"Research is to see what everybody else has seen, and to think what nobody else has thought."

-Albert Szent-Gyorgyi

University of Alberta

Behaviour of cold-adapted *Listeria monocytogenes* under conditions representative of meat processing plants

by

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For my mother, whose lifelong search for knowledge inspires my own.

Abstract

The objective of this study was to examine filamentation characteristics of cold-adapted *Listeria monocytogenes* under conditions representative of a meat processing plant. Initial analysis of 16 strains found three strains with a wide range of filamentation characteristics and these were used in subsequent experiments. *L. monocytogenes* were grown at 3°C in Tryptic Soy Broth (TSB), TSB with 4% or 8% NaCl, TSB adjusted to pH 5.5, 6 or 8.5, and TSB with 4% NaCl adjusted to pH 6. Filamentation characteristics were determined with microscopy and flow cytometry. Filamentation in saline conditions peaked early in the logarithmic phase whereas in acidic conditions filamentation increased steadily over time. In alkaline media filamentation peaked twice during the growth cycle. A combination of acidic and saline conditions resulted in an initial peak, followed by gradual increase of filamentation over time. The length of filaments increased with an increase in stressful conditions.

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List of Abbreviations

°C: degree Celcius

µL: microliter

µm: micrometer

ATP: adenosine triphosphate

a_w: water activity

BC: bacteria counting

BHI: brain-heart infusion broth

CFU: colony forming units

Csp: cold-shock protein

DNA: deoxyribonucleic acid

DPS: DNA-binding protein

GTP: guanosine-5'-triphosphate

h: hour

MAP: modified-atmosphere packaging

MHB: Mueller-Hinton broth

min: minute

mL: milliliter

M: molar

MPa: megapascal

OD: optical density

PBS: phosphate-buffered solution

RNA: ribonucleic acid

RTE: ready-to-eat

TSA: tryptic soy agar

TSB: tryptic soy broth

VBNC: viable but non-culturable

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1. Introduction and Literature Review

Listeria monocytogenes are pathogenic bacteria that has an optimum growth range of 30-37˚C but grows at temperatures as low as 4˚C (Batt 1999). A recent outbreak in Canada of *L. monocytogenes* led to 57 confirmed cases and 22 confirmed deaths (Health Canada 2009). This bacteria is found both in the environment and in ready-to-eat (RTE) food products like meat (Bĕrzins, Terentjeva, Korkeala 2009; Fenlon, Wilson, Donachie 1996). Companies that produce RTE meats utilize a variety of different techniques to reduce the risk of *L. monocytogenes* including modified-atmosphere packaging (MAP), pH control or high salt. However, *L. monocytogenes* has an unusual tolerance to many of these stress factors (Doyle 1988; Sorrells and Enigl 1989). Moreover, is has been recently discovered that the process of cell division itself is altered in response to these stresses (Bereksi and others 2002; Giotis, Blair, McDowell 2007; Jydegaard-Axelsen and others 2005).

1.1 Formation of Filamentous Cells

Since the first discovery of filament formation in *Escherichia coli* in 1968, research on filament formation of pathogenic bacteria has been of interest to the food industry (Shaw 1968). *E. coli, L. monocytogenes* and *Salmonella enterica* all produce filaments near their respective minimum growth temperatures (Jones, Gill, McMullen 2003; Jydegaard-Axelsen and

others 2005; Kieboom and others 2006). Filament formation is largely believed to be caused by problems in cell division as well as gene and protein expression (Bereksi and others 2002; Kieboom and others 2006; Margolin 2000). This belief was strengthened through the discovery of a number of nucleoids evenly spaced throughout the filamentous cells (Mattick and others 2000; Shaw 1968). Furthermore, septa are visible along the length of elongated cells of *Listeria* with fluorescence microscopy (Hazeleger, Dalvoorde, Beumer 2006). Once filamentous cells are placed in less stressful environments, filaments divide into individual cells (Bereksi and others 2002; Jones, Gill, McMullen 2002; Kieboom and others 2006). These filamentous cells are longer than single cells, but have approximately the same width (Giotis, Blair, McDowell 2007). Some studies suggest that osmotic shock prevents cell division (Jørgensen, Stephens, Knøchel 1995), but this theory is contradicted by studies involving cell filamentation free of osmotic stress (Giotis, Blair, McDowell 2007; Jydegaard-Axelsen and others 2005).

The creation of filamentous cells could be a type of reversible stress response, occurring during pH, osmotic, atmospheric and temperature stress (Bereksi and others 2002; Jydegaard-Axelsen and others 2005). Filamentation has also occurred when bacteria are exposed to toxic substances (Imlay and Linn 1987). The results of a study performed in 1987 suggest that filamentation occurs in order to repair DNA lesions caused by stressful environments (Imlay and Linn 1987). In a recent study, *L.*

monocytogenes lacking the *yneA* gene, a gene involved in the SOS stress response (a response caused by DNA damage), did not filament while the wild-type cells elongated (van der Veen and others 2010). This gene is regulated by the SOS repressor LexA, and during the SOS response YneA accumulates within the cell. In *Bacillus subtilis,* accumulation of this protein causes filamentation, irrespective of other genes expressed or the environment (Kawai, Moriya, Ogasawara 2003).

Filamentous cells are a potential safety issue for food manufacturers as they form single colonies when plated on agar media, causing current assessment models to underestimate the food safety risk of a given food product (Gill, Badoni, Jones 2007). While many studies on sub-lethal stress and filament formation have been done on *E. coli*, limited studies have been done on both *L. monocytogenes* and *S. enterica* serovar Enteritidis, especially at refrigeration temperatures. Discovery of the specific response of pathogens in response to various stresses in conditions representative of manufacturing plants is essential to maintaining food safety.

Filament formation capabilities vary between strains of bacteria, and within the strains themselves; even single strains lack homogeneity (Gill, Badoni, Jones 2007; Jones, Gill, McMullen 2003). The percentage of cells that form filaments in a given culture is dependent on the type and severity of the stress. Increased stress leads to an increase in filament formation (Isom and others 1995). Understanding the optimal filament-forming conditions for a given bacteria are necessary to avoid this morphological adaptation.

While a great deal of research has been done on filamentous *E. coli* and the conditions in which filamentation occurs at low temperatures and on *S. enterica*, few studies examine these same effects on *L. monocytogenes*. This is possibly because *L. monocytogenes* has a lower minimum growth temperature than both *E. coli* and *S. enterica.* As the conditions for filament formation are dependent on the genera of bacteria in question, a systematic investigation of filamentation by *L. monocytogenes* would be beneficial to the food processing industry.

The objective of this review is to examine the different conditions which result in filamentation and to determine what is known about the extent of filamentation in *L. monocytogenes.*

1.2 Filament Formation in Response to Sub-Lethal Stress

1.2.1 pH and NaCl-Induced Filamentation

While *L. monocytogenes* is known for its resistance to NaCl, it forms filaments at 30˚C in the presence of 1.5 M of NaCl when grown in both tryptic phosphate and Mueller-Hinton broth (MHB) (Farber, Coates, Daley 1992; Jørgensen, Stephens, Knøchel 1995). *Listeria* also forms filaments in tryptic soy broth (TSB) at 37˚C and 22˚C, and in brain heart infusion broth (BHI) when over 1 M (5%) of NaCl is present (Bereksi and others 2002; Hazeleger, Dalvoorde, Beumer 2006; Isom and others 1995). Several strains of *S. enterica* filament in low water-activity environments caused by NaCl, forming

filaments as long as 200 μm when over 4% NaCl is present (Kieboom and others 2006; Mattick and others 2000). Moreover, the water activities (a_w) were 0.94-0.95 for those strains, regardless of temperature (Kieboom and others 2006; Mattick and others 2000). The combination of NaCl and extreme pH values increases filamentation among cells of *Listeria*, and cause filamentation in conditions not shown to cause filamentous cell formation with one variable alone (Bereksi and others 2002). This suggests a possible synergistic relationship between the two variables (Bereksi and others 2002).

Increased NaCl stress produces cells with elevated thermotolerance (Jørgensen, Stephens, Knøchel 1995). When cells are placed in more optimal environments, the filamentous cells divide and thermotolerance is lost (Jørgensen, Stephens, Knøchel 1995). A study by Bereksi *et al*. (2002) discovered filamentation in *Listeria* grown at 22°C at both 5 and 10% salt and at a pH of either 5 or 7 (Bereksi and others 2002). This raises the question of to what degree filamentation occurred, and in what stage of growth.

Research should be conducted into the effect of temperature on filamentation of *L. monocytogenes* at various pH and saline environments to fully examine the resistance to temperature. Most studies examine *Listeria* at temperatures in its optimal growth range, but *Listeria* can grow in temperatures as low as 4˚C.

Many RTE foods have a high salt content or are brined, a practice that slows the growth of *Listeria*. Filamentation of cells of *Listeria* and *Salmonella* occurs in saline environments (Hazeleger, Dalvoorde, Beumer 2006). Filamented cells are of concern to quantification procedures used in the RTE food industry; knowledge of filamentation characteristics in these environments will help the industry understand the impact of filamentation on food safety.

Hurdles to bacterial growth in RTE meats often include acidic environments. Preservatives such as ascorbic, lactic or citric acid are often included into RTE meat, or meat containing products, to reduce growth. Ascorbic acid has been found to reduce the formation of N-nitrosamine from nitrites, a human carcinogen (Kim, Lee, Sung 1997). Nitrites are used frequently in RTE-meats as a means of preventing the outgrowth of spores of *Clostridium botulinum*. In the RTE meat industry as with other areas of the food industry, multiple hurdles to bacterial growth are often added to a single product. RTE meats are likely to not only be slightly acidic, but also be refrigerated and have a high salt content in the case of meats such as ham. Many cleaners and sanitizers used by the industry are strongly alkaline and if cells or biofilms of *Listeria* remain post-cleaning/sanitizing, information on filamentation of *Listeria* in these conditions is equally important. The order with which the stress is applied may play a role in filamentation (Hill and others 2002).

In its optimal growth range (30-37˚C) *Listeria* filamentation occurs at a pH of 5-6 and at a pH over 9 when pH is adjusted with citric acid and NaOH (Giotis, Blair, McDowell 2007; Isom and others 1995). As the pH becomes less neutral and the stress increases on the cells, filamentation and mean cell length increases. In a study by Giotis et al. (2007) the mean length of *Listeria monocytogenes* 10403S at pH 9 was 1.851 μm, compared to 1.600 μm at pH 7.4 after 3 h of incubation in MHB at 30˚C. As the pH was raised to 9.7, mean length of cells increased to 2.218 μm (Giotis, Blair, McDowell 2007). Other conditions, such as the matrix the bacteria are in, may also affect the ability of *L. monocytogenes* to form filaments. In a study involving various Italian cheeses exposed to lethal acidic conditions, bacteria isolated from crescenza cheese consisted primarily of filaments, while filaments were not found on any of the other cheeses (Cataldo and others 2007). Furthermore, *Listeria* isolated from crescenza displayed high tolerance in acidic conditions; however, bacteria from mozzarella exhibited a similar resistance and did not contain filaments, so cell morphology does not necessarily increase cell resistance to acid (Cataldo and others 2007).

1.2.2 Filamentation Induced by Temperatures Near the Minimum for Growth

Few studies have examined the effects of the morphology of *Listeria* cells at temperatures near its minimum for growth, nor are there reports in the literature that compare the behaviour of *Listeria* at different low

temperatures. Models tend to lose predictive power as conditions approach the minimum of growth, therefore research into the behaviour of pathogens at minimum temperatures is essential to the food industry (McMeekin and others 2002). *L. monocytogenes* on cheeses at 4˚C contained filamentous cells, but other stresses, such as acid, were also a determining factor in this study (Cataldo and others 2007). Furthermore, while the presence of filamentous cells was determined, quantification and mean cell length was not calculated and therefore cannot be compared to similar studies (Cataldo and others 2007).

Contrary to the limited information available on *L. monocytogenes*, much research has been done on the behaviour of *E. coli* at temperatures near its minimum of growth (Gill, Badoni, Jones 2007; Jones, Gill, McMullen 2002; Jones, Gill, McMullen 2004). At a constant temperature of 2 and 4˚C, below the 7˚C minimum growth temperature for *E. coli*, no filamentation occurs, while at 6˚C substantial filamentation occurs (Gill, Badoni, Jones 2007; Jones, Gill, McMullen 2002; Jones, Gill, McMullen 2004). At fluctuating temperatures around the minimum, cells elongated at 2 and 4˚C only when temperatures fluctuated above 6˚C (Jones, Gill, McMullen 2004). This suggests that the process of filamentation can only occur at or above the minimum growth temperature of the given microorganism, but that filamentous cells, once formed, survive in temperatures below the minimum for growth.

1.2.3 Filamentation Induced by High CO_2 Environments

Large amounts of CO_2 (over 70%) in the headspace of a food product inhibit the growth of *L. monocytogenes* (Farber, Cai, Ross 1996). However at 70% CO_2, the limit for sustained growth, CO_2 has been reported to cause elongation of cells of *Listeria* (Besnard, Federighi, Cappelier 2000; Jydegaard-Axelsen and others 2005; Li J. and others 2003; Nilsson and others 2000). Filaments formed in a CO_2 saturated environment were approximately 1.2 times longer than regularly-grown *Listeria*, and divided into individual cells within a few days (Jydegaard-Axelsen and others 2005). Similar results were achieved when *Aeromonas hydrophila*, a psychrotroph like *Listeria*, was exposed to a 100% CO_2 environment (McMahon, Blair, McDowell 1998). This suggests that filamentation in response to CO_2 is not a *Listeria*-specific phenomenon.

1.2.4 Other Filamentation-Causing Environments

Cells of *E. coli* became filamentous after treatment at 75 MPa for 90 min (Kawarai and others 2004). Similar to that observed with other forms of sub-lethal stress, the filamentous cells separated upon removal of high-pressure stress (Kawarai and others 2004). Little is known as to the effect of pressure on filamentation and morphology of *L. monocytogenes.*

1.3 Mechanism of Formation of Filamentous Cells

While the mechanisms of filament formation in *E. coli* are becoming clearer through new research, little research has been done on the mechanisms involved with *Listeria*. However, evaluation of the general stress responses for *L. monocytogenes* under types of stress also known to cause filamentation (acid, pH, low water activity) can be of use in determining a possible starting point for mechanism-related research.

1.4 General stress response

The main challenge in examining the stress response of bacteria is that while the functions of different mechanisms involved in the stress response have been well studied, in practice these responses work together with a myriad of other biochemical processes within the cell. As a result the "general" stress response is difficult to define, as results can be confusing and difficult to attribute to any single particular process or combination of processes.

1.4.1 Cold-Shock Proteins

Cold-shock proteins (Csp's) are small, highly conserved proteins that bind to nucleic acids and regulate many processes in the bacterial cell(Ermolenko and Makhatadze 2002). They are believed to bind to DNA and RNA and may aid in translation, transcription and regulation of cells, acting

as chaperones (Ermolenko and Makhatadze 2002). The function of Csp's in cells under stress is more extensively known in *E. coli* and *Bacillus subtilis* than in *L. monocytogenes* (Goldstein, Pollitt, Inouye 1990; Graumann and others 1997; Lee and others 1994; Wang, Yamanaka, Inouye 1999; Weber and and Marahiel 2002). Three families of Csp proteins (CspA, CspB, and CspD) are found in *L. monocytogenes* and their roles differ in importance depending on the type of stress the cell is under (Glaser and others 2001; Schmid and others 2009). Under cold-temperature stress, mutants with deletions of the *csp* genes demonstrated that CspB is dispensable at cold temperatures, but without CspA growth is impossible, and without CspD growth at 4˚C is significantly impaired (Schmid and others 2009). Conversely, under NaCl-induced osmotic stress a loss of the CspD protein impairs cell tolerance to salt (Schmid and others 2009).

1.4.2 SigmaB Response

SigmaB (σ^B) is an alternative sigma factor that works with the core RNA polymerase to regulate the expression of genes under conditions of increased stress (Ferreira, O'Byrne, Boor 2001). In *Bacillus subtilis*, σ^B aids in the transcription of numerous genes that translate into general stress proteins (Gsp's) (Antelmann and others 1996; Antelmann and others 1998). The *SigB* operon in *L. monocytogenes* is similar to that in *B. subtilis*, the gene order of *SigB* in *L. monocytogenes* being identical to the last 5 genes of the *SigB* operon in *B. subtilis* (Wiedmann and others 1998). *Staphylococcus aureus* has a σ^B-dependant tolerance to heat, acid and oxidative stress (Chan

and others 1998). The effect of σ^B on the tolerance of bacteria to adverse conditions is similar in *L. monocytogenes*. Strains of stationary-phase *L. monocytogenes* grown at 37˚C are more resistant to certain types of stress than strains containing a *sigB* mutant (Becker and others 1998; Ferreira, O'Byrne, Boor 2001). The Δ*sigB* strain of *Listeria* is almost 10,000 times more susceptible to acid stress; however, when pre-exposed to acidic (pH 4.5) conditions for one hour Δ*sigB* became 1000 times more resistant compared to non-treated Δ*sigB* (Ferreira, O'Byrne, Boor 2001). Ferreiria *et al.* (2001) reported that following a 12-hour incubation in glucose-depleted medium viability of the Δ*sigB* strain was reduced by 85% while viability of the parent strain had a 25% reduction (Ferreira, O'Byrne, Boor 2001). The importance of these reductions is unclear as less than one log decrease in viability occurred. *SigB* is believed to be partially responsible for the psychrotrophic properties of *L. monocytogenes* as it is essential for accumulation of the cryoprotectants betaine and carnatine within the cell (Becker and others 2000); however, this is under dispute since as a Δ*sigB* strain grew normally at low temperatures (Chan, Boor, Wiedmann 2007).

