diacetate could have resulted in a reduced ability to control the growth of *L. monocytogenes* in the hams treated with these preservatives in this study.

Another preservative tested in this study was chitosan, a polycationic biopolymer commercially obtained from alkaline deacetylation of crustacean chitin (Rabea et al., 2003). While not officially recognized or approved as a preservative to be used in RTE foods in Canada, there are studies indicating the growth-inhibiting effects chitosan has on bacteria, and *L. monocytogenes* in particular. While the exact mechanisms of action are not fully elucidated, the effectiveness of chitosan relies on numerous factors, such as microbial, intrinsic, physical state, and environmental factors (Kong et al., 2010). It is also generally accepted that chitosan acts at

least in part on the cell wall and cellular membrane of bacteria, possibly altering permeability and chelating trace metals (Kong et al., 2010; Rabea et al., 2003). Another important factor in the use of chitosan is pH, as it is not only soluble in dilute organic acids, studies show that the antimicrobial activity is enhanced at lower pH, below the pKa of 6.3-6.5 (Kong et al., 2010; No et al., 2002; Rabea et al., 2003). Chitosan has demonstrated to effectively inhibit the growth of multiple bacteria species, but most studies have been done in nutrient broth-based systems (Chen et al., 1998; Díaz-Visurraga et al., 2010; Wang, 1992). Literature reports on the effects of chitosan on the growth of *Listeria* spp. have been mixed, with some indicating no inhibition at all (Wang, 1992), some requiring a sufficiently low pH (No et al., 2002), and some requiring additional preservatives to observe inhibition of growth (Ye et al., 2008). Hu (2019) found that incorporation of chitosan into the meat batter of hams (similar to what was done in the current research) resulted in a small bactericidal effect compared to a control with no chitosan. Chitosan was able to inhibit the growth of *L. monocytogenes* during storage of ham at 4°C better than sodium lactate/sodium diacetate. They also found that the combination of chitosan with bacteriocins from *C. maltaromaticum* UAL307 limited the growth of *L. monocytogenes* to less than one log (CFU/g) during 4 weeks of storage. This is in contrast to the results of the current study were the addition of chitosan did not suppress the growth of *L. monocytogenes* over 120 d of storage. While the effectiveness of chitosan on inhibition of the growth of *L. monocytogenes* requires further exploration, the nontoxic and biodegradability of chitosan make it a potential preservative for use in RTE meats.

The current research adds to the contemporary knowledge regarding the effects of chitosan on growth of *L. monocytogenes* in RTE meats. However, similar to the results for sodium lactate and sodium diacetate, in the presence of chitosan in ham was not able to inhibit

the growth of *L. monocytogenes* compared to the growth on hams with no antimicrobials (Figure 3.3). This result is somewhat similar to those found by Ye et al. (2008), as they found no inhibition of the growth of *L. monocytogenes* on ham steaks with chitosan alone. However, it is important to note that they applied the chitosan via coated plastic strips, whereas this study incorporated the chitosan directly into the formulation of the hams (0.5% v/w). Additionally, while pH testing was not carried out on the hams throughout the study, the pH of all ham treatments was determined before the third rep of testing, and indicated that all hams were at pH > 6.0. As stated by No et al. (2002), 0.03% chitosan in broth completely suppressed *L. monocytogenes* growth at or below pH 5.5. It may be that the pH of the hams was not sufficiently low for chitosan to exhibit a strong inhibitory effect on the growth of *L. monocytogenes*.

The final preservative examined in the current research were partially purified bacteriocins from *C. maltaromaticum* UAL 307. Bacteriocins are peptides produced by bacteria ribosomally, which may or may not be modified posttranslationally, are exported extracellularly, and exhibit antimicrobial effects (Alvarez-Sieiro et al., 2016). Lactic acid bacteria (LAB) are well known in terms of bacteriocin production, and those produced by LAB can be categorized into multiple classes, with Class IIa being particularly effective against *Listeria* spp. (Alvarez-Sieiro et al., 2016; Zacharof and Lovitt, 2012). The mechanisms of action of bacteriocins are varied and include pore formation, inhibition of cell wall biosynthesis, and potentially others that are yet to be elucidated (Alvarez-Sieiro et al., 2016). Studies have shown that multiple bacteriocins from different LAB species are effective at controlling the growth of *Listeria* spp. Schillinger et al. (1991) demonstrated the then-named *Lactobacillus sake* Lb 706 produced sakacin, which inhibits the growth of *L. monocytogenes* on minced meat. Bacteriocins used for controlling *L. monocytogenes* include nisin and pediocin, which can be applied to food