The mechanisms involved in *sigB*-dependent tolerance to stress are not completely understood. In one study involving the role of σ^B, a lack of *sigB* reduced the ability of *L. monocytogenes* to use carnitine and betaine as osmoprotectants and cryoprotectants (Becker and others 1998). Furthermore, while some reactions to stress are *sigB*-dependent, others are not. The method in which pre-adaptation to acid increases the tolerance of

Listeria to acidic conditions suggests that acid tolerance is only partially σ^B-dependent. Heat and ethanol stress in *L. monocytogenes* is also somewhat σ^B-independent (Ferreira, O'Byrne, Boor 2001). Research involving the mechanisms of stress tolerance is mostly done at optimal growth temperatures. Since *L. monocytogenes* grows at refrigeration temperatures, research into the function of σ^B at low temperatures would be of use to the food industry.

1.4.3 RpoS

RpoS is an alternative sigma factor that controls a variety of genes that play a role in general stress resistance in *E. coli*, *Salmonella* and other Gram negative organisms (Hengge-Aronis 1996; Nickerson and Curtiss III 1997). More than 70% of *Salmonella* cells inoculated in chicken meat extract were filamentous after 4 days of incubation at 8°C for both *rpoS* expressing strains and *rpoS* mutants (Mattick and others 2003). Several reviews discuss the role and regulation of *rpoS,* which encodes the transcription factor σ_s, that regulates over 60 genes, and how those genes respond to osmotic, acidic, heat and starvation stress (Hengge-Aronis 1993; Loewen and others 1998). A smaller fraction of *Salmonella rpoS* mutant cells formed filaments in chicken meat extract than the strains expressing *rpoS*, suggesting that the activity of RpoS is partially responsible for filamentation in *Salmonella* (Mattick and others 2003). Despite knowledge of the general role of *rpoS* in the cell, how *rpoS* and its expressed proteins affect filamentation is largely

unknown as, depending on the type of stress involved, filamentation is either dependant or independent of *rpoS* (Jones, Johns, Gill 2008; Mattick and others 2003).

1.4.4 Energy Requirements

Jones et al (2006) suggested that induction of the stringent stress response attributed to a depletion in energy may be the cause of filamentation at low temperatures in *E. coli* (Jones and others 2006). The stress response itself is connected to the energy requirements of cells, with cells in stressful environments requiring more energy than their counterparts in more optimal conditions. At 6˚C *E. coli* ceases growth upon formation of filaments until cells re-enter a more optimal environment (Jones, Johns, Gill 2008). Conversion into this reduced-energy state causes the cells to become more susceptible to further stress due to energy constraints (Jones, Johns, Gill 2008). In this reduced-energy state cells stop growing, lowering the energy requirement and allowing cells to survive until conditions become more favourable (Jones, Johns, Gill 2008).

Proton motive force has recently been found to be involved in protein localization throughout the cell, involving MinD and FtsA proteins from Gram positive and negative cells (Strahl and Halmoen 2010). When the proton motive force is malfunctioning, filamentation occurs (Strahl and Halmoen 2010). In order for cell division to occur in Gram negative bacteria, production of FtsA is required immediately before cell separation (Donachie

and others 1979). When *ftsA,* a gene found in Gram negative bacteria located upstream of *ftsZ*, is absent, filamentation occurs (Margolin 2000; Tormo, Martínez-Salas, Vicente 1980). MinCD, a component of the *min* system that is responsible for choosing the division site within a cell, inhibits FtsZ ring formation in Gram negative bacteria such as *E. coli* as well as preventing localization of FtsZ (Bi and Lutkenhaus 1993; Rothfield and Zhao 1996). In order for cell division to occur, these proteins need to localize in specific sections of the cell, a process requiring proton motive force (Strahl and Halmoen 2010). While the *ftsZ* gene is present in *L. monocytogenes*, few studies have examined its role in filamentation in *Listeria* cells.

Despite this gap in the research literature, dissipation of the proton motive force in Gram positive *Bacillus subtilis* produces filamentation, suggesting that protein localization may not be the only factor affecting cell division (Strahl and Halmoen 2010). The proton motive force is one of the main producers of ATP within the cell; dissipation of this force leads to a significant decrease in the amount of available energy and may be a factor affecting filamentation.

1.5 Strain Variation in *L. monocytogenes*

Filamentation occurs to differing degrees, dependent on the type of growth conditions (Bereksi and others 2002; Jones, Gill, McMullen 2002; Jones, Gill, McMullen 2003). There is also significant differentiation among strains (Arguedas-Villa, Stephan, Tasara 2010; Bereksi and others 2002).

This could be due to other differences between strains, such as *L. monocytogenes* L028 having reduced adhesion abilities compared to the Scott A strain at a pH of 7 (Bereksi and others 2002).

To eventually understand the genetic causes of filamentation, it is important to examine the degree of filamentation among strains of filamentous bacteria under similar conditions, and to determine conditions of importance in the food industry. Genetic differences between strains could potentially be linked to their ability to filament.

It is equally important for the food industry to examine strains that show a range of differing filamentation characteristics in order to fully understand the risk associated with this phenomenon.

1.6 Objectives

The objectives of this study were to 1) determine the degree of filamentation and comparative growth rate of 16 different strains of *L. monocytogenes* at different cold room (ready-to-eat meat plant) temperatures; 2) investigate the degree of filamentation of 3 strains of *L. monocytogenes* in saline conditions representative of RTE meat plants at 3°C; 3) investigate filamentation of *Listeria* in either acidic or alkaline conditions at 3°C; and 4) investigate filamentation of *Listeria* with three growth hurdles, a low temperature acidic and saline environment.

2. Materials and Methods

2.1 Bacterial Strains

Sixteen strains of *L. monocytogenes* were used. Fifteen, FS1 to FS15, were isolated from ready-to-eat meat products in a study by Bohaychuk et al. (2006) and the 16th strain (CDC7762) was the strain responsible for the large *L. monocytogenes* outbreak in 1998 (Mead and others 2006). All 16 strains were used in evaluating growth characteristics at different cold-room temperatures. All further experimentation was done with only *L. monocytogenes* FS2, FS12 and CDC7762 due to the variety of filamentation characteristics, determined by optical density (OD), fold change and microscopic image data.

2.2 Screening of 16 strains of *L. monocytogenes* at Various Cold Temperatures

2.2.1 Pre-Treatment Growth

Tryptic Soy Broth (TSB; Difco, Becton, Dickinson and Company, Sparks, MD, USA) was used as the growth medium. *L. monocytogenes* FS2-FS12 and CDC7762 were cold adapted by subculturing from a frozen stock into TSB and incubated overnight at 37°C, then subculturing into fresh broth and incubated overnight at 25°C, then into fresh TSB and incubated for 48 h at 15°C prior to use in experiments.

2.2.2 Treatments and Sampling

One microliter of log phase cold-adapted *L. monocytogenes* was added to 10 mL of TSB pre-cooled to the temperature of interest. This was done for all 16 strains at 3°C 3.5°C, 4°C, 4.5°C and 5°C in a RM 6 RMS cold-temperature circulating water bath (MGW Lauda, Lauda-Königshofen, Germany) filled with diluted ethylene glycol.

Optical density measurements were taken for all strains incubated from 3°C to 5°C on day 7 or 8, and day 10. Optical density measurements were performed using a GeneQuant Pro spectrophotometer (Biochrom Ltd., Cambridge, UK).

On days when optical density (OD) was determined 1 mL of each strain was fixed with 110 µL of 10% formaldehyde for microscopy at a later date. Samples were refrigerated at 4°C and used for microscopy within seven days. For flow cytometry samples were diluted with 0.22 µm filtered phosphate buffered solution (PBS) and 0.5 µL of Syto® BC dye (Molecular Probes, Eugene, OR, USA) was added prior to analysis on FACScan (Becton, Dickenson and Company, Sparks, NJ USA). Flow cytometry analysis of samples incubated at 3.5 and 3°C were not completed as samples were left overnight after dilution, and filament breakdown occurred. Samples (100 µL) were serially diluted in 0.9 mL of sterile 0.1% peptone water (Difco) and 100

µL of dilutions of interest were plated in duplicate on Tryptic Soy Agar (TSA) and incubated at 37°C.

Microscopy was performed using a Zeiss microscope with Axiocam MRm (Carl Zeiss Inc., Oberkochen, Germany). Fixed cultures of strains grown at 3 and 3.5°C were wet-mounted (10 µL) on a slide for viewing and images were taken for cell length comparison.

2.2.3 Analysis of Flow Cytometry Results

Analysis was performed using CellQuest Pro software (Becton, Dickenson and Company). Cells were gated using **forward scatter** and **side scatter**. Cell shift was determined by comparing the value at the 90th percentile of the control (15°C cold adapted cells), M1, to the percentage of cells held at that same point in the treatment samples. Fold change was determined by dividing the mean value of the 10% longest cells of the sample by the mean value of the 10% longest cells in the control (Jones, Gill, McMullen 2003).

2.3 Growth and Filamentation of Cold-Adapted Log Phase *L. monocytogenes* in Saline, Cold-Room Environments

2.3.1 Pre-Treatment Growth

L. monocytogenes FS2, FS12 and CDC7762 were cold adapted according to the process explained in 2.2.1.

2.3.2 Treatments and Sampling

Each strain of log phase cold-adapted *L. monocytogenes* was inoculated (1 µL) into 10 mL of each treatment broth incubated at 3°C in a RM 6 RMS cold-temperature circulating water bath (MGW Lauda, NJ, USA) filled with diluted ethylene glycol. TSB and TSB containing 4% total NaCl (40 mL of each broth) was aliquoted after inoculation into 15 mL aliquots for incubation; 50 mL of TSB with 8% NaCl was inoculated rather than 40 mL. Growth and filamentation in TSB or TSB with 4% NaCl was repeated in triplicate with freshly adapted strains of *L. monocytogenes*. Due to time constraints and equipment limitations the growth and filamentation in broth containing 8% NaCl was not replicated.

Strains grown in TSB and TSB with 4% NaCl were sampled daily until they began to enter stationary phase (as determined by plate counts). Strains grown in 8% NaCl TSB were sampled every three days until log phase was established by enumeration on TSA. On days 0 to 3 for the TSB and 4% NaCl TSB trial and days 0 to15 for 8% NaCl TSB, 3 mL of sample was fixed in a 1% formaldehyde solution and refrigerated for later flow cytometry analysis. On days 4 to 8 for TSB and 4% NaCl TSB, and days 16 to 33 for 8% NaCl, 2 mL of sample was fixed, and 1 mL of each strain was fixed for the remaining days for all treatments. On sampling days, 100 µL of sample was diluted in 1% peptone water and enumerated on TSA and incubated at 37°C for 24 h.

Flow cytometry samples that were less than 10^6 CFU/mL were concentrated by centrifugation at 2700 x *g* for 5 min. All but 100 µL of

supernatant was removed, the remaining sample vortexed and diluted 1/10 with filter (0.22 μm) sterilized - phosphate-buffered saline (PBS) to a total volume of 0.5 mL. Samples containing more than 10^6 CFU/mL of *Listeria* were diluted to 10^6 using filter sterilized PBS to a total volume of 0.5 mL. Within 20 min prior to analysis on the Facscalibur flow cytometer (Becton, Dickenson and Company) 0.5 μL of Syto® BC dye was added.

Microscopy was performed at 100 x magnification using an Axiocam MRm connected to a Zeiss microscope (Carl Zeiss Inc., Oberkochen, Germany). Fixed cells (10 μL) were immobilized on a slide with a drop of 1% agarose solution and a coverslip. Images were taken and lengths measured using Axiocam software.

2.3.3 Analysis of Flow Cytometry Results

Analysis was performed using CellQuest Pro software (BD, NJ USA). Cells were gated based on their **fluorescent reading** and their **forward scatter**. "Cell shift", the proportion of cells in the sample population that shifted in length was designated as the proportion of treated cells that fall under the forward scatter reading of the 90^{th} percentile of the control samples (15°C cold adapted cells). Fold change was determined by dividing the mean signal of the 10% longest cells of the sample by the mean signal of the 10% longest cells in the control, and is the main determinant in determining the degree of filamentation in the sample (Jones, Gill, McMullen 2003). A sample calculation is shown at the end of the Appendix.

2.3.4 Statistical Analysis

Statistical analysis was performed to compare fold change among strains using the Wilcoxen test for non-parametric data.

2.4 Growth and Filamentation of Cold-Adapted Log Phase *L. monocytogenes* in Acidic or Alkaline conditions at Cold-Room Temperature

2.4.1 Pre-Treatment Growth

L. monocytogenes FS2, FS12 and CDC7762 were cold-adapted to 15°C in TSB prior to use in studies.

2.4.2 Treatments and Sampling

One microliter of each strain of log phase cold-adapted *L. monocytogenes* was inoculated into 10 mL of each treatment broth at 3°C. Treatments consisted of 50 ml of TSB acidified to a pH of either 5.5 or 6 with citric acid, or adjusted to pH 8.5 with NaOH. Samples were aliquoted after inoculation into 15 mL tubes for incubation. An additional 10 mL was inoculated per strain for use in microscopy. Experiments for samples adjusted to pH 6 were completed in triplicate while experiments at pH 5.5 were performed in duplicate.

Strains grown in TSB adjusted to pH 6 were sampled daily until day 13; strains in TSB adjusted to pH 5.5 were grown for 27 d and were sampled on day 2, 5, 9, 14, 19, 23 and 27, and strains grown in TSB adjusted to pH 8.5 were sampled on day 0 and every two days until day 18. During sampling a

portion of cells were fixed in 1% formaldehyde solution and refrigerated for flow cytometry. In addition, 100 µL was serial diluted in 0.1% peptone water and plated on TSA and incubated at 37°C for 24 h prior to enumeration..

For flow cytometry, cells were adjusted to 10^6 CFU/mL either through dilution with sterile 0.22 µm-filtered PBS or by concentrating the cells by centrifugation at 2700 x *g* for 5 min, removing all but 100 µL of the supernatant, vortexing, and diluting 1/10 with filtered sterilized PBS. The dilution step with a filtered solution ensured a sample with few debris. Half of a microliter of Syto® BC dye was added to samples within 20 min prior to analysis on the Facscalibur flow cytometer (BD, NJ, USA).

Microscopy was performed at 100 x magnification using an Axiocam MRm connected to a Zeiss microscope (Carl Zeiss Inc., Oberkochen, Germany). Samples (10 mL) were centrifuged at 2700 x *g* for 10 min and 9.5 mL of supernatant was removed prior to vortexing. Ten microliters of fixed cells in the remaining sample were immobilized on a slide with a drop of 1% agarose solution and a coverslip. Images were taken and lengths measured using Axiocam software and strain comparison was performed.

2.4.3 Analysis of Flow Cytometry Results

Samples were analysed as described above in 2.3.3.

2.5 Growth and Filamentation of *L. monocytogenes* in Both an Acidic and Saline Environment

2.5.1 Pre-Treatment Growth

L. monocytogenes FS2, FS12 and CDC7762 were cold-adapted to 15°C in TSB prior to inoculation.

2.5.2 Treatments and Sampling

One microliter of cold-adapted log phase *Listeria* was added to 10 mL of TSB with 4% NaCl that had been adjusted to pH 6 with citric acid prior to autoclaving. Testing with pH paper revealed the post-autoclave pH to be 6. In total, 40 µL was inoculated for each strain per trial, with an additional 10 mL inoculated for use in microscopy. This treatment was performed in triplicate with freshly cold-adapted strains.

The strains were sampled on day zero and every two days thereafter until the growth rate began to decrease. At each sampling 100 µL of each strain was serially diluted in 0.1% peptone water, plated on to TSA and incubated at 37°C for 24 h for enumeration. A portion of cells were fixed in 1% formaldehyde and refrigerated for use in flow cytometry. Three milliliters were fixed the first 4 sampling dates, 2 mL the following four, and 1 mL on the remaining sampling dates.

Flow cytometry samples were adjusted to 10^6 CFU/mL and prepared as previously described in 2.4.2, as was microscopy.

2.5.3 Analysis of Flow Cytometry Results

Samples were analysed as described above in 2.3.3.

3. Results

3.1 Growth of *L. monocytogenes* at Cold Temperatures

To assess the variability in growth at low temperatures among the 16 strains of *L. monocytogenes*, the increase in OD between 7 and 10 d of incubation was determined. In general, as temperature decreased so did the increase of OD between 7 and 10 days of incubation (Figure 3.1). All strains grew at all temperatures between day 7 and day 10 of incubation (Figure 3.1). At 3°C *L. monocytogenes* CDC7762, FS2 and FS10 had the largest increase in OD between 7 and 10 d of incubation, while FS5, FS6 and FS8 had the smallest change in OD (Figure 3.1). At 4.5°C *L. monocytogenes* FS8, FS13 and CDC7762 had the least change in OD and FS2, FS11 and FS12 had the greatest change (Figure 3.1). Comparatively, *L. monocytogenes* FS12 grew very well from 4 to 5°C and *L. monocytogenes* CDC7762 grew poorly from 3.5 to 4.5°C (Figure 3.1).

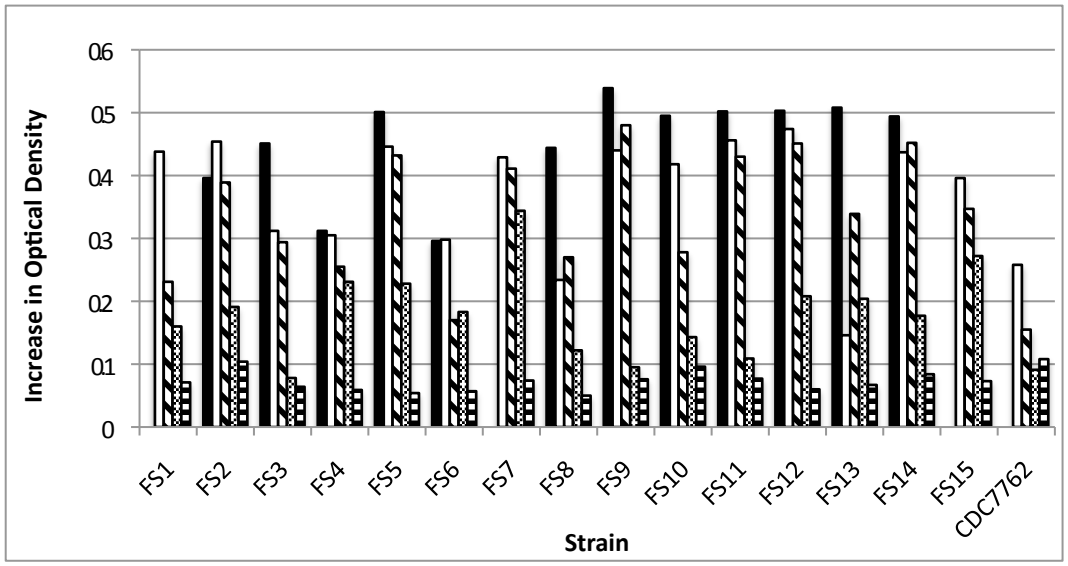

Figure 3.1. Change in optical density of 16 cultures of *L. monocytogenes* from 7 to 10 days of incubation at decreasing temperatures of 5 (black), 4.5 (white), 4 (diagonal line), 3.5 (hatched), and 3°C (horizontal line) **(n=1).**

When incubated at 4.5°C all strains increased their degree of filamentation from 7 to 10 d with the exception of *L. monocytogenes* FS8 (Figure 3.2). This was also observed for the majority of strains incubated at 4 and 5°C (Figures A.1 and A.2). The fold change of *L. monocytogenes* FS4 and FS14 increased while it decreased for all other strains from 10 to 11 d of incubation at 5°C (Figure A.1). At 4.5°C, the degree of filamentation for eleven of the 16 strains decreased from 10 to 11 d of incubation, and at 4°C, the degree of filamentation for nine of the 16 strains decreased between 10 and 12 d (Figures 3.2 and A.2).

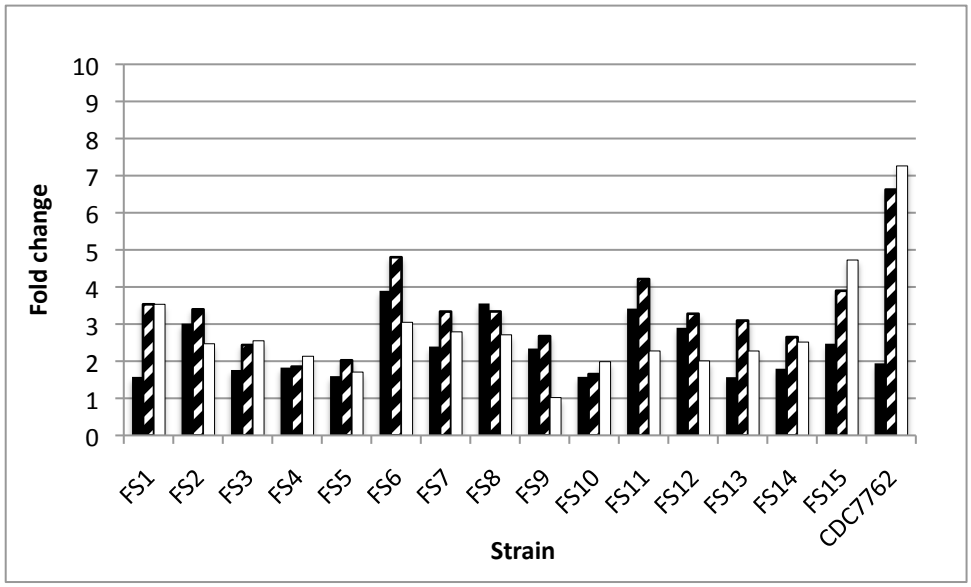

Figure 3.2. Fold change in the cell length of strains of *Listeria monocytogenes* at 7 (black), 10 (diagonal line) and 11 (white) days of incubation at 4.5°C **(n=1)**.

Figures 3.2 and 3.3 illustrate an inverse relationship between percentage of cells gated at the 90th percentile of the control and the fold change of the 10% longest cells of a sample. After 7 d of incubation at 4.5°C, the percentage of cells gated for *L. monocytogenes* FS1 (Figure 3.3) was high, while the fold change was low (Figure 3.2). This inverse relationship was confirmed by strains grown at other temperatures. All strains incubated at 4°C had a relatively low fold change, varying between one and three, while the percentage gated for all strains incubated at 4°C was relatively high, with the percentage falling under the forward scatter reading of the 90th percentile of the control representing 50 to 70% of the entire population (Figures A.2 and A.3).

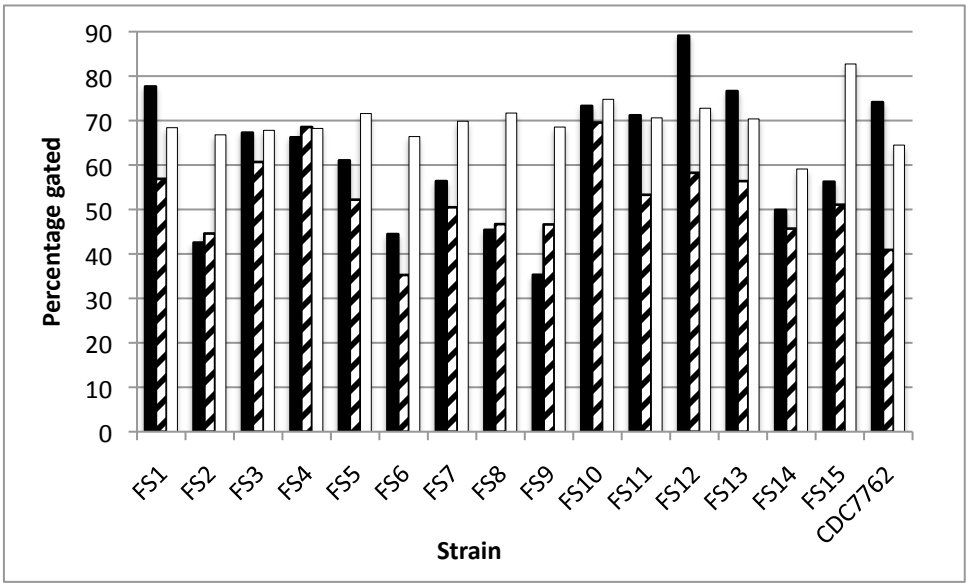

Figure 3.3. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples on 7 (black), 10 (diagonal line) and 11 (white) days of incubation at 4.5°C for multiple strains of *Listeria monocytogenes* **(n=1)**.

Microscopic images revealed very large filaments in the culture of *L. monocytogenes* CDC7762 grown at 3.5°C, while no filamentation was observed in images obtained for *L. monocytogenes* FS12 (Figure 3.4). Images of the other strains revealed a degree of filamentation between that observed for *L. monocytogenes* CDC7762 and that observed for *L. monocytogenes* FS12. Representative images of all strains grown at 3°C for 7 days are found in the appendix (Figures A.5 to A.7).

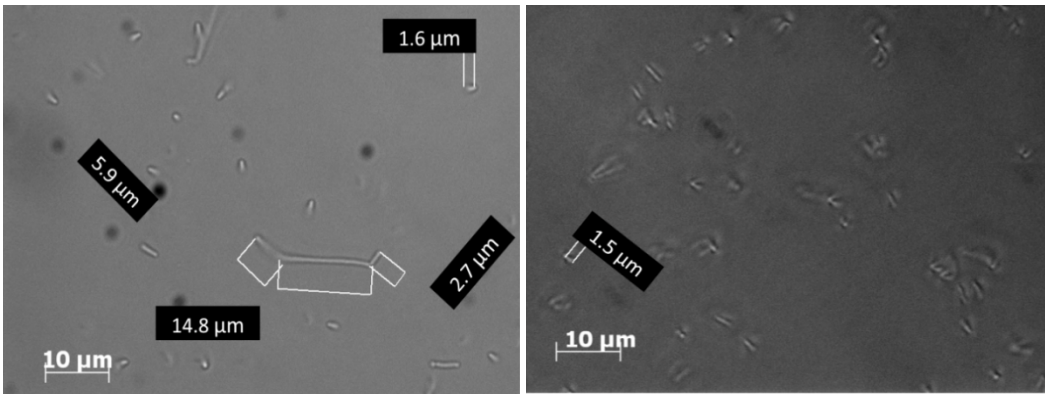

Figure 3.4. Microscopic images of cells of *L. monocytogenes* CDC7762 (left) and FS12 (right) after 10 d of growth in TSB at 3.5°C. Figures in boxes are the length of the cells.

3.2 Growth of *L. monocytogenes* in TSB with and without added NaCl at Cold-Room Temperatures

Increasing the salt concentration of the media increased the fold change in cell length. Figure 3.5 shows the growth (log CFU/mL) and fold change for *L. monocytogenes* FS2, FS12 and CDC7762 grown in TSB broth at 3°C with no additional NaCl added to the broth. There were no differences among the strains in the cell numbers and the fold change in cell length during incubation. In contrast, when strains were grown in TSB with 4% NaCl, the fold change in the cells increased over the first 4 days of incubation and then decreased over the remaining 16 d of incubation at 3°C (Figure 3.6). There were no significant differences ($p>0.05$) in fold change among the strains throughout storage in TSB broth with 4% NaCl. During storage, the fold change for *L. monocytogenes* CDC772 returned close to the original values but the fold change for *L. monocytogenes* FS2 and FS12 remained at 5

times what was observed when the cultures were grown in TSB with no added NaCl and what was observed at the beginning of storage in TSB with 4% NaCl.

When cultures were grown in TBS with 8% NaCl, the cell numbers increased to just above log 7 CFU/mL over storage at 3°C (Figure 3.7). The fold change in the 10% longest cells in the culture was 10 to 20 times larger at their peak compared to the fold change observed at the beginning of storage at 3°C. The fold change for *L. monocytogenes* FS12 was much higher than that observed for the other two cultures. Fold change values for the 10% longest cells decreased after the initial peak as the log phase continued but they did not return to original values.

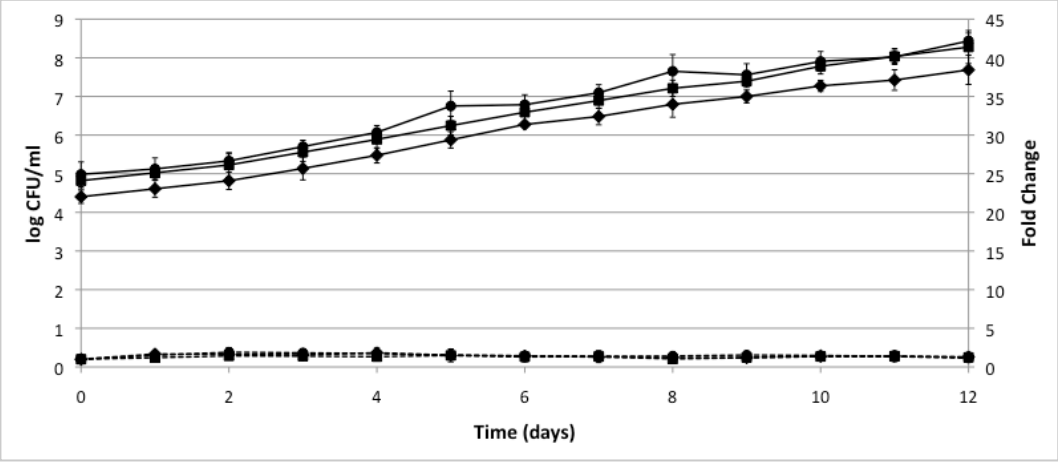

Figure 3.5. Log (CFU/ml) (solid lines) and fold change (dashed lines) of *Listeria monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) grown at 3°C in TSB (0.5% NaCl). Error bars are standard deviations **(n=3)**.

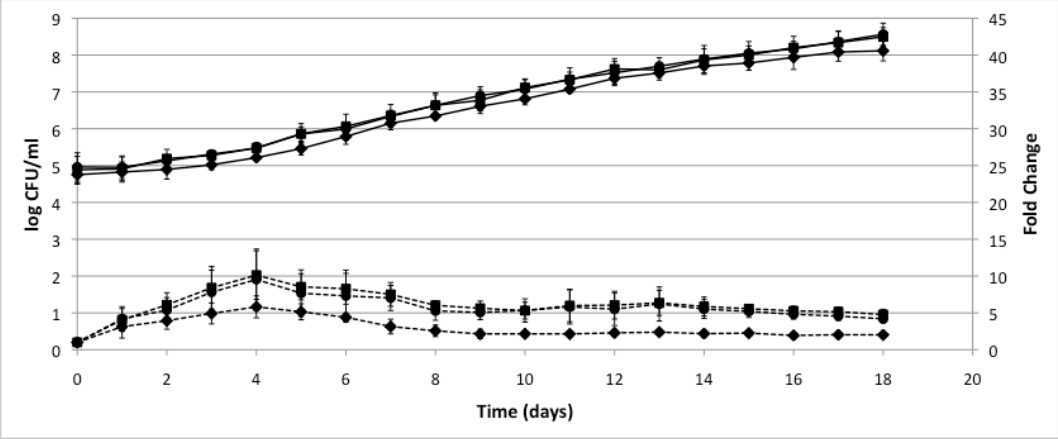

Figure 3.6. Log(CFU/mL) (solid lines) and fold change (dashed lines) of *Listeria monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) at 3°C in 4% NaCl TSB. Error bars are standard deviations **(n=3)**.

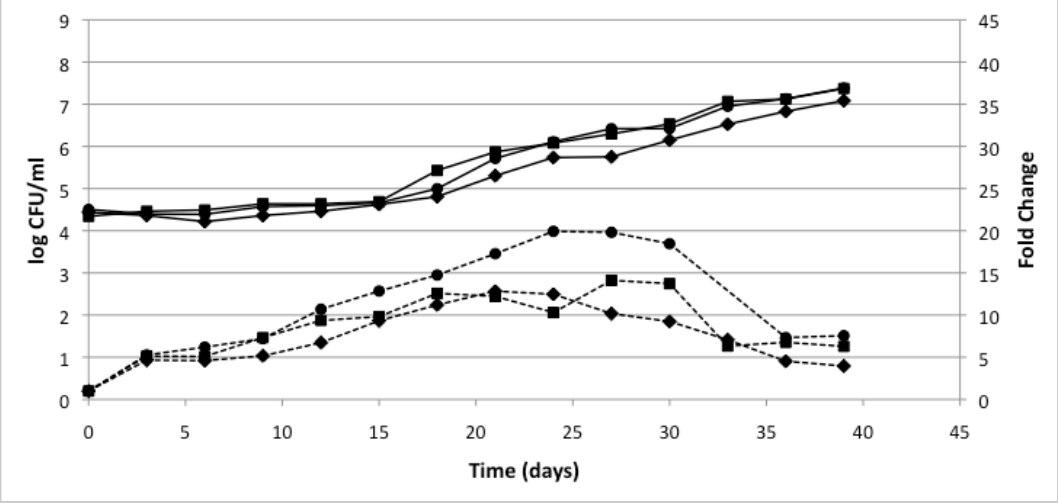

Figure 3.7. Log(CFU/mL) (solid lines) and fold change (dashed lines) of *Listeria monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) at 3°C in 8% NaCl TSB. **(n=1)**.

Figure 3.8 shows images of *L. monocytogenes* FS2 grown at 3°C in TSB and in TSB with 4 or 8% NaCl. The longest cells were observed when cultures were grown in TSB with 8% NaCl.