packaging materials in meat products or added during production of ricotta-type cheeses (Davies et al., 1997; Ming et al., 1997). There is even evidence that bacteriocins may be able to be used in preventing bacterial biofilm formation on industrial surfaces in processing plants (Pérez-Ibarreche et al., 2016). Health Canada regulations currently recognize *C. maltaromaticum* CB1 as an additive to control *L. monocytogenes* that can be used on vacuum-packaged RTE meats, such as wieners and sliced roast beef and cooked ham (Health Canada, 2012). Due to their effectiveness against *L. monocytogenes* on RTE meats as either whole bacteria additives or as partially purified solutions, bacteriocins offer potentially powerful natural biopreservatives.

For the purposes of this study, the bacteriocins used during formulation of specifically treated hams were partially purified from *C. maltaromaticum* UAL307, which is the same strain as *C. maltaromaticum* CB1. This bacteriocin solution contains piscicolin 126 and carnobacteriocin BM1, both Class IIa, and carnocyclin A, a circular bacteriocin. These bacteriocins have been characterized previously, and generally all act through insertion into the cell membrane and forming pores or anionic channels (Gong et al., 2009; Gursky et al., 2006; Jack et al., 1996; Martin-Visscher et al., 2008; Quadri et al., 1994). However, of the antimicrobials used in this study, the use of bacteriocins alone did not reduce the growth of *L. monocytogenes* when compared to the growth on the control hams (Figure 3.3). While this study demonstrated that these bacteriocins have little to no ability to inhibit *Listeria* spp. growth on treated hams, previous research has exhibited their effectiveness. It is possible that when introduced into ham formulation, bacteriocin effectiveness is reduced versus being applied to the surface of the product or incorporated into the packaging that is in contact with the surface of the product. Further research would help clarify these results and help reconcile them with other literature.

Use of each of these preservatives individually did not have a significant effect in delaying the growth of *L. monocytogenes* on hams treated with these preservatives. However, preservatives often are used in combination with each other in order to produce synergistic effects that prove to be more efficacious than the individual preservatives on their own. In 1993, Schlyter et al. used combinations of sodium diacetate with nitrite, sodium lactate, or pediocin to treat turkey slurries inoculated with *L. monocytogenes*, and found increased antilisterial activity with the sodium diacetate/sodium lactate and sodium diacetate/pediocin combinations. Sodium lactate also has additional inhibitory effects against the growth of *Listeria* spp. when used in combination with the nisin in smoked rainbow trout (Nykänen et al., 2000), and the combination of sodium lactate and sodium diacetate with Micocin® (*C. maltaromaticum* CB1 partially purified bacteriocins with the live organism on the product surface) also significantly inhibits the growth of *L. monocytogenes* on wieners compared to each antimicrobial alone (Carlson et al., 2007). Chitosan also has an ability to perform better as a preservative against *L. monocytogenes* when used in combination with other preservatives. When incorporated into plastic films, combining chitosan with the bacteriocin divergicin M35 had an additive effect against the growth of *L. monocytogenes* verified by agar diffusion (Benabbou et al., 2009). Additionally, in testing multiple preservatives used with chitosan films on ham steaks, Ye et al. (2008) found that sodium lactate used with chitosan had the greatest antilisterial effect over long-term storage. Overall, combining preservatives potentially offers increased protection against *L. monocytogenes* on food products, including RTE products.

In the course of testing hams treated with preservatives in this study, two combinations were used, sodium lactate and sodium diacetate with partially purified bacteriocins and chitosan with partially purified bacteriocins. The combination of chitosan and partially purified

bacteriocins did not result in greater inhibition of the growth of *L. monocytogenes* compared to the growth on the control ham and the inhibition was similar to that observed on ham with other antimicrobials, including the treatment which included chitosan without bacteriocins. Chitosan did decrease counts of *L. monocytogenes* 08-5923 on PCA and PALCAM agars after 2 days of storage, but this was not maintained during further storage. *L. monocytogenes* does produce chitinases (Paspaliari et al., 2015), which may account for the inability of chitosan to control the growth of *L. monocytogenes* during storage.