Percentage of cells gated in analysis of data collected by flow cytometry compared to 90% of the control sample (Figure 3.9) maintained an inverse relationship with fold change. A high fold change corresponded to a small percentage of cells shifted, meaning the population remained a similar size compared to the control, also represented by a "percentage gated" close to 90 (the 90^{th} percentile of control). The degree and pattern of cells gated inversely matches the results for the fold change for all strains, independent of the broth used.

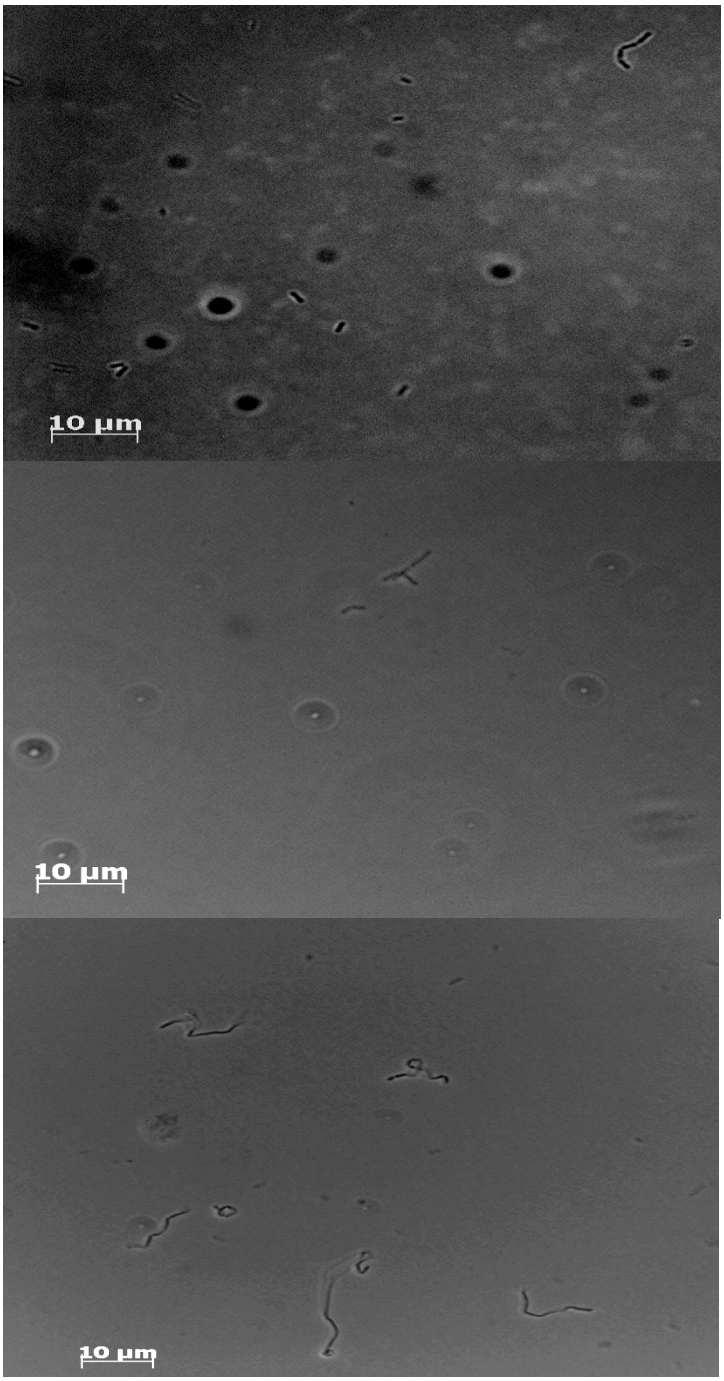

Figure 3.8. Microscopic images of *L. monocytogenes* FS2 at 3°C grown for two days in TSB (top), grown for 4 days in TSB with 4% NaCl (middle) and grown for 27 days in TSB with 8% NaCl (bottom).

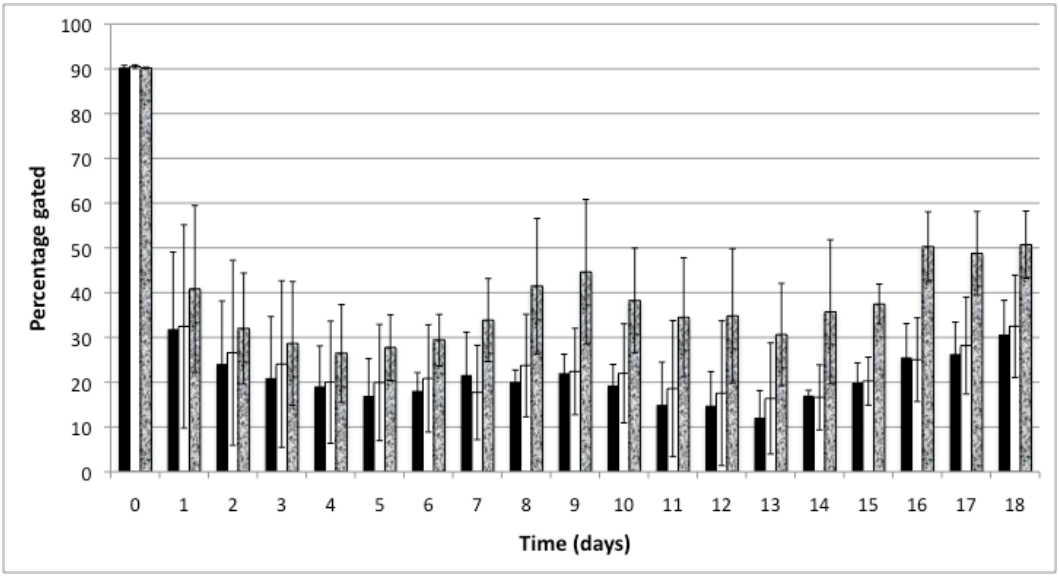

Figure 3.9. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples for FS2 (black), FS12 (white) and CDC7762 (grey patterned) at 3°C in 4% NaCl TSB **(n=3)**.

3.3 Growth of *L. monocytogenes* in Acidic or Basic pH Conditions at Cold-Room Temperature

To determine the impact of acidic or basic pH on the filamentation of *L. monocytogenes*, cells were grown in TSB adjusted to different pH values. Cells increased from 5 to 7 log(CFU/mL) when they were grown in TSB adjusted to pH 6 with citric acid (Figure 3.10). During that time, the fold change appeared to increase from a starting point of close to 0 to between 2 and 3. However, there was large variability in the fold change values. The fold change in cells of *L. monocytogenes* FS12 and CDC7762 was much greater when cells were grown in TSB adjusted to pH 5.5 (Figure 3.11). In contrast, when cells were grown in TSB adjusted to pH 8.5, the number of cells

increased but the fold change remained low and relatively constant throughout the experiment although two small peaks with questionable significance were evident (Figure 3.12).

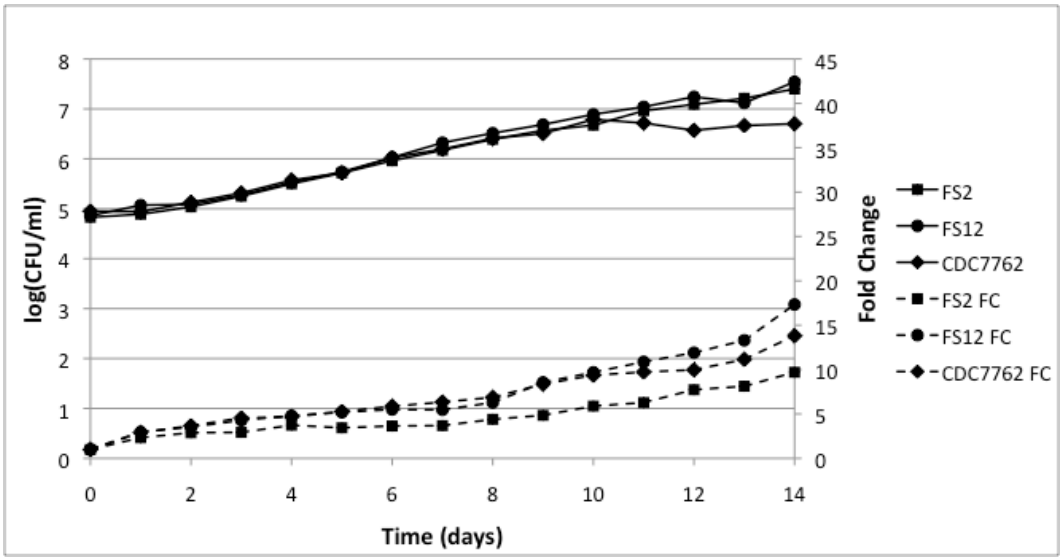

Figure 3.10. Log(CFU/mL) (solid lines) and fold change (dashed lines) of *L. monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) grown at 3°C in TSB adjusted to pH 6 with citric acid (**n=2**).

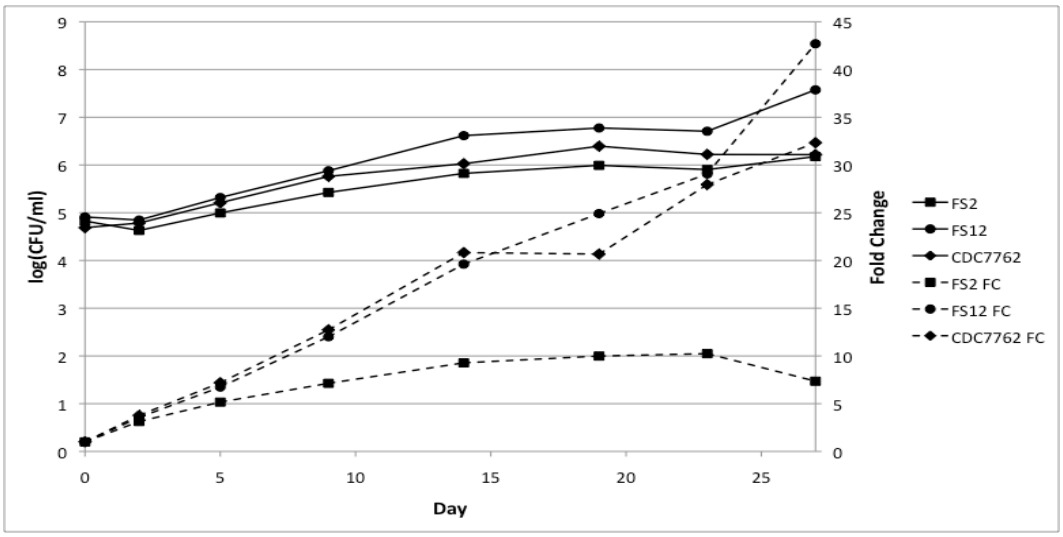

Figure 3.11. Log(CFU/mL) (solid lines) and fold change (dashed lines) of *L. monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) grown at 3°C in TSB adjusted to pH 5.5 with citric acid (**n=2**).

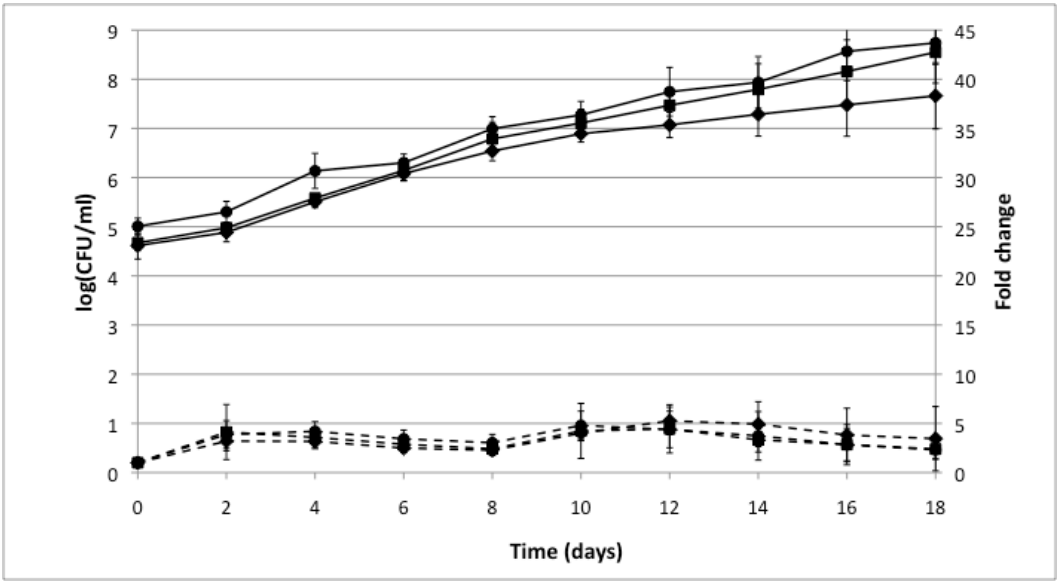

Figure 3.12. Log(CFU/mL) (solid lines) and fold change (dashed lines) of *L. monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) grown at 3°C in TSB adjusted to pH 8.5 (**n=3**).

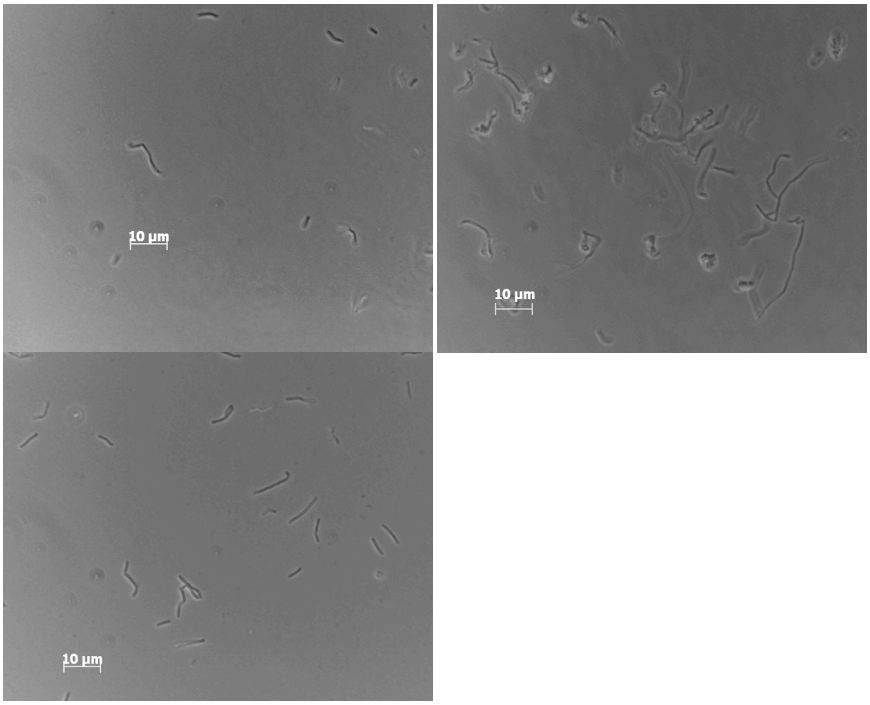

Figure 3.13. Microscopic images of *L. monocytogenes* FS2 (top left), FS12 (top right) and CDC7762 (bottom left) grown for 27 days at 3°C in TSB adjusted to pH 5.5 with citric acid.

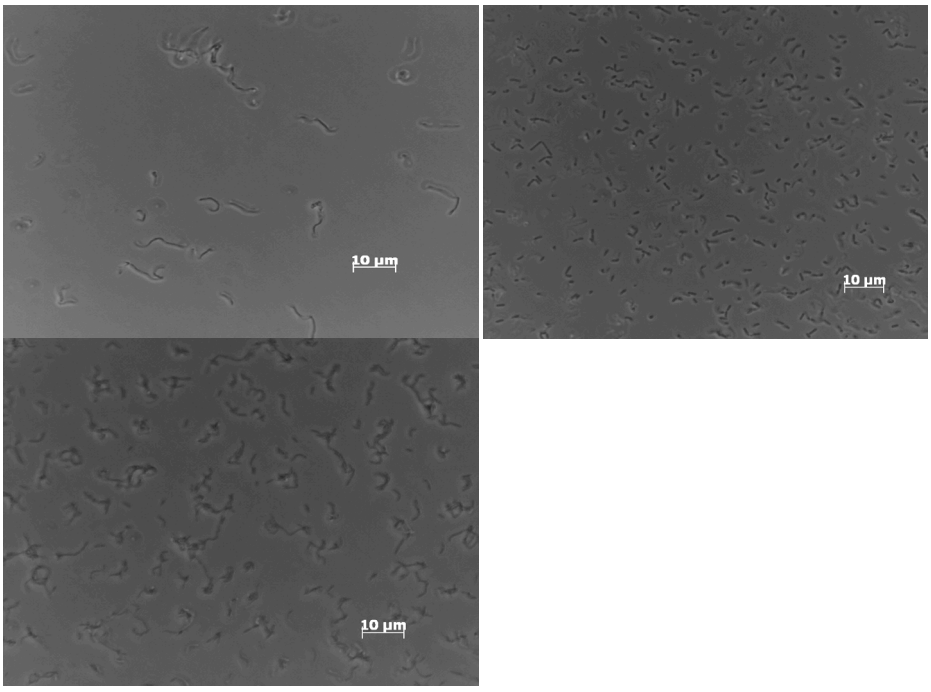

Figure 3.14. Microscopic images of *L. monocytogenes* FS2 (top left), FS12 (top right) and CDC7762 (bottom left) grown for 18 days at 3°C in TSB adjusted to pH 8.5.