While the results of this study correlate with research concerning synergistic effects of preservatives with sodium lactate and diacetate, the results of this research show a marked differentiation with regards to the combination of preservatives with bacteriocins. Most interesting is the work done by Carlson et al. (2007) that specifically showed that combining the bacteriocins from *C. maltaromaticum* CB1/UAL307 with the live organism and sodium lactate and sodium diacetate kept *L. monocytogenes* levels at or below inoculum levels for up to 70 days of storage at 4°C. These conditions were similar to the work done in this study, with the same concentrations of sodium lactate and sodium diacetate, and similar storage temperature. However, the current work did not include *C. maltaromaticum* CB1/UAL307 on the surface of the ham slices, which could have accounted for the differences in the inhibition of *L. monocytogenes* during storage.

The data for bacterial counts from the current study did show some differences in the inhibition of the two strains of *L. monocytogenes*. The growth of *L. monocytogenes* 08-5923 was inhibited up to 30 d of storage whereas there was a slight increase in growth of *L. monocytogenes* FS12 during this time. The reason for this difference was unclear but it seems that *L.*

monocytogenes 08-5923 was initially more susceptible to antimicrobial effects of sodium lactate/diacetate and bacteriocins than *L. monocytogenes* FS12.

The inhibition of *L. monocytogenes* with chitosan in combination with other antimicrobials differed drastically from results reported of Ye et al. (2008), and Benabbou et al. (2009). This may be a result of the type of application of the chitosan, with previous studies using plastic films to administer the chitosan versus adding the chitosan directly to the meat batter prior to cooking. Adding chitosan directly to a meat batter during formulation may result in interactions of chitosan with the components of meat and the high pH may interfere with the antimicrobial action. Application of chitosan as a surface treatment may be important to the effectiveness of chitosan both alone or in combination with other preservatives and could increase its potential as an antilisterial preservative.

In summary, this study found that only hams treated with a combination of sodium lactate and sodium diacetate with bacteriocins from *C. maltaromaticum* UAL307 had significantly reduced counts of *L. monocytogenes* after storage at refrigeration temperatures over an extended time. The use of sodium lactate and diacetate on their own, chitosan either with or without bacteriocins, and bacteriocins on their own were not able to reduce the growth of *L. monocytogenes* counts. However, while plate counts are sufficient in determining whether preservative treatments and combinations can control the growth of *L. monocytogenes*, more informative methods are required to examine the morphological changes these bacteria undergo under these conditions.

Flow cytometry as a technique to analyze cell size and morphology has been reviewed previously (Cossarizza et al., 2017; Picot et al., 2012; Veal et al., 2000), and more specifically with regards to application in the food industry (Comas-Riu and Rius, 2009). Generally, flow

cytometry is used for its rapid processing speed, high throughput, and automation over other methodologies such as microscopy, and its ability to utilize fluorescent probes to examine multiple cell populations or targets within individual cells.

Flow cytometry can be applied to examine multiple aspects of cells, including their morphology. At its most basic usage, flow cytometry can give information on cell size (forward light scatter) and internal complexity (side light scatter) based on light scatter effects. These measurements, specifically the forward light scatter, have been used to examine filamentation in bacteria. Wickens et al. (2000) observed filamentation of *E. coli* after ciprofloxacin exposure using forward light scatter plots. Similarly, Jones et al. (2003) applied flow cytometry to examine the mean length of the longest 10% of *E. coli* cells grown near the minimum temperature for growth. This same methodology has been applied to examining filamentation of *L. monocytogenes* exposed to various salt concentrations, different pH, and minimum growth temperatures (Liu et al., 2014b; Vail et al., 2012). While flow cytometry data offers quick analysis of the morphology of many "events", or cells, visualization of the cells and filaments can offer additional confirmation of the cell structure. While this would normally require further analysis and preparation of microscopy samples, there are technologies available to help expedite the process.

Imaging flow cytometry combines the rapid, high throughput capabilities of flow cytometry with the ability to view each individual cell that passes through the cytometer with fluorescent microscopy images. The Amnis® ImageStream® cytometer has been reviewed previously in detail (Cossarizza et al., 2017; Picot et al., 2012). This technology has been applied in a wide variety of clinical studies where imaging of samples, as well as flow analysis, was required. More specifically, the combined cytometry and fluorescent imaging has proven useful

in examining morphological changes in cellular systems, such as filamentation. Some of these include observing the relationship between virulence and pathogenicity with filamentation in *Candida albicans* (Peroumal et al., 2019), or examining the role of Spo0M in cell division of *Bacillus subtilis* (Vega-Cabrera et al., 2017). However, the current literature is lacking with regards to the use of imaging flow cytometry with food microbiology and filamentation. The research presented in this thesis represents potentially one of the first uses of this technology to examine the effects of food preservatives on the morphology of a pathogen like *L. monocytogenes*.

Using the Amnis® ImageStream® Mark II imaging flow cytometer, the influence of multiple preservatives on filamentation of *L. monocytogenes* inoculated onto the surface of treated hams was assessed. Inclusion of chitosan and chitosan with partially purified bacteriocins in hams had dramatic effect on the morphology of *L. monocytogenes*. Cells harvested from hams formulated with chitosan had a significantly lower percentage of their *L. monocytogenes* population filamented than the other preservative treatments or the control.

It should be noted that the data generated in Figures 3.6 and 3.7 are the result of both subjective and objective observation. The gating used to determine whether the cells harvested from the hams were determined using *L. monocytogenes* grown in TSB with 10% NaCl to produce filaments (Figure 3.5). When these control samples were analyzed in the imaging flow cytometer, a gate was generated around the events representing filamented cells, and this gate was applied to all of the other plots generated for the harvested samples. However, using the fluorescent images generated by the machine, the filamented cells in the harvested samples extended beyond the gate generated by the control samples. As such, the gate was manually adjusted to include as many filamented cells as possible while excluding “typical”, or

unfilamented, cells. While this was subjectively done based on fluorescent images, the adjusted gate was applied to every other plot generated from the harvested samples, maintaining a level of objectivity across all samples. This may affect the true amount of filamented cells captured by the gating process, but the examination of the fluorescent images indicates the gating was sufficient to represent the filamented population of each sample.

While previous research into the effect of chitosan on bacterial cell division is present, mechanisms are still being explored. The use of chitosan-Ag(I) films was observed to disrupt cell division, causing filamentation and cell death in *Staphylococcus aureus* (Díaz-Visurraga et al., 2010). Beyond this study, no others have studied the morphological effects chitosan may have on foodborne pathogens, such as *L. monocytogenes*. Even the mechanism of action of chitosan has not been fully elucidated. As stated above, the effectiveness of chitosan relies on a multitude of factors, and generally works at the surface of the cell membrane (Kong et al., 2010; Rabea et al., 2003). The molecular mass of chitosan may also alter how chitosan interacts with the cell wall, with low molecular mass molecules targeting cell permeability and growth, while the higher molecular mass variants form a barrier to block nutrient uptake (Benabbou et al., 2009). Nevertheless, the exact result of chitosan application on bacterial growth and the mechanism of its action still require further research.

A possible explanation for the results observed in this study involving treatment with chitosan is the presence of chitinases and other chitin-active enzymes potentially produced by *L. monocytogenes*. An extensive review of chitinases and other enzymes capable of modifying chitosan and its derivatives has been recently published by Kaczmarek et el. (2019). Chitinases and chitosanases are both enzymes that catalyze the hydrolysis of glycosidic bonds of either chitin or chitosan, respectively. These enzymes work generally by oxidizing the C1 or C4 of the

glucopyranose ring, and have been found in several bacterial species. Another chitin-active enzyme found in bacteria includes lytic polysaccharide monooxygenases (LPMOs), which differ from the glycoside hydrolases (GHs) like chitinase and chitosanase by directly cleaving the glycolytic bonds of highly crystallized chitin (Kaczmarek et al., 2019). Both of these enzymes can be used to break down chitin and chitosan in to simpler oligosaccharides, mainly N-acetyl-D-glucosamine $(GlcNac)_n$ for chitin, and D-glucosamine $(GlcN)_n$ for chitosan. These chitooligoshaccharides can be used in bacterial metabolism.