Micrographs of cells grown in TSB adjusted to pH 5.5 and pH 8.5 are shown in Figure 3.13 and 3.14, respectively. Cells grown in TSB adjusted to pH 5.5 lacked regular morphology, all cells in the images were filamentous. At a pH of 8.5, all cells were filamentous but filaments did not appear to be as large as those visualized from cultures grown in TSB adjusted to pH 5.5.

The proportion of cells that fell under the forward scatter reading for the 90th percentile of the control in acidic conditions (Figures 3.15 and 3.16) had an inverse relationship with the observed fold change (Figures 3.10 and 3.11), where a higher fold change corresponded to a lower percentage gated, as low as 0.86% for *L. monocytogenes* FS12 after 23 days of growth in TSB

adjusted to pH 5.5. In contrast, when *L. monocytogenes* was grown at 3°C in TSB adjusted to pH 8.5, the percentage of cells gated compared to the control (Figure 3.17) remained relatively stable from day 2 to day 8 of incubation.

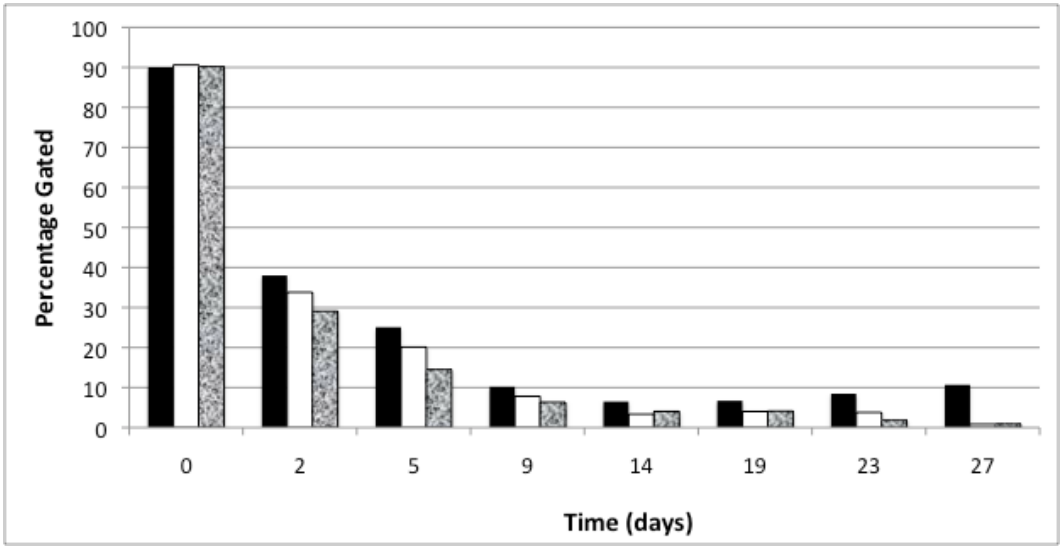

Figure 3.15. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples for *L. monocytogenes* FS2 (black), FS12 (white) and CDC7762 (grey patterned) grown at 3°C in TSB adjusted to pH 5.5 (**n=2**).

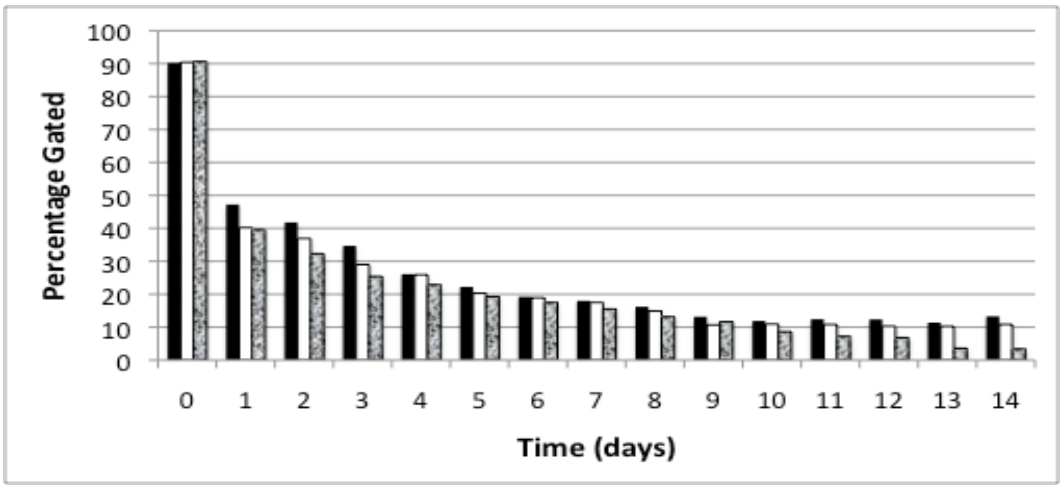

Figure 3.16. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples for *L. monocytogenes* FS2 (black), FS12 (white) and CDC7762 (grey patterned) grown at 3°C in TSB adjusted to pH 6 (**n=2**).

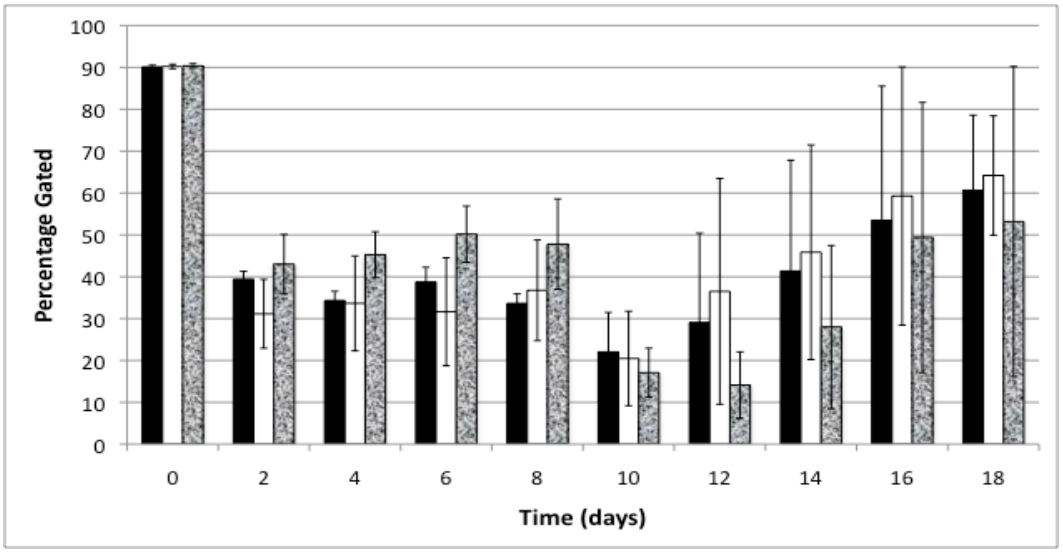

Figure 3.17. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples for *L. monocytogenes* FS2 (black), FS12 (white) and CDC7762 (grey patterned) grown at 3°C in TSB adjusted to pH 8.5 (**n=3**).

3.4 Impact of the Combination of Saline and pH on *L. monocytogenes* Grown at 3°C

To determine the impact of a combination of saline and lower pH conditions on the growth of *L. monocytogenes*, strains were grown at 3°C in TSB with 4% NaCl and adjusted to pH 6. All three strains reached a maximum population between log (CFU/mL) 7 and log (CFU/mL) 8 during storage (Figure 3.18). Cells reached a maximum in fold change by 4 days of storage which was maintained throughout storage. Although the fold change was maintained the percentage of the population gated compared to the 90th percentile of the control decreased during storage (Figure 3.19).

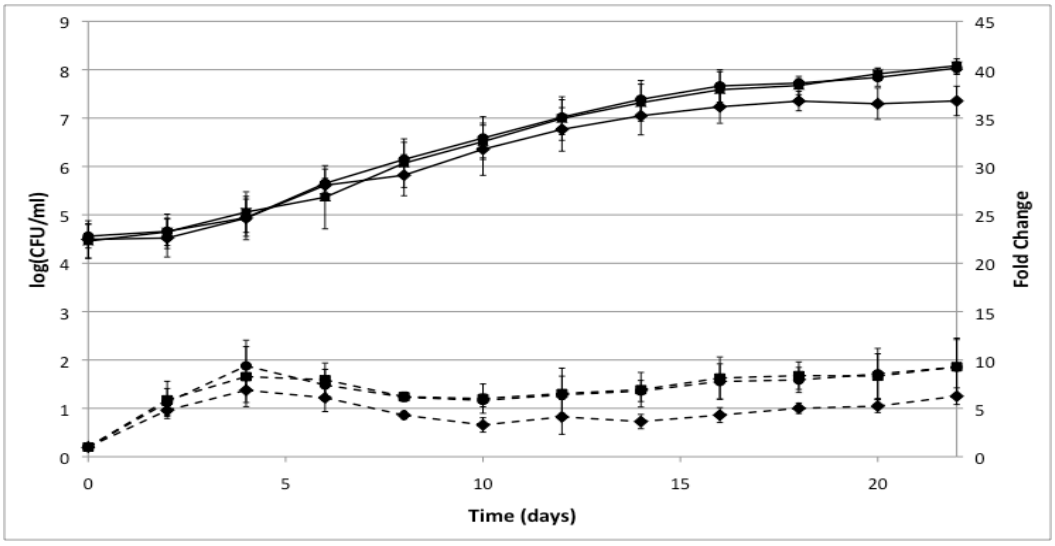

Figure 3.18. Log(CFU/mL) (solid lines) and fold change (dashed lines) of *L. monocytogenes* FS2 (■), FS12 (•) and CDC7762 (◆) grown at 3°C in TSB with 4% NaCl and adjusted to pH 6 with citric acid (**n=3**).

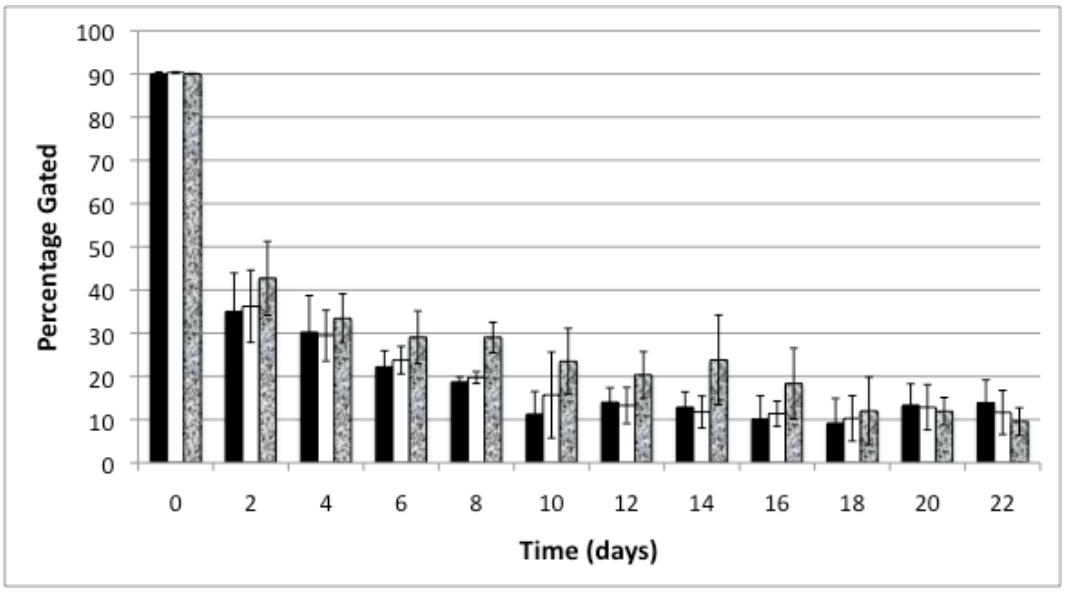

Figure 3.19. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples for FS2 (black), FS12 (white) and CDC7762 (grey patterned) at 3°C in 4% NaCl TSB adjusted to pH 6 with citric acid (**n=3**).

4. Discussion

As environmental conditions become less optimal, growth of bacterial cells is hindered. Refrigeration temperatures can cause stress that can lead to the decreased growth of bacteria (White 2000). Cells of *E. coli* may enter a reduced-energy (and therefore reduced growth) state to survive until more optimal conditions are achieved (Jones, Johns, Gill 2008). At low temperatures several mechanisms are involved, and the efficacy and variability of these mechanisms between strains contributes to the growth variability among different strains of *L. monocytogenes.*

This research is one of the first studies that examined filamentation in *Listeria monocytogenes* at refrigeration temperatures; however, several studies concerning filamentation of *E. coli* at cold temperatures exist. Gill et al. (2007) found that filamentation was apparent in various strains of *E. coli* grown at 6, 8 and 10°C, which is near the minimum temperature for growth, (Gill, Badoni, Jones 2007). This is similar to the current results; however, unlike the current results, no set pattern of filamentation emerged among temperatures. In cells of *E. coli* grown at temperatures close to the minimum for growth, some strains had a greater tendency to form filaments than others (Gill, Badoni, Jones 2007). Insight into the growth phase of the individual strains may aid in explaining these results.

A number of different factors may be involved in filamentation of cells at temperatures close to the minimum for growth. Csp's are small highly

conserved proteins that regulate many processes in the bacterial cell by binding to nucleic acids (Ermolenko and Makhatadze 2002). These proteins are induced by cold temperatures and their primary protective function is to chaperone RNA (Csermely and Vign 2007; Hunger and others 2006). Fatty acid composition of the membrane is important to cell survival at low temperatures. *L. monocytogenes* has been shown to alter its fatty acid composition at lower temperatures, with chain lengths becoming shorter and branching switching from iso to anteiso as the temperature lowers (Annous and others 1997). This altered composition allows the membrane to remain in the liquid-crystalline state allowing for proper transfer of nutrients and enzymes through the cell. Solute balance within the cell is equally important as it prevents excess turgor of the cell wall. *L. monocytogenes* transports compatible solutes effectively at low temperatures, which act as osmoprotectants and maintain an isotonic environment (Yancey and others 1982). Glycine betaine porter I (BetL) and II (GBu) as well as OpuC the carnitine transporter have been found responsible (Angelidis and Smith 2003).

It may be of interest to the food industry to determine the levels of the above components that contribute to cryoprotection in the various *L. monocytogenes* strains so that correlations between growth and strain, and the cause of filamentation, can be more fully understood.

In the current study, all strains grew below the common refrigeration temperature of 4°C. For the food industry this means that since growth of

cold adapted *Listeria* is not inhibited it is important to maintain a strict cleaning regimen and/or employ the use of other hurdles to bacterial growth (such as high salt content, pH control, etc.) in order to maintain the safety of RTE meats.

All of the strains used in this research were derived from food products. Knowledge of the extent of growth at varying temperatures allows for a better cost-benefit analysis. Refrigeration in a food processing plant is a major cost to the company; the lowest growth temperature observed in this study was 3°C. The shelf life of product and total costs including those for refrigeration should be considered against the degree of growth of *Listeria* strains within a food processing plant. Future work would ideally replicate these experiments, making the results more reliable to future modeling, a factor also important when determining the degree of filamentation at different temperatures.

Various studies have suggested that filamentation is induced as a response to osmotic, atmospheric, acidic or temperature stress (Bereksi and others 2002; Jydegaard-Axelsen and others 2005). These studies correlate with the results of the current research that illustrated an increased average cell length (higher fold change) compared to the controls (cold-adapted cells at 15°C). Furthermore, a trend exists among most of the strains, where cell length increases over time at 3.5°C. It is possible that longer exposure to the stress of low temperature possibly leads to increased filamentation. This is supported by Jones *et al.* (2003) who found that cells of *E. coli* grown at 7°C

increased in cell length over a period of 8 days while the length of cells grown at the 10°C remained constant over the same time period (Jones, Gill, McMullen 2003).

At higher temperatures of 4 to 5°C the opposite phenomenon occurs, with filamentation having no relation to temperature. This is potentially due to the fact that due to the increased growth rate at these temperatures, samples grown at higher temperatures entered into the stationary phase of growth between 7 and 11 days while cells grown at 3°C remained in the logarithmic phase of growth. While in stationary phase cells have an increased ability to adapt to their environment, which may lead to a decrease in filamentation (White 2000).

From the flow cytometry data, the percentage gated is an indication of the overall cell size in a sample compared to the cell size of a control, where the reference value is gated at 90%. Values less than 90% indicate an increase in overall cell size in a sample, as cells within the population shift to a longer length. Cell length of the 10% longest cells in a sample, denoted as fold change, increased slightly at lower temperatures for most strains (Figures 3.2, A.1 and A.2), and the percentage gated decreased thereby supporting the fold change data (Figures 3.3, A.3 and A.4).

It is important to note that gating for flow cytometry events for the temperature-dependent treatment with 16 strains was based purely on the size of the event (forward scatter) and granularity (side scatter). The gating

method is independent of nucleic acid content, and similarly sized debris could be included in the analysis.

At lower temperatures more stress is placed on the cell; therefore, along with a decrease in growth compared to higher temperatures there is also an increase in cell death when compared to cells growing for the same length of time at higher temperatures. It is therefore likely that at lower temperatures flow cytometry samples contain an increased proportion of debris caused by dead cells, potentially skewing results. Current results are inconsistent with results obtained with *E. coli* at decreasing temperatures, where mean length at 7°C was significantly larger over time than mean length at 8 or 9°C (Jones, Gill, McMullen 2003). At 4.5 and 5°C, fold change data representing cell length of *L. monocytogenes* was larger than at for cultures grown at 4 and 3.5°C. This could potentially be due to increased debris in the cell cultures (Figures 3.2, A.1, A.2, A.8). In subsequent experiments an alternate flow cytometry method for gating cells for analysis was used due to the increased accuracy allowed by nucleic acid binding Syto® BC dye.

Despite the potentially inaccurate gating method used for this portion of flow cytometry, there are clear distinctions between strains vis a vis their degree of filamentation at low temperatures. Flow cytometry results for *L. monocytogenes* CDC7762 at 4 and 4.5°C showed significantly larger cells than other strains at 4.5°C (Figure 3.2) and comparable length at 4°C. Furthermore, representative microscopic images of this strain grown at 3

and 3.5°C revealed extremely large filaments compared to that observed for the other strains (Figures 3.4 and A.7). This potentially indicates that this strain, the strain isolated from the Bil Mar *L. monocytogenes* outbreak in 1998, has the greatest ability to filament under stress compared to other strains tested. *L. monocytogenes* FS12 has radically different characteristics, with images at 3.5°C revealing no filamentation whatsoever (Figure 3.4). This could be due to any one of the strain variation factors listed earlier in this discussion. Cell length for this strain remained small compared with other strains at higher temperatures, but again this may be due to inaccurate analysis.

If a proper method is utilized, flow cytometry leads to faster and more accurate analyses. Flow cytometry is a method of measuring cell length by means of light diffracted off individual cells. Lengths are compared quantitatively to controls by comparing the mean forward-scatter fluorescent signal. Utilizing a fluorescent dye such as Syto® BC green allows for increased specificity of the analysis, binding specifically to nucleic acid content and in this way excluding non-bacterial particles from interpretation by gating events based on fluorescence. By setting a minimum fluorescent signal required, broken cells and free-floating DNA are excluded. This more specific gating method was utilized to measure fold change in experiments to determine the impact of saline, acidic and alkaline conditions on filamentation in *L. monocytogenes*.

The primary advantages of flow cytometry is the potential for incredibly specific and reliable measurements, as well as the speed of measurement; 10,000 events can be measured in less than 15 seconds, a feat that would take hours or days through microscopic analysis.

Flow cytometry has been used to determine the degree of filamentation in *E. coli* and *Salmonella*, but has not been used previously to examine filamentation in *L. monocytogenes* (Gill, Badoni, Jones 2007; Jones, Gill, McMullen 2004; Kieboom and others 2006). Previous studies involving the filamentation of *Listeria* in saline environments measure filamentation by the rough percentage of cells longer than normal, or in other equally qualitative terms (Bereksi and others 2002; Hazeleger, Dalvoorde, Beumer 2006). Quantitative measurement of filamentation in conditions representative of meat products or processing plant conditions is important to correct for potential underestimation of plate counts. Furthermore, it allows for comparison among bacterial strains and environmental conditions.

A limitation of most studies concerning filamentation is that the growth phase of the bacteria in question is not considered or related to the degree of filamentation. Furthermore, regular sampling of the degree of filamentation during growth is lacking (Bereksi and others 2002; Hazeleger, Dalvoorde, Beumer 2006). In saline, neutral-pH conditions at 3°C filamentation of *L. monocytogenes* peaked at the beginning of log phase for the three strains evaluated in this research. This indicates a potential role of

the growth phase in filamentation, therefore an explanation of the growth phases of bacteria would be beneficial for future considerations.

During lag phase numerous adaptations are occurring, from recovery from toxic products cells may have accumulated prior to subculture, to the synthesis of new enzymes, to adaptation required for the new environment (White 2000). In new and more stressful environments bacteria such as *Listeria* require a degree of adaptation to grow in sub-optimal conditions. Previous studies show that filamentation increases with an increase in environmental stress (Bereksi and others 2002; Jones, Gill, McMullen 2003), suggesting a possible cause for the increase of filamentation in the log phase. Results of this study showed little increase in filamentation in the first few days of growth; however, during this lag phase, there is no net growth, which limits the degree to which the cells can divide and therefore the degree of filamentation. Cell division increases substantially in the exponential phase of growth, immediately following the lag period. It is at this interphase between lag and log phase of growth where the combination of increased cell adaptation occurring coupled with exponential growth could cause the creation of large filaments.

The combination of adaptations within the cell and positive net growth may be the cause of the initial peak in filamentation observed for *L. monocytogenes* grown in TSB with 4% and 8% NaCl. In addition, the acclimatization of cells to their environment during the exponential growth phase could account for the subsequent decrease in filamentation over time.

This decrease is not present in acidic conditions, suggesting that a different mechanism may be at work. Few other studies link filamentation with the early growth phase of bacteria, a limitation in an industry where processing environments are thoroughly cleaned on a regular basis.

Results of *Listeria* growth at 4 and 8% NaCl concur with filamentation observed in *Listeria* grown at 22°C with varying salt concentrations; as the concentration of salt increased, so does filamentation (Bereksi and others 2002). Unfortunately, the effect of the change in temperature cannot be compared due to the qualitative nature of the results of the study ay 22°C by Bereksi *et al.* (2002). Flow cytometry is only recently being used in filamentation studies, a limitation when comparisons are being made to the more common, qualitative studies.

At the barrier between lag and log phase, cells are completing the initial adaptations required for growth in a new medium and exponential growth is beginning. Innumerable studies involving bacteria and filamentation use OD measurements as a tool to track the growth of bacteria (Bereksi and others 2002; Isom and others 1995; Jones, Gill, McMullen 2002; Jones, Gill, McMullen 2004). However, for *Listeria* at 3°C, this method was found to be unacceptable during the preliminary screening of strains due to the high limit of detection (>10^7CFU/mL compared with plate counts) as the cells, regularly 2 μm, decreased in size under additional stresses. Plate counts were therefore the primary data used for growth analysis in this study, with only OD used in preliminary experiments. Despite other work suggesting that

filamentation can cause underestimation of the counts, our results, while potentially inaccurate in number, served the purpose of linking the general growth phase to the degree of filamentation (Gill, Badoni, Jones 2007).

When a cell adapts to new environments through processes such as gene upregulation and enzyme production, energy within the cell such as ATP and GTP is required to fuel them (Cashel and others 1996). The peak in filamentation observed in the current research could be due to the degree of stress, which may have caused the cell to enter a reduced energy state as suggested by Jones and others (2008) concerning filamentation of *E. coli* at low temperatures. Another possibility is that energy and resources normally spent on cell division are being re-appropriated towards cold temperature- and osmotic adaptations such as upregulation of solute transporters, cold-shock proteins and membrane adaptations (Bayles and Wilkinson 2000; Schmid and others 2009; Wememkamp-Kamphuis and others 2004). When cells are grown in TSB with 8% NaCl more solutes are being actively taken into the cell than when grown in 4% NaCl or at 3°C, suggesting a higher use of resources for active transport of solutes, gene upregulation and protein production (Ko, Smith, Smith 1994).

Many other studies concerning *Listeria* and other bacteria note that with increased stress including salt, water activity and temperature comes increased filamentation (Bereksi and others 2002; Jones, Johns, Gill 2008; Kieboom and others 2006). The current data substantiate this claim.

In acidic environments, adaptation must occur to prevent and reduce the damage of undissociated acid entering the cell, dissociating and lowering the pH within the cytoplasm (Jeong and others 2008; Lindahl and Nyberg 1972; Smith 2003). An acidic environment within the cell can lead to enzyme denaturation, DNA depurination and disruption of membrane potential (Jeong and others 2008; Lindahl and Nyberg 1972; Smith 2003). For Gram positive organisms such as *Listeria*, adaptations in response to increased acidity include alteration of the membrane fatty acid content designed to limit entry of acid into the cytoplasm by creating a more hydrophobic environment and decreasing the physical space between acyl chains (Chang and Cronan Jr. 1999; Mastronicolis and others 2010). Furthermore, in *Listeria*, *E. coli* and *S. enterica* DNA-binding protein (Dps) binds to DNA, preventing acid from damaging, or "nicking", the strands (Calhoun and others 2010; Jeong and others 2008). These different adaptation mechanisms may account for the different trend in filamentation observed during the growth of *Listeria* in acidic versus growth in saline environments.

Increased fold change occurred in acidic pH ranges, for all strains. There was significantly less filamentation in *L. monocytogenes* FS12 and CDC7762 at pH 6 (Figure 3.10) than at pH 5.5 (Figure 3.11), while growth of the strains remained similar. Fold change in TSB at a pH of 5.5 and 6 was higher than the fold change in regular TSB for all strains (Figures 3.5, 3.10, 3.11). At pH 5.5 *L. monocytogenes* FS2 and FS12 achieved a higher fold change than in saline environments.

The percentage of cells gated compared to the 90th percentile of the control decreased dramatically in the acidic conditions used in this study. Less than 1% of the entire population of cells of *L. monocytogenes* FS12 and CDC7762 were gated compared to 90% for the control by day 27 when cells were grown in TSB adjusted to pH 5.5, and under 5% for all three strains at pH 6 (Figures 3.15, 3.16). This is significantly more than the 40 to 60% shift observed when cells were grown in TSB with 4% NaCl (Figure 3.9). Therefore, in addition to acidic conditions causing larger filaments in the 10% largest cells of the population, a larger proportion of the population shifted to a larger size in the acidic samples as compared to samples grown in saline environments.

Formation of filaments in acidic conditions was discovered in 1939 in *Klebsiella pneumoniae* and several infectious but non-foodborne strains such as *Salmonella paratyphi* and *Shigella* spp. (Wahlin and Almaden 1939). Results of the impact of pH on filamentation in the current study conform to results from a study by Isom et al. (1995) that examined *Listeria* at 37°C at a pH of 5-6. Unfortunately, few studies involve a quantitative filamentation data on *Listeria* at single, or multiple acidic growth environments. In a study involving various Italian cheeses acidified to a pH of 3.5, causing 30% of the culture to die after 2 hours of exposure, bacteria isolated from crescenza cheese consisted primarily of filaments, while filaments were not found on any of the other cheeses (Cataldo and others 2007). The specific food matrix

and other factors such as salt concentration, which the study did not specify, may have been factors in the degree filamentation that occurred.

Filamentation of *Lactobacillus helveticus*, a bacterium used to ferment whey, occurred in supplemented whey permeate medium with a pH of 5.1-6.3 that was acidified with HCl (Norton, Lacroix, Veillemard 1993). The lengths of cells were between 4-100 μm, irrespective of a pH range of 4.4-6.3 (Norton, Lacroix, Veillemard 1993). This differs from the results of this study, which showed an increase in fold change (cell length) over time and in increasingly acidified conditions. A limitation to the study on *L. helveticus* may be that researchers failed to examine or report the proportion of cells that filamented within the population, and the change in that proportion in different acidified environments.

A study that has similar results to the current research involves a commercial culture of *L. alimentarius* BJ33 that formed filaments in the presence of gluconic or citric acid at sublethal doses of 100-110 mM and 50-55 mM, respectively and when both acids were used in combination (Lemay and others 2000). As with *Listeria* in our study, filamentation increased with increased acid concentrations (Lemay and others 2000).

With the exception of *L. monocytogenes* FS2, filamentation increased dramatically over time in acidic environments, compared to a peak in the early growth phase of *Listeria* grown in saline environments (Figures 3.6, 3.7, 3.9, 3.10). Filamentation of FS2 was extremely low. A potential explanation could be the slower growth rate observed for this strain. Despite this

anomaly, the pattern of increased stress causing increased filamentation is well established among results of other treatments and within the acid treatment itself. Furthermore, the cell population data (Figure 3.15) confirm the results as, despite the low filament size in the 10% longest cells, close to 80% of the population of *L. monocytogenes* FS2 shifted to a larger size when grown in TSB adjusted to pH 5.5 as compared to the control (Figure 3.15).

Once equilibrium is achieved little additional adaptation would occur. In the case of acid adaptation, Dps would continually be released to protect DNA, especially as DNA is duplicated during cell division. These adaptations are perhaps more lengthy and occur over a longer period of time, which may account for the continual increase in filamentation.

Citric acid, $H_3C_6H_5O_7$, has 3 different pKa values, with the highest (for the release of the third proton), being 7 at 25°C, a value that decreases slightly with temperature (due to citric acid being a weak acid) (Petrucci, Harwood, Herring 2002). Therefore, at the pH values of 5.5 and 6 used in this study, less than half of the citric acid was in its completely undissociated form. As primarily only undissociated acid can cross the hydrophobic lipid membrane, this provides a benchmark as to the strength of the acid. For a relatively weak acid, significant filamentation occurred. As the pH decreased and approached the pKa of the acid, an increased proportion of acid was able to penetrate the bacterial membrane and acidify the interior of the cell, and filamentation increased. Isom et al. (1995) reported that at a pH of 5-6 *Listeria* filamentated at 37°C in citric acid, but not in hydrochloric, acetic or

lactic acid which all have lower pKa values at 25°C (Isom and others 1995; Petrucci, Harwood, Herring 2002). Therefore at a pH of 5-6 citric acid would have the highest proportion of undissociated ions compared to the other acids. The pKa value of the acid used should be taken into consideration in food products where *Listeria* is a risk, especially if the degree of filamentation is to be considered. Future studies into the effects of different acids on filamentation of *Listeria* could provide further insight.

Multiple nucleoids were found in *L. monocytogenes* cells exposed to pH 9 at 30°C, suggesting that the mechanism of filamentation for this type of stress occurs in a later stage of binary fission, but very little is currently known about the effects of alkaline pH on the bacterial cell (Giotis, Blair, McDowell 2007). Winslow and Shaughnessy (1923) reported that when bacterial cells are placed in an alkaline medium, they initially secrete acidic compounds and create a buffer of a lower pH surrounding the cell; over time, this buffer capability is exhausted (Winslow and Shaughnessy 1924). Cells of *L. monocytogenes* that underwent alkali-shock had growth characteristics similar to that of the control. However, alkali-adapted cells had a substantially increased lag phase, perhaps due to the upregulation of the acidic compounds released upon exposure to alkaline environments (Taormina and Beuchat 2001). The lack of an extended lag phase when cells were grown in TSB adjusted to an alkaline pH in this study suggests that the buffering capacity of TSB was quickly exceeded (Figure 3.12); future trials involving a broth with a larger buffering capacity would be beneficial as an

extended lag phase benefits industry by possibly extending the shelf-life and safety of a given product. If cleaning and sanitizing are insufficient to completely eliminate *Listeria* within a food plant, cells that have survived are likely alkali-shocked due to the high pH of many commercial cleaners and sanitizers. If cleaners and sanitizers are not properly rinsed off, cells may become alkali-adapted.

Exposure to alkaline environments has also been found to induce cross-protection to heat treatments in *L. monocytogenes* (Taormina 2001), which may affect heat treatment of products exposed to alkali-adapted strains of *Listeria*. Future research into the effects of an alkaline pH on the bacterial cell is of extreme importance in order to understand and potentially mitigate this cross-protection.

When *Listeria* is grown at 3°C in TSB adjusted to pH 8.5 with NaOH there are two distinct peaks in filamentation that occur over the growth period for all three strains, showing an overall increase over time (Figure 3.12). This could potentially be due to the buffering capacity being reached midway through growth, causing the pH of the medium to decrease over the course of growth as acidic compounds are produced and released, as suggested by Winslow and Shaughnessy (1924). On average, the pH lowered a full point over the course of growth in samples, from pH 8.5 to 7.5. In a study by Giotis *et al.* (2007) *Listeria* stressed in buffered media showed an increase over time in both filament length and density within a sample, whereas *Listeria* grown in unbuffered alkaline media filamented initially, but

these filaments decreased in length and number as growth continued and the pH approached neutral (Giotis, Blair, McDowell 2007).

Floors of processing facilities are usually kept dry between cleaning and sanitizing and so *Listeria* cells are more likely to be in an unbuffered or mildly buffered environment, especially when transferred to different locations in the plant by worker or product movement. However, due to the rise in acidity of the broth indicative of an exceeded buffer capacity, results of this study cannot be used to conclusively show the filamentation of *L. monocytocenes* in alkaline environments for a *prolonged* period of time and may not represent the filamentation characteristics of all the *Listeria* present in a food manufacturing operation. Another limitation is that presence and spacing of nucleoids was not examined; in combination with the limited information available on the effect of alkali on the cell, this limits any inferences that could be made to the mechanism of filamentation involved.