L. monocytogenes has two chitinases (ChiA and ChiB) and a chitin-active LPMO (*Lm*LPMO10) (Paspaliari et al., 2015). The chitinases are readily secreted by *L. monocytogenes* when grown in the presence of colloidal chitin, while the LPMO is not, indicating a potentially different trigger for its transcription. Nevertheless, the chitinases with or without the LPMO can degrade environmental chitin into potential carbon and nitrogen sources for metabolism. This could potentially explain the results seen in this study regarding the hams treated with chitosan. If *L. monocytogenes* can produce and secrete these enzymes to break down chitosan, this could prevent the chitosan from reaching and interacting with the cell membrane, thus leading to the lower proportions of filamentation seen compared to the other preservative treatments (Figures 3.6, 3.7). Also, if the chitosan was reduced to substrates useful for growth and metabolism, this may account for why the chitosan treatments showed no lowering in growth compared to the control treatment (Figures 3.1, 3.2. 3.3).

However, the preservative used in this study was chitosan, whereas *L. monocytogenes* secretes chitinases. Chitinases and chitosanases are substrate-specific, so it is unlikely that the chitinases in *L. monocytogenes* would act upon the chitosan on the hams. The current literature on the chitinolytic system of *L. monocytogenes* does not indicate the presence of chitosanases.

Therefore, it cannot be concluded definitively that the chitinases present in these *L. monocytogenes* strains were responsible for the results seen in the chitosan treatments. Further examination into the presence/absence of chitosanases in *L. monocytogenes* or potential cross-substrate action may be useful in further elucidating this phenomenon.

While this study cannot give insight into the exact mode of action for chitosan or how it may interact with the cell division machinery in *L. monocytogenes*, it can offer a look at the results of its use as a food preservative. Hams treated with chitosan, regardless of the presence or absence of partially purified bacteriocins, had a much lower percentage of the population that was filamented compared to the other preservative treatments and controls. Conversely, the use of other preservatives resulted in little to no difference from control hams in terms of the proportion of the population that was filamented.

Similar to chitosan, there are few papers that look specifically at the relationship between the preservative action of sodium lactate or sodium diacetate and filamentation. While the activity and mechanism of these preservatives has been discussed previously, few have sought to link the action of these salts with morphological changes in *L. monocytogenes*. Liu et al. (2017) examined filament formation of *L. monocytogenes* 08-5923 when exposed to sodium lactate, sodium diacetate, or a combination of the two. They found that a complex response network might be responsible for the reaction of *L. monocytogenes* to salt-based preservatives, with one of the resulting reactions being a down regulation of genes related to cell division and increased filamentation. While this seems to corroborate that results obtained in this study, it does not explain why the amount of filamented cells measured for the sodium lactate and sodium diacetate treated hams was comparable to the control ham, which did not contain any preservative. It is possible that the salt content of the control ham was enough to elicit a

filamentation response from the *L. monocytogenes* present, as has been demonstrated in previous research (Hazeleger et al., 2006; Liu et al., 2014b; Pratt et al., 2012). In addition, the methodology used to determine the population of filamented cells was very different than that used in the current research. Liu et al. (2014b) examined only the 10% of the longest cells in the population where the current research examined the total population of cells. This may have accounted for differences in results. Further work on the effects of sodium salt preservatives on filamentation may help explain the effect, or lack thereof, seen here.

In terms of bacteriocins, some research indicates that different bacteriocins may elicit filament formation in bacteria. Jones et al. (2013) review on filament formation in foodborne bacteria mentions some of these studies. However, work done specifically to examine the reaction of *L. monocytogenes* to sublethal doses of carnocyclin A showed no effect on filament formation (Liu et al., 2014a). The results of the current study are mixed, as when treated with bacteriocins alone or in combination with sodium lactate and sodium diacetate, *L. monocytogenes* harvested from the hams had high proportions of filamented cells. Conversely, there was reduced filamentation of *L. monocytogenes* regardless of the presence or absence of the partially purified bacteriocins of *C. maltaromaticum* UAL307. Whether or not the presence or absence of these molecules truly affect the proportion of filaments in the *L. monocytogenes* population harvest from hams requires further examination to reach a definite conclusion.

In summary, using fluorescent imaging flow cytometry, the proportion of *L. monocytogenes populations* harvested from preservative treated hams that was filamented was analyzed. Only hams treated with chitosan, either with or without partially purified bacteriocins, had significantly lower levels of filamented cells. This may represent a previously

uncharacterized property of chitosan action on *L. monocytogenes*, but will require further examination to confirm.

Understanding how preservatives work to limit growth of harmful bacteria on ready-to-eat meats is the first step in developing strategies to ensure the safety of consumers. *L. monocytogenes* is of particular note as a foodborne pathogen due to its high mortality rates in susceptible individuals and its ability to survive in a wide range of circumstances, including refrigeration temperatures. These characteristics help this pathogen cause notable outbreaks, which have caused massive costs to both consumers and the food industry (Buchanan et al., 2017; Olanya et al., 2019; Thomas et al., 2015). Specifically in Canada, the consequences of *Listeria* outbreaks have led to increased awareness and regulation of meat processing procedures and policy.

To briefly review, the policy for *L. monocytogenes* in RTE foods used by Health Canada divides these food products into three distinct categories based on the ability of *L. monocytogenes* to grow on them, and these categories define the type of testing required, the limit of bacteria accepted, and the level of response if these limits are exceeded. Category 1 foods include those where *L. monocytogenes* can grow during the stated shelf life, requires 5 x 25g analytical units examined by enrichment, and no *L. monocytogenes* must be detected within the total 125 g tested (Health Canada, 2011). Category 2A foods include those that allow limited *L. monocytogenes* growth, require 5 x 10 g analytical units examined by direct plating, and *Listeria* levels cannot exceed 100 CFU/g in tested units (Health Canada, 2011). Finally, category 2B foods include those that do not allow growth of *L. monocytogenes*, and follow the same testing parameters as category 2A foods (Health Canada, 2011). Detection of *L. monocytogenes* in category 1 foods registers as a Health Risk 1, a probable risk of serious or life-threatening

complications requiring action at both the producer and consumer level, while detection in category 2A or 2B foods is considered a Health Risk 2, which represents temporary or non-life threatening complications requiring action only at the distribution level. As a result of this policy on *L. monocytogenes* detection in RTE foods, Health Canada also has recommendations on the use of additives in these products to help control the growth of *L. monocytogenes*.

Under Health Canada's policy on food additives that may be used as preservatives to control *L. monocytogenes* in RTE foods, sodium lactate and sodium diacetate can currently be used in meat and poultry products (Health Canada, 2012). As detailed above, these sodium salts have been validated to have inhibitory effects on *L. monocytogenes*, in addition to other properties that make them beneficial to the production of meat foodstuffs.

The current study confirms that the use of sodium lactate and sodium diacetate helps to inhibit the growth of *L. monocytogenes* on RTE hams treated with the preservatives. Only the combination of sodium lactate and sodium diacetate with partially purified bacteriocins from *C. maltaromaticum* UAL307 significantly reduced numbers compared to the control. It should also be noted that this reduction was greater with *L. monocytogenes* FS12 compared to *L. monocytogenes* 08-5932, an outbreak strain,, and the effect was reduced beyond 90 days of storage (Figure 3.3). From these results, it would seem that the current regulations are sufficient, albeit with recommendations to use these preservatives in combination with bacteriocins, looking into strain specificity, and observing their effectiveness over longer storage times. However, the validation that these preservatives have undergone through other studies indicates that they are effective at controlling *Listeria* growth on RTE products.

This study also implies that the use of sodium lactate and sodium diacetate has no effect on filamentation of *L. monocytogenes* on RTE ham. While this corroborates evidence from Liu et

al. (2017), whether it is relevant to regulatory procedures remains to be determined. It has been observed that bacterial filaments can septate into multiple viable cells, potentially leading to misleading plate counts and potentially causing increased risks for consumers (Gill et al., 2007; Jones et al., 2003; Vail et al., 2012). However, if the inhibitory effect of lactate and diacetate salts outweighs the effect to cause filamentation, then the use of these preservatives may still result in safer RTE meat products. It may be of interest for future studies to examine the inhibitory effect of sodium lactate and sodium diacetate versus their filament formation ability, and determine whether this would cause an increased risk to consumers.