The gradual decrease in pH from 8.5 to 7.5 is clearly the cause of the final decrease in filamentation towards the latter end of growth in this trial portion of the study, as confirmed by the increase in the size of the general population as shown by the increase in percentage of cells gated by flow cytometry compared to control (Figures 3.12, 3.17), However, this fails to explain the presence of multiple peaks in filamentation length over time. During the first peak in filament length on approximately day 4, the population as a whole continues to shift larger, not reflecting the sudden increase in size (Figures 3.12, 3.17). This trend between the two flow

cytometry variables is similar to that in the peak reflected in the 4% NaCl trial (Figures 3.6, 3.9, 3.12, 3.17) and could potentially be caused by the upregulation of genes and protein production required to adapt to the alkaline environment. The presence of peaks in the alkaline trial compared to the acidic trial suggest completely different mechanisms and interactions within the cell (Figures 3.6, 3.12). However this cannot be confirmed until the effects of an alkaline pH on the bacterial are more fully researched and understood. Furthermore, the similar initial peak placement and fold change that occurred in both 4% NaCl and the alkaline trial may have no relation until the alkaline mechanisms are brought to light (Figures 3.6, 3.12).

Giotis *et al.* (2007) exposed *L. monocytogenes* to alkaline environments of pH 9 or higher for up to 12 hours at 30°C and collected data on filamentation (Giotis, Blair, McDowell 2007). Mean cell length increased from 1.6 μm to 2.2 μm over the 12 hour period at pH 9, a fold-change ratio of 1.4, demonstrating a relatively immediate increase in cell length, a phenomenon observed in the current study (Giotis, Blair, McDowell 2007). However, at this point growth would have been much further advanced due to the higher temperature, and results are not comparable without the growth curve of their treatments. Future studies should include information on the growth of bacterial cultures along with data on filamentation to make data more comparable.

When grown in both slightly acidic and saline environments (4% NaCl at a pH of 6), the trend of filamentation over time appears to be a

combination of the trends observed for filamentation in saline and acidic conditions (Figures 3.18, 3.6, 3.10). There was an initial peak in filamentation when cells were grown in saline conditions followed by a gradual increase over time, which is typical of acidic conditions. The initial peak occurs around 4 d of incubation which is similar to what was observed in the cultures grown in broth with added NaCl (Figures 3.18, 3.6). This "combination trend" that occurs when *Listeria* is grown under multiple applied stresses strengthens the hypothesis that the mechanisms of filamentation for *Listeria* grown in saline or acidic conditions are different. It is possible that each separate mechanism causes filamentation at different stages of growth.

While the trend for changes in cell filamentation among experiments was similar, the degree to which filamentation occurred was equally comparable to the experiment where cells were grown in TSB with 4% NaCl at the initial peak in filamentation but was not comparable to the experiment where cells were grown in TSB adjusted to pH 6 towards the latter end of growth, where the increase in filamentation was not as large (Figures 3.6, 3.10, 3.18). The difference in fold change by the end of growth was 5-10, which could be due to some interaction between the two stress factors. However, despite differences in magnitude the pattern of filamentation still suggests mechanisms that are largely, if not completely separate.

A consideration for this portion of the study was the order of stress applied for experiments involving salt and acid. In this case stresses were

applied simultaneously. Applying the stresses simultaneously avoided further sub-culturing, which could alter strain characteristics; a potential study limitation. To best represent conditions that cells might encounter in meat processing plants, the stress should likely be applied in the same order as applied in industry, as brining of meat at the addition of acidic preservatives are not necessarily a single-step process. Additional studies that examine the effects of the order of application of different stress on filamentation would be useful. Despite the potential for error, the similarity of results between the experiments done in TSB with 4% NaCl adjusted to pH 6 and those done under acidic and salt conditions as individual stressers, lend validity to this method.

One theoretical result of these numerous stress-response adaptations is that less energy and therefore resources are available for cell division, leading to increased filamentation. This theory is strengthened by the connection existing between filamentation and the proton motive force; when the proton motive force is hindered less ATP is available and bacteria have been shown to filament (Strahl and Halmoen 2010).

While general theories concerning energy requirements have been suggested, no actual mechanism of action has been determined as the cause of filamentation at low temperatures. Areas of research include *RpoS* regulated genes in Gram negative bacteria such as *E. coli*, and research on *sigmaB,* an operon that controls stress-related genes in both *Bacillus subtilis* and *L. monocytogenes* (Becker and others 1998; Leenanon and Drake 2001).

More research linking protein expression to filamentation is required to more fully understand this phenomenon.

Future work in this research area should include examination of the role of osmoprotectants, their transporters, acid, and alkaline adaptation mechanisms in filamentation at low temperatures. Other factors examined in tandem with low temperatures that have relevance to the food industry, such as modified atmosphere and acidic environments (evaluation of multiple environments/acids found in food products and packaging), would provide additional information concerning the importance of filamentation of *L. monocytogenes* to the food industry. Testing for the presence of multiple nucleoids spaced throughout the filaments would provide additional information on what stage of cell division filamentation occurs. Finally, multiple stresses reflective of food plant and product conditions and the resulting filamentation should be examined in order to gain better understanding of this phenomenon in a more accurate setting. This will allow for more accurate modeling of the overall degree of filamentation of *L. monocytogenes* likely to be found in meat processing plants. With accurate models, the potential of plate counts to inaccurately represent food safety risks of filamentous microorganisms could be reduced or eliminated, making ready-to-eat food products safer for the consumer and lowering the potential for recalls, saving food companies money.

5. Conclusions

In this study, strains of *L. monocytogenes* were examined to determine their respective degree and nature of filamentation in conditions representative of meat processing plants. Post-processing contamination of *L. monocytogenes* is a constant threat in RTE meat products that do not involve additional cooking by consumers, and which are refrigerated for weeks prior to consumption. Filaments appear as single colonies on plate count agar, which may lead to underestimation of a products' bacterial risk (Giotis, Blair, McDowell 2007). While many causes of filamentation have been proposed, no one mechanism has been discovered, and very little research exists into gene and protein expression in Gram positive organisms such as *Listeria*.

A preliminary study was completed involving 16 strains of *L. monocytogenes,* strains FS2-FS15 that were isolated from ready-to-eat meats in Edmonton, AB (Bohaychuk and others 2006), and one strain, CDC7762, was obtained from the Center for Disease Control (Atlanta, Georgia, USA) and was the cause of an outbreak in ready-to-eat meat products in 1998. In the preliminary portion of this study, temperatures ranging from 3-5°C were used and filamentation characteristics for the 16 strains determined. Results of this study determined that *L. monocytogenes* FS2, FS12 and CDC7762 showed a wide range of filamentation characteristics, and that filamentation increased at decreased temperatures. A temperature of 3.5°C was chosen for all future experiments as this study attempted to imitate plant conditions

that, while being reasonable, also promoted the degree of filamentation and the length of filaments.

L. monocytogenes FS2, FS12 and CDC7762 filamented in saline, acidic and alkaline conditions as well as in an acidic saline medium. Different filamentation patterns over time suggest different mechanisms are involved with saline and acidic stress. In acidic conditions the length and proportion of filaments within a population increased over time, while in saline conditions these characteristics peaked early in the growth phase and decreased for the remainder of growth. When a combination of acidic saline conditions was applied, both of these trends were evident, all strains had a minor filamentation peak early in growth similar to saline conditions alone, followed by a slow increase in filamentation over time characteristic of acidic conditions. The more extreme the stress, the more filamentation was evident within the population. An increase in filamentation may be linked to moments of increased cellular adaptation when there is an increased strain on resources.

The alkaline medium used was insufficiently buffered, so while the pH decreased throughout the length of the experiment, the filamentation over time and the proportion of cells that were filamented shows unexplained results leading to multiple peaks over the length of growth. Future work into the effect of alkaline conditions on the bacterial cell is essential to understanding this trend, as little information is available on alkaline stress responses within the cell.

Future work in this area would include investigating the degree of filamentation in modified atmosphere conditions as well as other ready-to-eat meat plant conditions not examined in this study. Analysis of protein and gene expression of *L. monocytogenes* at increased moments of filamentation may be of importance. Additional information on cellular stress responses, especially in regards to alkaline stress, would help to further determine and explain the mechanisms behind filamentation and the trends that occur in the filamentation of *L. monocytogenes* over time. Knowledge of protein expression of Gram positive organisms including but not limited to *Listeria* would further benefit the food industry.

This study can be utilized to understand which conditions aggravate filamentation of *Listeria* in food plants, and can be used as a tool to minimize filamentation in RTE meat products, or account for filamentation in current enumeration practices and procedures and adjust methods and dilutions accordingly.

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Appendix

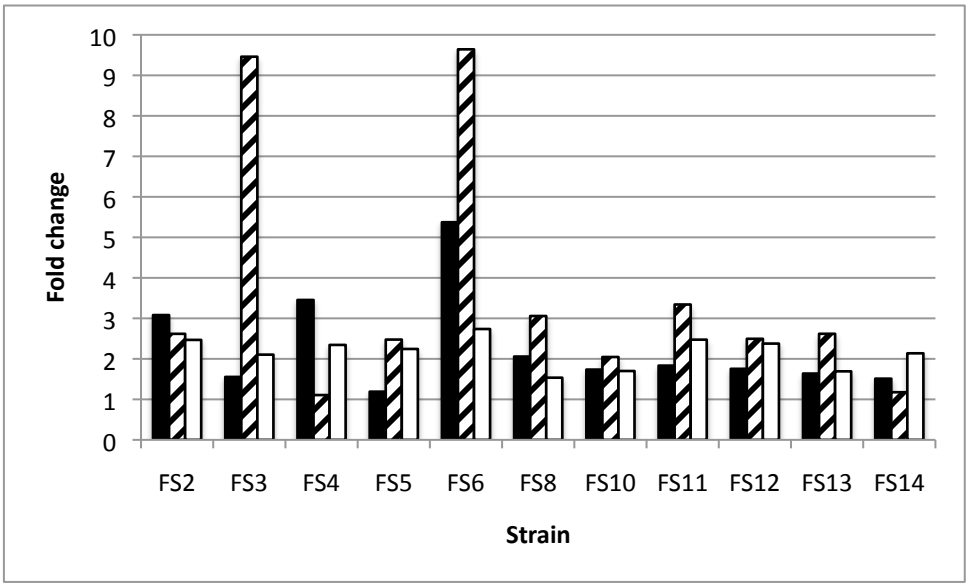


Figure A.1. Fold Change of strains of *Listeria monocytogenes* on days 7 (black), 10 (diagonal line) and 11 (white) of incubation at 5°C.

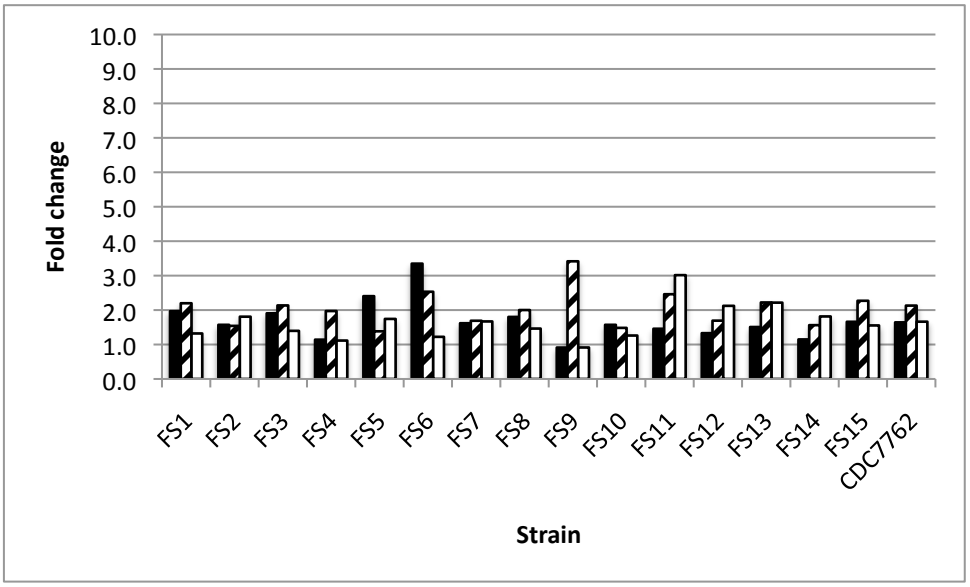


Figure A.2. Fold Change of strains of *Listeria monocytogenes* on days 7 (black), 11 (diagonal line) and 12 (white) of incubation at 4°C.

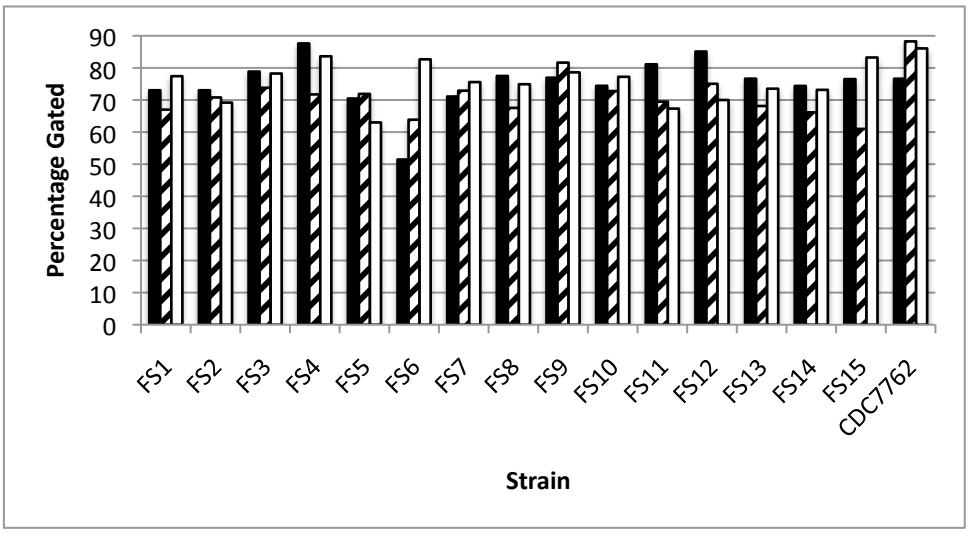


Figure A.3. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples on days 7 (black), 11 (diagonal line) and 12 (white) of incubation at 4°C for multiple strains of *Listeria monocytogenes*.

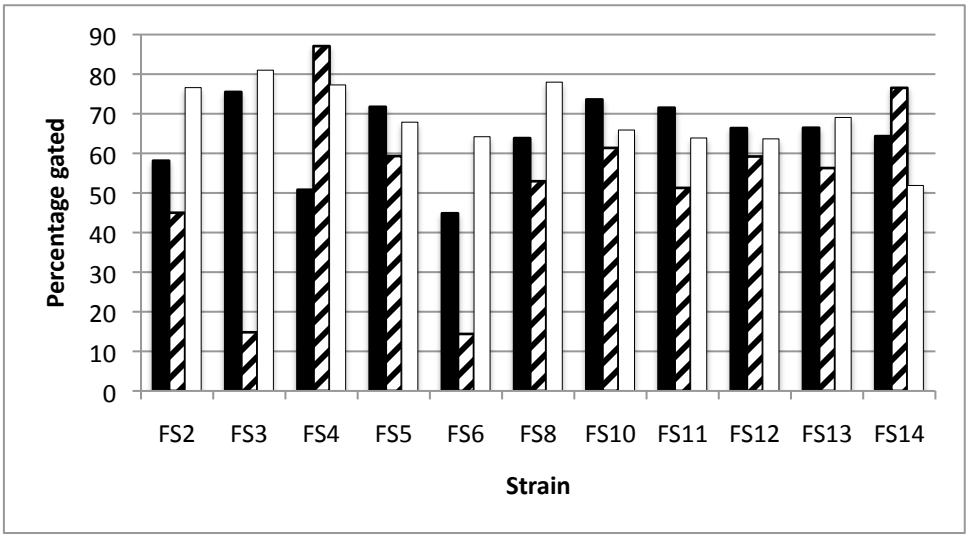


Figure A.4. Proportion of treated cells that fall under the forward scatter reading of the 90th percentile of the control samples on days 7 (black), 10 (diagonal line) and 11 (white) of incubation at 5°C for multiple strains of *Listeria monocytogenes*.