Conversely, the effects of chitosan on the *Listeria* harvested from the hams showed different results. Unlike sodium lactate and sodium diacetate, chitosan is not approved by Health Canada for use to control the growth of *L. monocytogenes* on RTE foods (Health Canada, 2012). The current research would agree with this decision, chitosan did not inhibit the growth of *L. monocytogenes* in ham. However, the antilisterial activity of chitosan molecules has been examined, as detailed above. These antimicrobial effects, as well as properties such as biodegradability and nontoxicity, may necessitate further examination of chitosan for inhibitory effects on *L. monocytogenes*.

The effect of chitosan of *L. monocytogenes* filamentation observed in this study may have interesting consequences for *Listeria* policy and regulation in Canada with regards to the limit of 100 CFU/g. The results represent one of the only examinations of chitosan on filamentation of *L. monocytogenes*, and indicate that the use of it in hams can effectively reduce the proportion of the population that filaments (Figure 3.6 and 3.7). However, as with sodium lactate and sodium diacetate, it remains to be seen if filamentation truly affects food safety and food safety policy. If chitosan truly inhibits filamentation but does not effectively control the growth of *L.*

monocytogenes, its usefulness as a preservative would be extremely limited in the food industry. With such little research done into the effects of chitosan on bacterial morphology, further examination would be recommended before changes to food production policy are made.

A mixed set of results was seen with the bacteriocin-treated hams used in this study. In Canada, *C. maltaromaticum* CB1 , which is known as *C. maltaromaticum* UAL307 in our laboratory is currently approved as an additive to control *L. monocytogenes* on RTE meats (Health Canada, 2012). These bacteriocins on their own did not inhibit growth or decrease in amount of filamentation of *L. monocytogenes* harvested from the hams (Figures 3.3, 3.6, 3.7). However, combining them with sodium lactate and sodium diacetate resulted in reduced growth, and combining them with chitosan saw a reduction in the proportion of filamented cells detected by imaging flow cytometry.

These results may have implications for current regulations on the use of bacteriocins for controlling *L. monocytogenes* in RTE foods. Multiple studies detailed previously have shown that bacteriocins can hinder *L. monocytogenes* growth, as well as cause cells to elongate. If the results of the current examination were true, it would indicate that the bacteriocins from *C. maltaromaticum* UAL307 could be used for either growth or inhibition filament formation of *L. monocytogenes*, depending on the other preservatives used along with them. Combinations of bacteriocins with other preservatives have shown synergistic effects in terms of controlling growth (Alvarez-Sieiro et al., 2016; Benabbou et al., 2009; Schlyter et al., 1993). Further testing to observe the combination effects of bacteriocins with other preservatives may be beneficial for regulations on recommended additives and mixtures for formulating RTE foods safe for consumption.

To review, sodium lactate, sodium diacetate, and *C. maltaromaticum* and its bacteriocins are currently approved by Health Canada for use in controlling the growth of *L. monocytogenes* in RTE foods. This study confirms that the use of sodium lactate and diacetate with bacteriocins can help reduce *L. monocytogenes* growth on RTE hams, but did not find this same effect with either preservative on its own. The results also showed that while chitosan may disrupt filament formation, it does not control growth of *L. monocytogenes*. As regulations are more concerned with limiting the number of cells consumers are exposed to, this would indicate that sodium lactate and sodium diacetate with bacteriocins would be preferred over chitosan as additives for RTE foods. However, some concern has arisen due to the possibility of filaments of *L. monocytogenes* breaking apart into individual cells upon being introduced into less harsh conditions. This would increase the load of bacteria actually present on the foods, which may not be noticed with traditional plate counts, which are used for RTE food testing. However, to determine whether or not the current regulations regarding testing for *L. monocytogenes* on RTE foods is beyond the scope of this study, and instead the results may help to inform future researchers on the effects of the tested preservatives on the growth and morphology of this foodborne pathogen.

In terms of potential future avenues of research, many have already been suggested in this discussion. These include focusing on the effects of chitosan to further elucidate a mode of action and possible effects on filamentation. The current literature on chitosan as a preservative is lacking, and would benefit from a dedicated insight into how it would affect aspects of foodborne pathogens such as cell morphology. Another approach for the future includes observing the synergistic capabilities of combining bacteriocins with other preservatives. The current research shows potentially interesting interactions between the bacteriocins of *C.*

maltaromaticum and sodium lactate and sodium diacetate or chitosan. Previous research also reports combinational effects of preservatives with bacteriocins (Benabbou et al., 2009; Mbandi and Shelef, 2001; Ye et al., 2008). It may be beneficial to further examine these interactions, and the effects they have on pathogens such as *L. monocytogenes*. Beyond re-examining the effects of these chosen preservatives on the growth and filamentation of *L. monocytogenes*, there are other topics that may provide the basis for further research.

While this study focused on preservative action and refrigeration temperatures, there are multiple other stressors that may have been examined in terms of eliciting filamentation in *L. monocytogenes*. Jones et al. (2013) reviewed multiple stressors that demonstrate the ability to induce filamentation in foodborne pathogens. These include pH, high pressure, low water activity, and elevated CO_2 levels. All of these stresses may be present in foods, and as such may increase the amount of filamented bacteria like *Listeria* spp. on these foods. It is also not uncommon for multiple stressors to be present in the same foodstuff. As such, examining combinations of stressors and their effect on the growth and filamentation of *L. monocytogenes* would benefit academia and industry, providing information as to which combinations of stressors may promote filamentation or which may increase growth inhibition. The use of technology such as the Amnis® ImageStream® Mark II imaging flow cytometer could prove beneficial in theses studies by providing detailed fluorescent images alongside flow data.

Another aspect that was not taken into account in the current study was genetics. Observing gene expression and performing genetic analysis for the presence/absence of specific genes is a powerful tool in microbiology. It also has a wide application with regards to filamentation in foodborne pathogens. These techniques have been used to help identify and isolate specific strains of *L. monocytogenes*, including in the context of outbreak scenarios

(Gasanov et al., 2005; Gilmour et al., 2010; McLauchlin et al., 2004). They have also been beneficial in the understanding of bacteriocins, their structure, and their activity (Martin-Visscher et al., 2008; Quadri et al., 1994). Genetics has also been utilized in finding alternate gene expression of *L. monocytogenes* in salt-induced filamentation and after exposure to sodium lactate and sodium diacetate (Liu et al., 2017, 2014b). However, a missing piece in this genetic research is the effect of chitosan on *L. monocytogenes* gene expression. As such, exposing *Listeria* spp. to chitosan on RTE hams and examining potential genetic effects may prove useful in elucidating a mode of antimicrobial action for chitosan, and will add to the understanding of how preservatives affect *L. monocytogenes*.

Overall, the research reported in this thesis offers valuable insight into the effects of preservatives on the morphology of a serious foodborne pathogen. The results show that current approved preservatives for RTE foods do inhibit the growth of *L. monocytogenes*, while another may demonstrate an ability to limit filament formation. It is also of relevance due to the fact that in current food culture, there is a push towards "clean label" ingredients and natural preservatives. As such, additives such as bacteriocins and chitosan will require further testing and scrutiny, as they can both be considered as natural and "environmentally friendly". If these preservatives prove to be effective at controlling growth, as well as potentially limiting the rate of filamentation, then the current policy on control of *L. monocytogenes* in RTE foods in Canada may need to be updated or revised.

Ultimately, the hypothesis put forward in this study was rejected. The combination of sodium lactate and diacetate, chitosan, and bacteriocins did not elevate the levels of filamentation of *L. monocytogenes* on RTE hams beyond those seen in control hams. However, it was discovered that the combination of sodium lactate and sodium diacetate with the

bacteriocins of *C. maltaromaticum* UAL307 could reduce the growth of *L. monocytogenes* on RTE hams compared to the control. It was also seen that treating RTE hams with chitosan, with or without bacteriocins present, could reduce the proportion of the *Listeria* population that formed filaments. Also, this paper provides additional information on the growth of *L. monocytogenes* on an RTE product for an extended storage time at refrigeration temperatures in the presence of multiple preservatives, further informing on the potential control of this pathogen. Finally, the use of imaging flow cytometry proved effective at combining the information gained by microscopy and flow cytometry at examining the phenomenon of filamentation in *L. monocytogenes*. This technology could prove useful in further studies into the effects of preservatives on the morphology of foodborne pathogens.

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