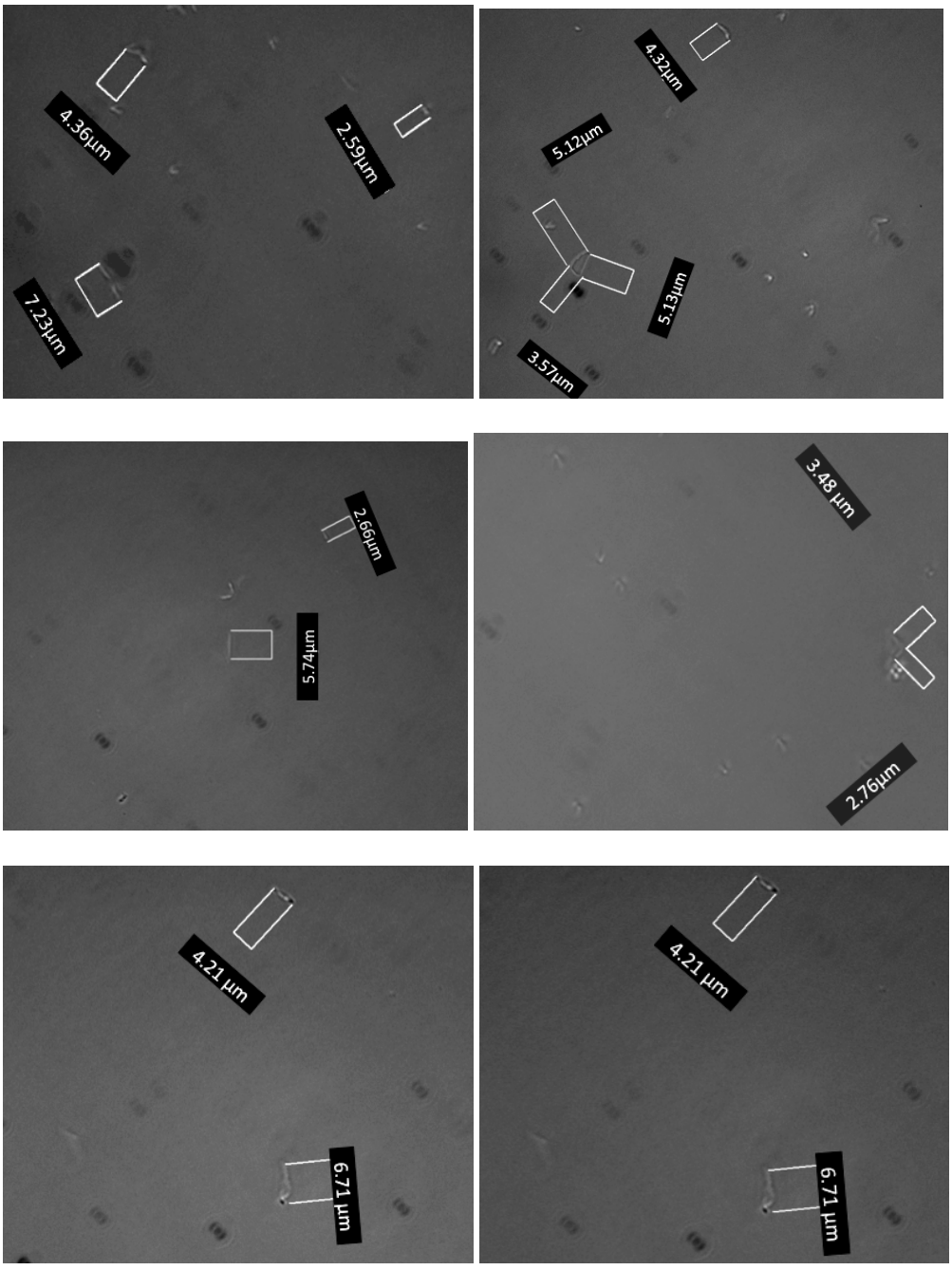


Figure A.5. Images of *L. monocytogenes* FS1 (top left), FS2 (top right), FS3 (middle left), FS4 (middle right), FS5 (bottom left) and FS6 (bottom right) grown in TSB incubated at 3°C for 7days (100x magnification).

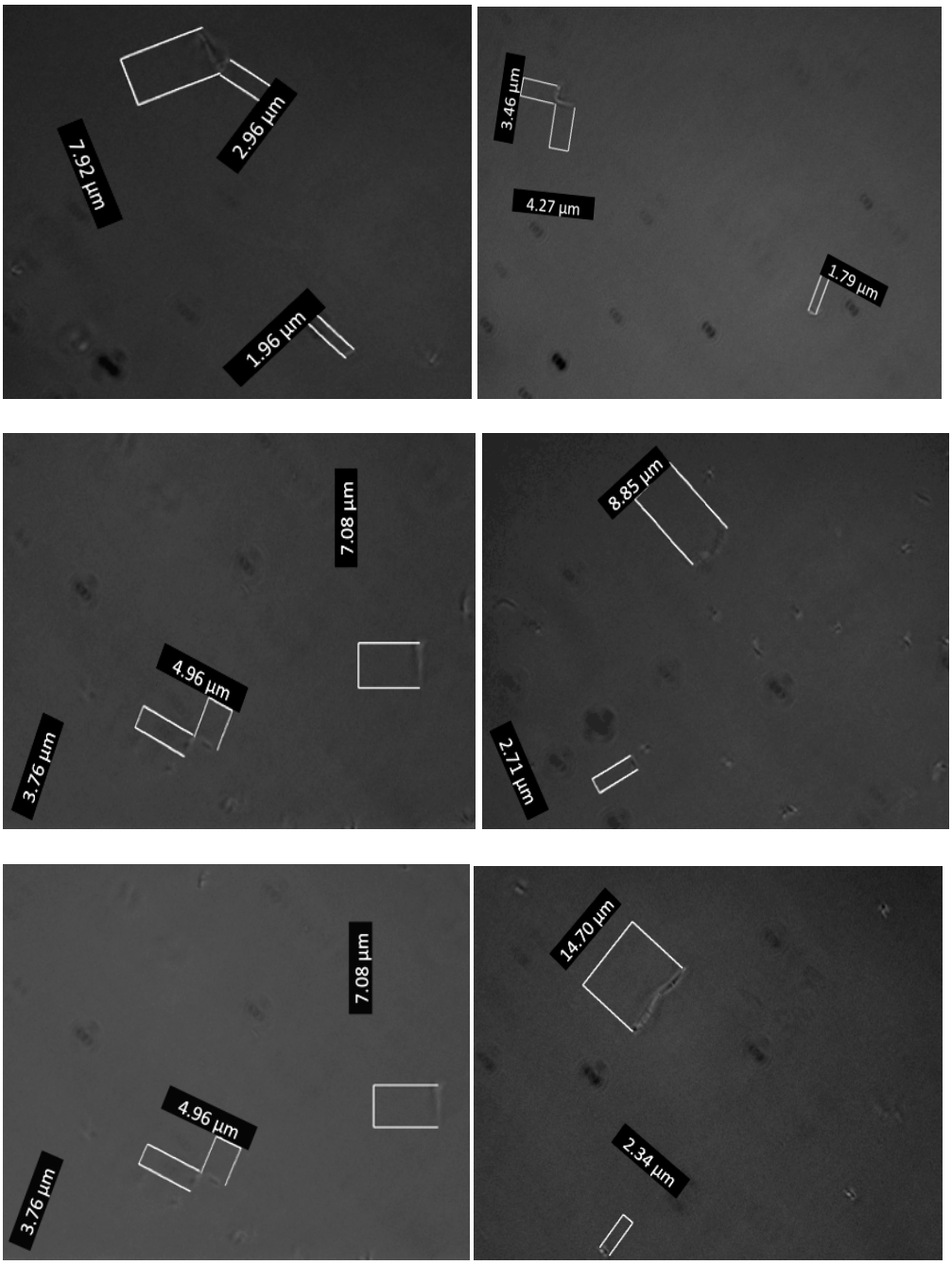


Figure A.6. Images of *L. monocytogenes* FS7 (top left), FS8 (top right), FS9 (middle left), FS10 (middle right), FS11 (bottom left) and FS12 (bottom right) grown in TSB incubated at 3°C for 7 d (100x magnification).

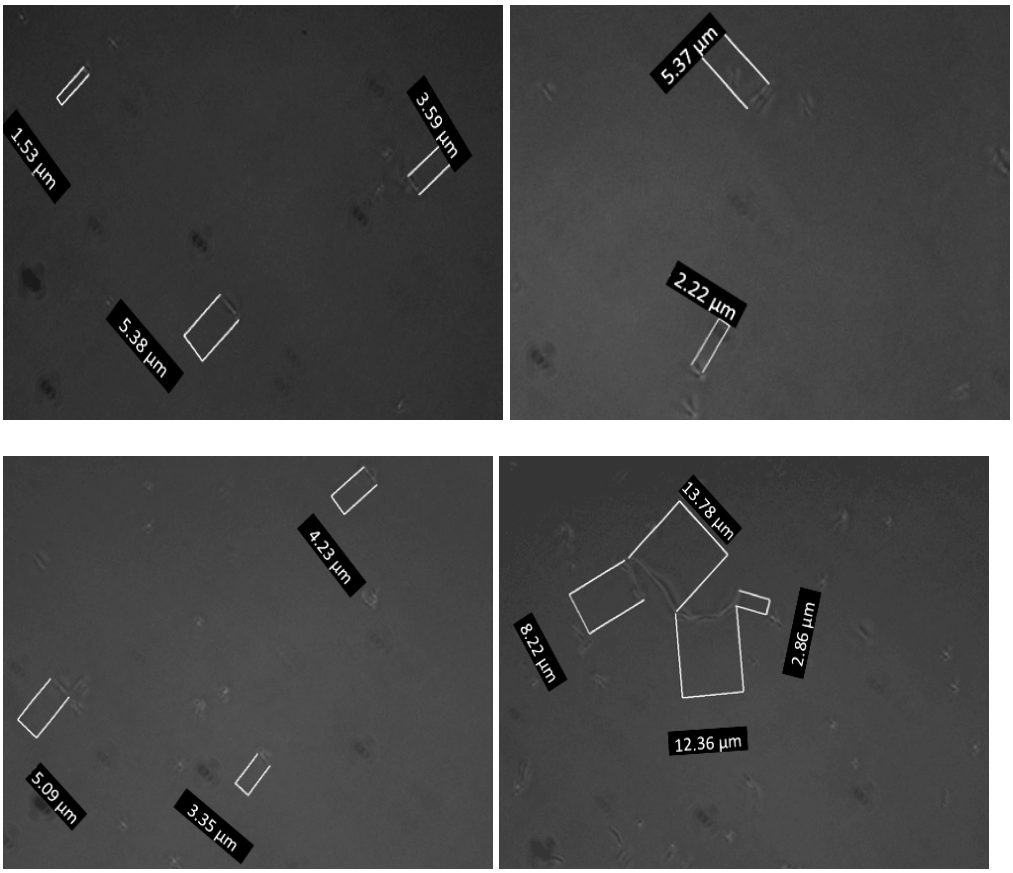


Figure A.7. Images of *L. monocytogenes* FS13 (top left), FS14 (top right), FS15 (bottom left) and CDC7762 (bottom right) grown in TSB incubated 3°C for 7 d (100x magnification).

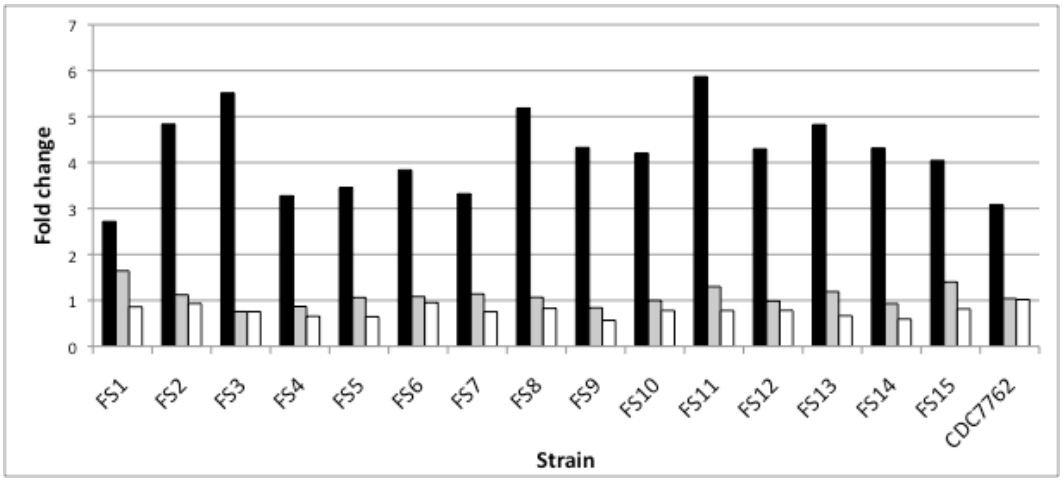
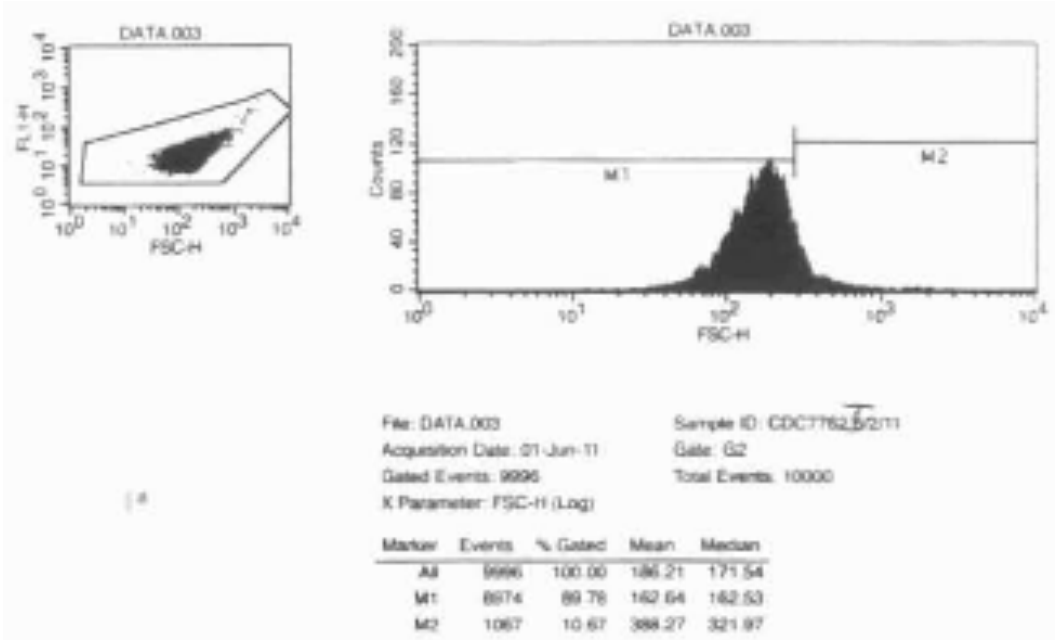


Figure A.8. Fold change of strains of *L. monocytogenes* on days 7 (black), 10 (diagonal line) and 11 (white) of growth in TSB incubated at 5°C.

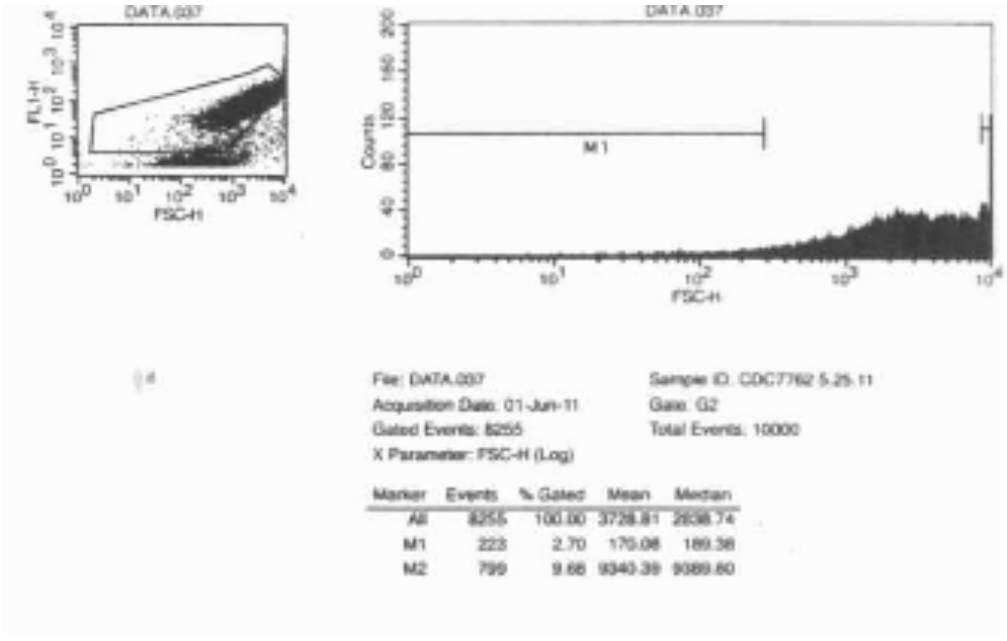
Sample calculations for fold change and proportion of forward scatter value that falls under the 90th percentile of the control

Control data- *L. monocytogenes* CDC7762, pH 5.5, trial 1



Marker	Events	% Gated	Mean	Median
All	9996	100.00	186.21	171.54
M1	8974	89.76	162.64	162.53
M2	1067	10.67	388.27	321.97

Day 27- *L. monocytogenes* CDC7762, pH 5.5, trial 1



Marker	Events	% Gated	Mean	Median
All	8255	100.00	3728.81	2638.74
M1	223	2.70	170.08	169.38
M2	799	9.68	9340.39	9389.60

Fold change calculation:

Fold change = (10% longest cells in sample)/(10% longest cells in control)

= 9340.39/388.27

= 24.06

Proportion of forward scatter value that falls under the 90th percentile of the control

1. Set M1 in the control to the 90th percentile (where % Gated = 90)

2. Without changing location of M1, read the % gated value in the sample

% gated value = 2.70%